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Platelet P-selectin and GPIIb/IIIa expression after liver transplantation and resection

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Abstract Platelet dysfunction contributes to haemostatic defects, possibly leading to bleeding complications. We hypothesised that liver transplantation and liver resection, together with portal clamping time, might be a potential stimulus for platelet activation. Therefore, we determined the expression of platelet GPIIb/IIIa and P-selectin, representing important platelet activation markers, and the thrombopoietin (TPO) serum level after transplantation and resection. Twenty patients [ten that had undergone orthotopic liver transplantation (OLT), ten with liver resection (LRX)] were included in the study. From sequential venous blood samples, surface expression of GPIIb/IIIa and P-selectin was quantified by flow cytometry, and TPO serum levels were determined by ELISA. Baseline GPIIb/IIIa receptor expression on circulating platelets was significantly reduced in the OLT group compared to the LRX group and healthy volunteers. GPIIb/IIIa expression after activation with TRAP-6 increased significantly ($P < 0.001$) in the LRX group but not in the OLT group. P-selectin expression after TRAP-6 stimulation increased significantly ($P < 0.001$) in the LRX group, being comparable to that in healthy volunteers, whereas only a very low increase in the OLT group was found. In the OLT group, TPO serum levels were in the lower normal range and rose above the upper limit of normal values 24 h after reperfusion. These data indicate that neither liver transplantation nor liver resection influences GPIIb/IIIa and P-selectin expression on circulating platelets. There was a lack of expression in cirrhotic patients and unimpaired baseline expression and functional reserve in non-cirrhotic liver-resection patients. After liver transplantation, increasing serum TPO levels, which indicated a recovering graft function, resulted in rising peripheral platelet counts.

Keywords Platelet · GPIIb/IIIa · P-selectin · Liver transplantation · Liver resection

Introduction

Platelets play a crucial role in haemostasis and inflammation, responding to tissue injury with changes in receptor expression. The damage related to liver reperfusion after transplantation or liver resection with portal clamping may be an effective stimulus for platelet activation. A previous clinical study has demonstrated platelet activation and consumption following reperfusion after liver transplantation [1]. In a rat model Cywes

et al. demonstrated that the percentage reduction in circulating platelets was dependent on the length of preservation time but not on the initial platelet concentration [2].

Platelet dysfunction may be reflected by quantitative abnormal membrane glycoproteins, of which glycoprotein IIb/IIIa (GPIIb/IIIa, CD41) and P-selectin represent the most prominent markers. GPIIb/IIIa is expressed on the surface of platelets and can be activated by thrombin. In its active form, this complex can bind to matrix macromolecules, such as fibrinogen and von Willebrand factor [3]. P-selectin (CD62P) is an adhesion molecule, which is synthesized by megakaryocytes and incorporated in platelet alpha-granules. After stimulation of platelets, P-selectin is transported to the cell surface by fusion of the alpha-granule membrane with the plasma membrane. P-selectin is responsible for the adhesion of activated platelets to endothelium and leukocytes. Platelet activation is characterized by the expression of "activation-dependent" antigens, e.g. P-selectin, and by an increase in the number of GPIIb/IIIa complexes on the platelet surface.

Thrombopoietin (TPO) is primarily produced in hepatocytes and is a regulator of platelet production [4]. Accordingly, TPO is likely to represent an important link between hepatic and platelet disorders. Moreover, in liver grafts, reperfusion leads to platelet activation and consumption, resulting in thrombocytopenia [5].

The fate of platelets after liver transplantation or resection has not yet been the subject of systematic analysis. For this reason the aim of our study was to investigate whether the platelet P-selectin and GPIIb/IIIa expression on circulating platelets increases after liver transplantation or liver resection. Furthermore, the study was intended to elucidate whether TPO serum levels correlate with platelet activation in the postoperative course. To answer these questions we prospectively compared the effects of liver transplantation or resection on glycoprotein IIb/IIIa (CD41) and P-selectin (CD62P) expression on circulating platelets and on thrombopoietin serum levels.

