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Title
Platelet dynamics in the early phase of post-ischemic liver in vivo

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ABSTRACT

**Background.** In liver surgery, ischemia/reperfusion (I/R) injury occasionally leads to liver failure by activating Kupffer cells (KCs) and leukocytes. However, few reports have demonstrated a relationship between KCs and platelets *in vivo*. This study investigated the relationship between these cells using intravital microscopy (IVM).

**Materials and Methods.** Male Wistar rats were divided into two groups: i) KC+ group, receiving 1 ml saline; and ii) KC- group, intravenously injected with liposome-encapsulated dichloromethylene disphosphonate (Cl₂MDP) for elimination of KCs. At 48 h after administration, 20 min of total normothermic hepatic ischemia was induced. Rhodamine-6G-labeled platelets and sinusoidal alterations were monitored using IVM up to 120 min after reperfusion. P-selectin, accumulated leukocytes and morphological damage, and alanine aminotransferase (ALT) were evaluated.

**Results.** In the KC+ group, numbers of adherent platelets increased significantly within 30 min after reperfusion. Endothelial cells of sinusoids in which KCs were mainly located were destroyed and the sinusoids were significantly constricted after reperfusion. Conversely, in the KC- group, adherent platelets in sinusoids were suppressed, and sinusoidal perfusion, endothelial cell damage and serum ALT levels were significantly improved. P-selectin on sinusoidal endothelial cells was not observed up to 120 min after reperfusion in either group.

**Conclusions.** Adherent platelets appear to reflect activation of KCs and lead to leukocyte accumulation, resulting in sinusoidal perfusion disturbance and liver failure. Evaluation of adherent platelets in the microcirculation offers an important marker of hepatic injury.

**Key Words:** adherent platelet; Kupffer cells (KCs); intravital microscopy (IVM); hepatic ischemia/reperfusion (I/R)

**Abbreviations:** Cl₂MDP, liposome-encapsulated dichloromethylene disphosphonate; ALT, alanine aminotransferase; CCD, Charge coupled device;
INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury is the major source of morbidity associated with liver resection under vascular occlusion, i.e., Pringle maneuver, or after liver transplantation [1] [2]. An excessive inflammatory response is a critical component of hepatic I/R injury. The early phase of inflammation is characterized by activation of KCs, which generate reactive oxygen species and aggravate the early injury occurring within 120 min after reperfusion[3] [4] [5] [6]. In addition, leukocytes accumulate in the post-ischemic liver and contribute to hepatocyte injury in the late phase of I/R, which appears longer than 6 h after reperfusion[7] [8]. Many studies have indicated roles for KCs and leukocytes in liver injury after I/R.

In this decade, some studies have focused on the role of platelets in hepatic I/R. Activated platelets have been considered to be involved in hepatic injury and contribute to the development of apoptosis in sinusoidal endothelial cells after I/R[9] [10]. Conversely, some studies have reported a role of accumulated platelets in hepatocyte growth and liver proliferation in the injured liver [11] [12]. In addition, contact of platelets with hepatocytes or endothelial cells plays an important role in cell growth under various stressful conditions [13]. Evaluation of the dynamics of platelets in the hepatic microcirculation is thus essential. Recently, a few studies have reported the dynamics of platelets in vivo after hepatic I/R [9] [14]. However the mechanisms underlying sinusoidal platelet accumulation remains unclear, and the relationship between platelets and KCs has yet to be precisely evaluated.

The aim of this study was to investigate platelet-endothelial cell interactions in vivo and to determine whether KCs are associated with platelet adhesion in the early phase of hepatic I/R using intravital microscopy (IVM).
MATERIALS AND METHODS

Animals

Male Wistar rats weighing 220-250 g were obtained from CLEA Japan (Tokyo, Japan). Animal experiments were carried out in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba, and in accordance with the Regulation for Animal Experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Experimental groups

Total normothermic hepatic ischemia was induced for 20 min by clamping the portal triad. Animals were divided into 2 groups 48 h before operation: i) KC+ group, in which animals were intravenously injected with 1 ml saline instead of liposome-encapsulated dichloromethylene disphosphonate (Cl₂MDP); and ii) KC- group, in which animals were injected with Cl₂MDP intravenously to eliminate KCs. The hepatic microcirculation was observed just before and 0, 30, 60 and 120 min after reperfusion. Platelets were observed at 30, 60 and 120 min after reperfusion (Fig.1).