Materials and methods

After having given written informed consent, according to the local Ethics Committee, ten patients who had undergone liver transplantation (seven male, mean age 45 ± 14.2 years) and ten patients with liver resection (six male, mean age 57 ± 14.0 years) were included in the study. In the liver transplantation group all patients had undergone orthotopic full-sized liver transplantation for end-stage liver disease (medical urgency criteria T_2/T_3). The grafts for liver transplantation had a cold ischaemic time of 9.6 ± 2.0 h and an anhepatic time of 0.49 ± 0.11 h. All grafts were preserved with HTK preservation solution (Dr. Köhler Chemie, Alsbach-Hähnlein, Germany).

In the liver resection (LRX) group all patients had undergone major liver resection for primary or secondary malignancies. A "Pringle" manoeuvre was carried out for the time of parenchyma dissection. The portal clamping time was 0.27 ± 0.12 h. None of the patients received medication known to alter platelet function.

Patients' characteristics are shown in detail in Tables 1 and 2.

Flow-cytometry analysis

Five venous blood samples (3 ml) were collected in sodium citrate disposable blood sampling tubes (0.3 ml, 0.106 mol l^{-1}, S-Monovette, Sarstedt, Nümbrecht, Germany) before induction of anaesthesia, 10 min, 1 h, 24 h and 1 week after reperfusion. Samples were taken from a peripheral vein through a 16-gauge needle without stasis. To avoid ex vivo platelet activation, blood samples were processed within 45 min of being drawn. All samples were taken, processed and measured by the same investigator. To achieve comparable preconditions for staining with saturating antibody concentrations, and to reduce non-specific background fluorescence, we adjusted platelet concentration to 20,000 μl^{-1} with 37°C pre-warmed phosphate-buffered saline (PBS, pH 7.2, without Ca^{2+} and $MgCl_2$; GIBCO BRL, Eggenstein,

Table 1 Characteristics of patients who had undergone OLT (*AIH* autoimmune hepatitis, *ALC* alcohol-induced cirrhosis, *CIT* cold ischaemia time, K-hepatitis B virus, *HCC* hepato-cellular carcinoma, *HCV* hepatitis C virus, *F* female, *M* male)

Patient no.	Gender	Age (years)	Diagnosis	Child–Pugh classification	CIT (h:min)	Anhepatic time (h:min)
1	F	58	HCV	B	08:00	00:40
2	M	64	ALC with HCC	A	13:44	00:40
3	M	34	HCV	A	13:44	00:45
4	M	60	HCV	A	08:46	01:00
5	M	23	AIH	B	11:30	00:50
6	M	33	AIH	A	08:20	00:35
7	M	40	HBV/HCV	B	12:30	00:55
8	F	65	ALC	B	08:40	01:00
9	M	42	HCV	B	08:40	01:05
10	F	33	HBV	B	08:00	00:45

Table 2 Characteristics of patients who had undergone LRX (*CCC* cholangiocellular carcinoma, *CRM* colorectal metastasis, *HCC* hepatocellular carcinoma, *Klatskin* hilar cholangiocellular carcinoma, *F* female, *M* male)

Patient no.	Gender	Age (years)	Diagnosis	Resection	Portal clamping time (h:min)
1	M	76	CRM	Right	00:15
2	M	33	CRM	Right	00:17
3	M	58	Klatskin	Extended left	00:45
4	F	52	CCC	Extended right	00:22
5	M	66	CRM	Extended right	00:36
6	M	59	CCC	Extended right	00:35
7	M	57	Klatskin	Extended left	00:43
8	F	69	CRM	Extended right	00:25
9	F	35	HCC no cirrhosis	Extended right	00:18
10	M	65	CCC	Extended left	00:22

Germany) and 1% bovine serum albumin (BSA; Roche, Mannheim, Germany).