Surgical procedure

Under anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), animals were tracheotomized. To reduce spontaneous breathing, animals were ventilated mechanically (KN-55, Natsume Co., Ltd, Tokyo, Japan) and additional pentobarbital (10 mg/kg) was administered intravenously approximately every 60 min. Animals were placed in a supine position on a heated pad to maintain rectal temperature at 37 °C. To monitor arterial blood pressure and allow continuous infusion of Ringer’s solution, polyethylene catheters (PE-50, 0.58/0.96-mm inside/outside diameters; Becton Dickinson, Sparks, MD) were inserted into the left carotid artery and left jugular vein, respectively. The left liver lobe was exteriorized on a specially designed phase to minimize movements caused by respiration and covered with cover glass. Hepatic ischemia was induced by clamping the portal triad, i.e., hepatic artery, portal vein and bile duct, for 20 min using a microclip (Aesculap, Tuttlingen, Germany).
Elimination of KCs

Cl₂MDP reportedly selectively depletes phagocytic macrophages in the liver and spleen, but does not affect dendritic cells or leukocytes [15]. A suspension of Cl₂MDP-liposomes was prepared as described by Van Rooijen et al. Briefly, a thin phosphatidylcholine and cholesterol membrane was formed in an evaporator and dispersed in Cl₂MDP solution. The suspension was kept at room temperature for 2 h under nitrogen gas, then sonicated for 3 min in a waterbath sonicator and kept for another 2 h. After 15 min of centrifugation at 10,000×g, the white band of liposomes was collected and washed using phosphate-buffered saline (PBS) at 30 min of centrifugation at 25000×g. Finally, the pellet was suspended in 4 ml of PBS. This suspension was injected intravenously via the tail vein (1 ml/rat) at 48 h before operation.

Platelet preparation

Platelets were isolated from whole blood of syngenic rats and labeled with rhodamine-6G (0.05%; 50 μL/mL whole blood: R-4127; Sigma, St. Louis, MO), as described by Massberg et al. [16]. Briefly, the collected blood was diluted with buffer after addition of prostaglandin E₁ and rhodamine-6G. After 2-cycle centrifugation, fluorescent platelets were resuspended in PBS. In our study, a total of 1×10⁸ fluorescence-labeled platelets, representing approximately 1% of all circulating platelets in the recipient rat, were injected intravenously at 5 min before IVM.

Intravital fluorescence microscopy

Intravital fluorescence microscopy was performed using a modified microscope (BX30 FLA-SP; Olympus, Tokyo, Japan) with a 100-W mercury lamp attached to a filterblock. The hepatic microcirculation was recorded using a Charge coupled device (CCD) camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) and a digital video cassette recorder (GV-D1000 NTSC; Sony, Tokyo, Japan) for offline evaluation. Using water-immersion objective lenses (W10×/0.3 to W20×/0.7; Olympus, Tokyo, Japan), magnification of 325× to 650× was achieved on the video screen. Microcirculatory analysis of platelet-endothelial cell interactions was performed using videotaped images.

Rhodamine-6G labeling platelets were infused intra-arterially at 30, 60 and 120 min after reperfusion, and 7-10 randomly chosen acini were visualized. The plasma marker fluorescein (2×10⁻⁳ M/kg; F-6377; Sigma, St. Louis, MO) was then injected intravenously to assess sinusoidal
perfusion before and 0, 30, 60 and 120 min after reperfusion.
Quantitative assessment of microcirculatory parameters was performed offline using WinRoof image software (version 5.0; Mitani Shoji, Tokyo, Japan).

Microcirculatory analysis

The following four parameters were analyzed. i) Number of adherent platelets; platelets firmly attached to the endothelium within sinusoid for longer than 20 s [14]. The number of adherent platelets was counted in scanned acini. Results were expressed as the number of adherent platelets per field (1 field = approximately 0.2 mm²). ii) Zonal distribution of platelets (Zone1+Zone2 and Zone3). iii) Microvascular diameter rate, defined as diameter after-ischemia / diameter before-ischemia in identical sinusoids and post-sinusoidal venules up to 120 min after reperfusion. iv) No-reflow sinusoid rate (%), calculated as the number of unperfused sinusoids after ischemia from among 10 pre-ischemic sinusoids in 5-7 acini, as an index of microcirculatory disturbance.

Immunohistochemistry

The distribution and elimination of KCs at 120 min of reperfusion were assessed immunohistochemically using ED-2 antibody (BM4001; Acris Antibodies GmbH, Hiddenhausen, Germany), which specifically recognizes KCs. Liver samples were obtained from each group and embedded in Optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Fine Technical, Tokyo, Japan). Frozen sections (10 μm) were prepared and fixed in 10% formalin for 1 min. Endogenous peroxidase was inactivated by incubating in 0.3% hydrogen peroxide in methanol for 5 min. After treatment with 3% bovine serum albumin for 30 min, sections were incubated with 2.5 μg/ml of ED-2 antibody at room temperature for 60 min. Primary antibody reactions of ED-2 were enhanced using Horseradish peroxidase (HRP)EnVision (Dako, Carpinteria, CA). The immunoreaction was visualized with 0.05% 3,3-diaminobenzidine (DAB) solution. After washing in distilled water, specimens were counterstained with hematoxylin for 2 min, then mounted. To assess expression of the P-selectin adhesion molecule, anti-human P-selectin (CD-62P) rabbit polyclonal antibody (No. 553716; BD Pharamingen, San Diego, CA) was used at 120 min of reperfusion. The method of sample preparation and staining was the same as for ED-2 staining.
Leukocyte population in liver specimens

After 120 min of reperfusion, liver samples were obtained from each group, fixed with 10% formaldehyde and embedded in paraffin. Thin sections (4 μm) were prepared and stained with hematoxylin and eosin (HE). Leukocytes were counted in 5 randomly selected high-power fields (×400).

Transmission electron microscopy (TEM)

To assess endothelial cell damage after hepatic I/R, electron microscopic findings were investigated. After 120 min of reperfusion, livers were quickly resected. Tissue samples from the right hepatic lobe, not observed by IVM, were cut into 1-mm³ cubes and stored in 2.5% glutaraldehyde. Specimens were postfixed with osmium tetroxide, dehydrated in graded alcohol series and embedded in Epon mixture. Ultrathin sections were prepared using an Ultracut S microtome (Leica Aktiengesellschaft, Vienna, Austria) and picked up on copper grids. Sections were treated using uranyl acetate and lead citrate to enhance contrast. Specimens were examined using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Serum ALT level

To assess damage to the hepatic parenchyma, serum ALT levels were measured using a Drychem 7000V autoanalyzer (Fujifilm, Tokyo, Japan). A blood sample was taken from the catheter of the carotid artery before ischemia and up to 120 min after reperfusion.

Statistical analysis

All data are expressed as mean ±standard deviation (SD). The Mann-Whitney test and analysis of variance (ANOVA) were used, followed by post hoc test. Values of p<0.05 were considered statistically significant.
RESULTS

Elimination of KCs by Cl2MDP

Cl2MDP successfully eliminated KCs from liver tissue by 120 min of reperfusion (data not shown). We did not detect any side-effects of Cl2MDP administration such as liver injury or depletion of platelets in recipient rats.

Number of adherent platelets in acini

In the KC+ group (saline-treated rats), the number of adherent platelets was significantly increased at 30 min of reperfusion compared with before ischemia. The number of adherent platelets increased in proportion to the duration of reperfusion, up to 120 min (Fig. 2A, B). Conversely, in the KC- group (Cl2MDP liposome-treated rats), platelet adhesion was significantly suppressed compared with the KC+ group (Fig. 2B).

Zonal distribution of platelets and KCs

In the KC+ group, about 80% of adherent platelets were located in Zones 1 and 2 after 120 min of reperfusion (Fig. 3A). The distribution of platelets correlated with the location of KCs. Conversely, in the KC- group, the number of adherent platelets in Zones 1 and 2 was significantly decreased compared with the KC+ group (Fig. 3B).

Effects of P-selectin on adherent platelets in early-stage reperfusion

In sinusoidal endothelial cells displaying adherent platelet interactions under IVM, no P-selectin was observed after 120 min of reperfusion in either group.

Effects of KC elimination on sinusoidal vasoconstriction and no-reflow sinusoids

We were able to perform repeated observations of identical microvessels during reperfusion[17]. The diameter rate of post-sinusoidal venules (after reperfusion/before-ischemia) was not significantly different between KC+ and KC- groups. However, sinusoidal diameter rate significantly decreased during reperfusion. Conversely, without KCs, sinusoidal diameter remained the same as that before ischemia (Fig. 4). In addition, no-reflow sinusoid rate as an index of microcirculatory disturbance was significantly increased in the KC+ group compared with the KC- group after 120 min of reperfusion.
Histological findings (HE and TEM)

The number of infiltrated leukocytes was increased in sinusoids in the KC+ group after 120 min of reperfusion on histological examination. In the KC- group, infiltrated leukocytes were significantly decreased compared with the KC+ group (Fig. 5A). Areas of necrotic hepatocytes were not observed in either group.