IgG isotype controls were applied to detect non-specific staining. For platelet identification and detection of glycoprotein IIb/IIIa (GPIIb/IIIa) expression, samples were stained with 6 µl anti-GPIIb/IIIa PE (phycoerythrin)-conjugated monoclonal antibody (CD41-PE, clone P2; Beckman–Coulter, Krefeld, Germany). To detect platelet activation we stained the samples with 6 µl fluorescein isothiocyanate (FITC) monoclonal antibody against P-selectin (CD62P-FITC, clone CLB/Thromb/6; Beckman-Coulter). Samples were incubated at 37°C for 10 min in the dark. To determine platelet reactivity we activated the tubes with 3 µmol/l thrombin receptor activating peptide (TRAP-6; Bachem, Heidelberg, Germany) for 10 min at 37°C before staining them with the monoclonal antibodies. After addition of 2 ml PBS containing 1% BSA, we stopped activation by transferring the samples onto ice.

The flow cytometer was equipped with an argon laser wavelength of 488 nm (Epics XL, Beckman–Coulter). For each sample a minimum of 25,000 platelets was analysed. The green fluorescence emission for CD62P was measured by the corresponding photomultiplier (FL 1, 525 nm). The photomultiplier for FL 2 (575 nm) was used for recognition of platelets (CD41-PE). The forward scatter, side scatter, FL 1 and FL 2 were assessed, using the logarithmic mode. Data files were stored in list mode, and mean channel fluorescence emissions were analysed in histograms and platelet–leukocyte aggregates in dot plots, with a PC software package (EXPO 2.0, Beckman-Coulter).

Whole-blood assays of platelets required correct identification and separation of platelets from erythrocytes or debris. Platelets were identified on the basis of their high density expression of the specific platelet antigen CD41-PE in the FSC/FL 2 dot plot. The platelets were gated by the setting of a polygonal gate and transferred to an FL 1 (CD62P-FITC) and FL 2 (CD41-PE) histogram. The mean channel expression of FL 1 and FL 2 was used to determine the activation-dependent expression density of GPIIb/IIIa and P-selectin according to TRAP-6 stimulation.

Thrombopoietin levels

In both groups, serum samples were taken before induction of anaesthesia, as well as 10 min, 1 h, 24, 72 h and 1 week after reperfusion of the transplanted liver or completion of resection. Samples were measured by the Quantikine human TPO-enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden, Germany), with the lowest TPO standard being 31.2 pg/ml. TPO-levels below 100 pg/ml are considered normal in healthy people [6]. The assay was performed according to the manufacturer's instructions. TPO level was assessed on the serum samples that had been stored at −70°C before being tested. Briefly, this assay uses the sandwich immunoassay technique. Standards and samples were pipetted into wells precoated with a murine monoclonal antibody specific for human TPO. TPO was captured after a 3 h incubation at 2–8°C. The wells were then washed to remove any unbound substances. To detect the amount of bound TPO, a horseradish peroxidase-linked, TPO-specific, monoclonal antibody was incubated within each well for 1 h at 2–8°C. After a 2nd thorough washing, the amount of TPO was quantitated by the addition of substrate (tetramethylbenzidine). The absorbance was read at 450 nm, and the background was corrected by the subtraction of the absorbance at 540 nm. This value was proportional to the amount of TPO bound in the initial step. The results were reported in picogrammes per millilitre.

Statistical analysis

All numeric flow-cytometry data are values of mean florescence units. The percentage of P-selectin expression on platelets after reperfusion was compared with the respective percentage before. All data showed a Gaussian distribution (Kolmogorov–Smirnov test). A multiple factor univariate analysis of variance (ANOVA) was used

to identify effects of different ischaemia and time (SPSS/PC V 10.0 software package, SPSS, Munich, Germany). To perform the multiple comparisons we used a one-factor univariate ANOVA followed by the Bonferroni post-hoc test or Games–Howell test if Levene's test for homogeneity of variance failed. We used the Mann–Whitney U test to compare data with only two groups. A probability of less than 0.05 was considered significant.

Results

Comparison of baseline and TRAP-6-stimulated GPIIb/IIIa receptor expression

Preoperative baseline GPIIb/IIIa antigen expression on circulating platelets was drastically reduced in the orthotopic liver transplantation (OLT) group, in contrast to healthy volunteers (9.8 vs 164.7, $P < 0.05$) and the LRX group (9.8 vs 122.2, $P < 0.05$), yet there was no statistically significant difference between healthy volunteers and the LRX group. Moreover, the time course of baseline GPIIb/IIIa receptor expression on circulating platelets was significantly reduced in the OLT group compared with the LRX group. After activation with TRAP-6, both groups differed significantly in their response. In the LRX group, GPIIb/IIIa expression increased significantly ($P < 0.001$) from the baseline value of 122.2 (±27.6) to 284.6 (±63.3). The increase after TRAP-6 stimulation was comparable to that in healthy volunteers and was statistically unchanged during the entire observation period. By contrast, only a weak and insignificant increase in the expression of GPIIb/IIIa from 9.8 (±1.2) to 13.6 (±4.3) was measured in the OLT group after TRAP-6 stimulation (Fig. 1).

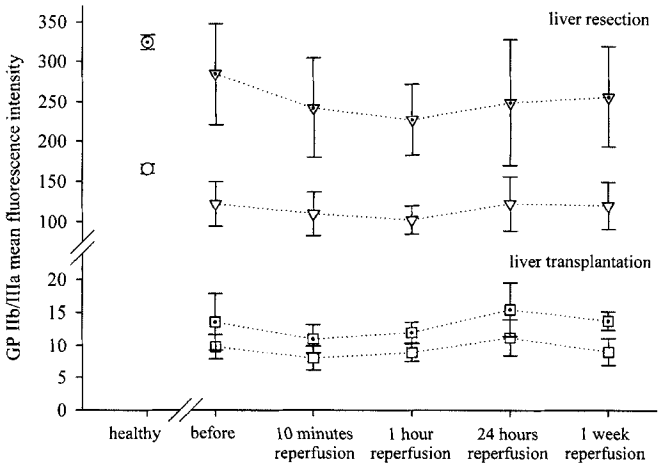

Fig. 1 Time course of the expression of GPIIb/IIIa on resting (*open symbols*) and TRAP-6-stimulated (*dotted symbols*) platelets from the liver resection group (*triangles*), the liver transplantation group (*squares*) and healthy volunteers (*circles*). The healthy volunteers served as controls until the time point before surgery for both groups. Values are given as means ± SEM of fluorescence intensity

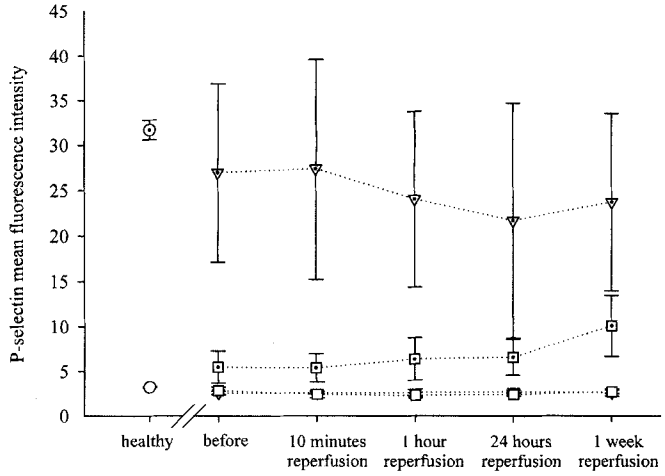

Fig. 2 Time course of the expression of P-selectin on resting (*open symbols*) and TRAP-6-stimulated (*dotted symbols*) platelets from the liver resection group (*triangles*), the liver transplantation group (*squares*) and healthy volunteers (*circles*). The healthy volunteers served as controls until the time point before surgery for both groups. Values are given as means ± SEM of fluorescence intensity

Comparison of baseline and TRAP-6 stimulated P-selectin receptor expression

On circulating platelets, we observed no difference in baseline P-selectin expression between healthy volunteers and both other groups, preoperatively, or during the entire observation period. After stimulation with TRAP-6, the expression of P-selectin in the LRX group increased significantly ($P < 0.001$) from 2.6 (±0.2) to 27.0 (±9.9) and was stable during the entire observation period (Fig. 2). There was no significant difference in the increased P-selectin expression between the LRX group and healthy volunteers (2.6 to 27.0 vs 3.2 to 30.5). However, after TRAP-6 activation there was only a very low increase in P-selectin expression in the OLT group, from 2.8 (±0.4) to 5.5 (±1.8), which was approximately only 25% of the response in the LRX group (Fig. 2).

In the OLT group we observed an increase to preoperative values of P-selectin expression after TRAP-6 activation during the first week after transplantation, which was significant at 1 week after transplantation (Fig. 3).

Peripheral platelet counts

In the OLT group, peripheral platelet counts were low, preoperatively, and decreased 1 h and 24 h after transplantation; yet, they had recovered after 1 week. In the LRX group, peripheral platelet counts were in the normal

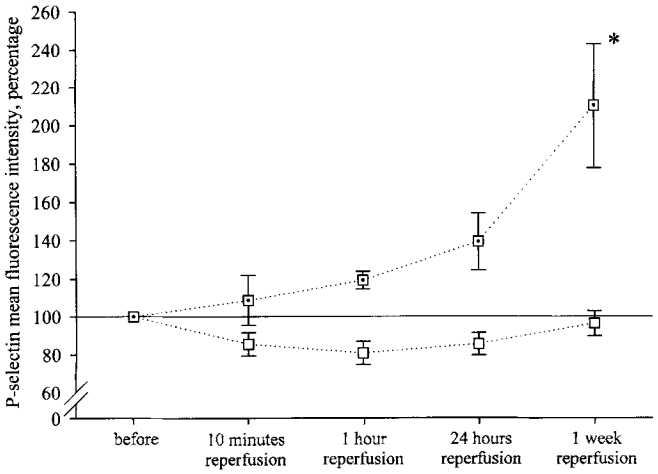

Fig. 3 Percentage of P-selectin expression on resting (*open symbols*) and TRAP-6-stimulated (*dotted symbols*) platelets from the liver plantation group (*squares*) compared with the respective initial values (set at 100%). Values are given as means ± SEM. *$P = 0.001$, one-factor univariate ANOVA, time course

Table 3 Platelet counts (platelets × 10^9/l)

Patient no.	Platelet count			
	Preoperatively	1 h after reperfusion	24 h after reperfusion	1 week after reperfusion
OLT group				
1	50	120	60	91
2	145	92	87	155
3	78	52	49	64
4	67	43	45	53
5	34	22	39	40
6	139	88	163	142
7	38	27	25	75
8	46	35	34	46
9	59	31	26	59
10	48	33	35	45
LRX group				
1	243	178	226	267
2	421	289	343	376
3	349	228	235	280
4	190	159	182	235
5	402	258	309	346
6	119	92	112	187
7	251	167	154	192
8	305	265	278	312
9	153	143	162	207
10	228	183	215	265

range but decreased 1 h after reperfusion, reaching preoperative values in the first week after resection (Table 3.

Thrombopoietin levels after orthotopic liver transplantation

Before induction of anaesthesia, 10 min and 1 h after reperfusion, TPO serum levels were in the lower normal range, below the limit of detection of 31.2 pg ml^{-1} (range 5.0–35.0). TPO serum levels rose above the upper limit of normal values of 175.4 pg ml^{-1} (range 136–202) at 24 h after reperfusion, with a transient peak at 72 h of 228.8 pg ml^{-1} (range 136–271), and declined, thereafter, to 188.0 pg ml^{-1} (range 74–235) (Fig. 4). In contrast, liver resection had no influence on the TPO serum levels. All TPO serum levels were within normal range during the observation period (range 43–86 pg ml^{-1}).

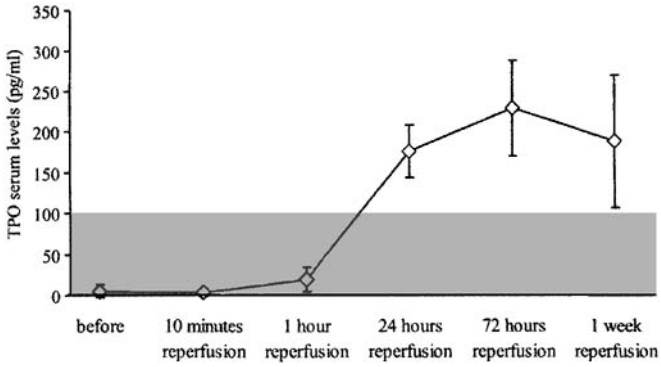

Fig. 4 TPO plasma levels before and after liver transplantation. The grey area represents the normal range of TPO, with an upper limit of 100 pg/ml. Values are given as means ± SEM

Discussion

The tendency for bleeding after major liver resection and liver transplantation remains a critical issue, despite refined surgical skills and improved peri- and postoperative management. Especially after liver transplantation, patients with progressive liver cirrhosis are well known to have an impaired coagulation system with cellular and plasmatic dysfunction [7, 8, 9, 10]. Conversely, from experimental studies, there is increasing evidence that platelet activation mediates hepatic injury and graft dysfunction [11, 12, 13, 14]. However, clinical data on platelet activation following liver transplantation or resection in humans are limited.

Our study deals with platelet receptor expression in patients undergoing liver transplantation (OLT group) and patients undergoing major liver resection for malignancies (LRX group). GPIIb/IIIa baseline expression and TRAP-6 stimulation on circulating platelets showed no significant difference between healthy volunteers and the LRX group. By contrast, in the OLT group GPIIb/IIIa baseline expression, as well as response after TRAP-6 stimulation, was significantly reduced in comparison with that of healthy volunteers and the LRX group. This finding is in accordance with the study published by Pihusch et al., who found a similar total amount of GP IIb/IIIa expression and only minimal reactivity of the GP IIb/IIIa receptor after TRAP-6 stimulation in cirrhotic patients [15].

With regard to P-selectin, there was only weak baseline expression in both groups. The response after TRAP-6 stimulation was strongly reduced in the OLT group, yet tendentially began to recover 1 week after transplantation. In a previous study, P-selectin expression after ex vivo stimulation was significantly lower in patients with liver cirrhosis than in healthy controls [16]. This may have been attributable either to reduced functional capacity of platelets or to adhesion of activated platelets that were not detectable in the circulation any more. Indeed, in that study, platelet activation after graft reperfusion was demonstrated, as measured by the increased secretion of platelet-derived beta-thromboglobulin [5, 8]. Since only circulating, and not adherent, platelets were measured, our data might contain a bias towards reduced platelet activation in both patient groups. Accordingly, since the significance of thromboglobulin as a marker of platelet activation needs further elucidation, the question of platelet activation in liver transplantation cannot be finally answered.

TPO is primarily produced by hepatocytes, whereby serum levels are regulated by platelet turnover [17]. In patients with advanced liver disease reduced platelets may be a result of decreased TPO production. After liver transplantation TPO levels increased, and with a delay of several days the peripheral platelet count was elevated [18]. We observed preoperatively low, and postoperatively increased, TPO levels, together with an increasing platelet count after liver transplantation, which is in accordance with the results of previous studies [8, 19].

In conclusion, our data demonstrate that circulating platelet GPIIb/IIIa and P-selectin expression is strictly related to the underlying disease. There was a lack of expression in cirrhotic patients, which was in contrast to the unimpaired baseline expression and functional reserve in non-cirrhotic liver resection patients. After liver transplantation increasing serum TPO levels,-indicating a recovering graft function, resulted in rising peripheral platelet counts. Previous data on platelet function following liver transplantation were based mostly on animal models, which are different from the clinical setting. Accordingly, the data obtained in this study may contribute to a better understanding of platelet pathophysiology and assessment of bleeding risk in liver surgery.

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