In the KC+ group, TEM findings of sinusoids revealed endothelial cell destruction and increased numbers of swollen KCs. In the KC- group, KCs were completely eliminated and structural integrity of the sinusoidal endothelium was well maintained (Fig. 5B).

Serum ALT level

Serum ALT, reflecting hepatic parenchymal injury, was immediately increased after reperfusion, but did not differ between groups up to 30 min after reperfusion (KC-: 136.5 ± 42.0 IU/L vs. KC+: 219.4 ± 147.5 IU/L; P=0.42). After 60 min of reperfusion, serum ALT was significantly improved in the KC- group (KC-: 255.4 ± 62.9 IU/L vs. KC+: 525.6 ± 145.1 IU/L after 120 min of reperfusion; P<0.01).
DISCUSSION

Hepatic ischemia and reperfusion injury are considered to result from proinflammatory cytokines released by KCs and accumulating leukocytes [18] [19]. Some recent studies have focused on the role of platelets in hepatic I/R [10] [20]. However, the dynamics of platelets and the relationship between platelets and KCs in vivo have not previously been precisely evaluated in the early phase of hepatic I/R. This study revealed 3 main findings in post-ischemic liver microcirculation, as follows. 1) The number of adherent platelets was significantly increased immediately after reperfusion and increased in proportion to reperfusion time. 2) Constriction of sinusoids and presence of activated KCs leads to platelet adhesion, whereas the P-selectin adhesion molecule does not affect platelet adhesion in the early phase of reperfusion. 3) Adherent platelets induce leukocyte accumulation in sinusoids, and these leukocytes cause sinusoidal perfusion disturbance and liver failure.

We have previously demonstrated in the same ischemia model an increment in adherent leukocytes in sinusoids by 60 min after reperfusion [17]. In general, platelets have been observed using electron microscopy in a rat model [21], and in mouse liver using immunohistochemical methods [22]. However, evaluating the precise dynamics of platelets or leukocytes is difficult using fixed specimens. The present study demonstrated the interaction of platelets with sinusoidal endothelial cells using an IVM system for visualization and evaluation. The increment of adherent platelets occurred immediately after reperfusion, and the number of accumulated leukocytes subjected to the platelet’s adhesion. Platelets thus seem likely to precede the accumulation of leukocytes, and modulate leukocyte accumulation and hepatic dysfunction.

KCs represent the largest population of resident macrophages in the body and activated KCs release various mediators in hepatic I/R injury [19] [6]. In general, the early phase of inflammation after hepatic I/R is mainly characterized by activation of KCs, which generate reactive oxygen species and aggravate early hepatic injury [3] [4] [5]. Leukocytes accumulate in the post-ischemic liver and actively contribute to hepatocyte injury in later phases, which appear several hours after reperfusion [8]. Destruction of KCs is reportedly associated with reduced I/R injury and improved outcomes for liver transplantation [23] [24]. KCs appear to cause the production of the cytokines that result in injury following hepatic I/R. However, Sindrum et al. reported that without platelets and leukocytes, the function of KCs is decreased [10]. KC alone thus does not cause all the injury in hepatic I/R. In this study, movement of platelets occurred as
the earliest reaction among hemocytes after hepatic I/R, and modulated leukocyte accumulation. Platelets thus appear to play an important role in mediating between KCs and leukocytes in the early phase of hepatic I/R.

The mechanisms of sinusoidal platelet accumulation remain unclear [20]. In general, accumulation of platelets after I/R has been considered to be induced by adhesion molecules like P-selectin, a key mediator of leukocyte accumulation during I/R as well as KCs [25] [26]. Concomitantly, platelets also are thought to interact with stimulated endothelial cells via P-selectin[27] [28]. However, in this study, while most platelets adhered to Zones 1 and 2, P-selectin was not detected on the endothelial cells of sinusoids within 120 min after reperfusion. This suggests the specificity of hepatic endothelial cells lacking Weibel-Palade bodies containing P-selectin and von Willebrand factor[29]. For this reason, adhesion molecules cannot be expressed on hepatic endothelial cells immediately after stimulation of the liver. Conversely, we demonstrated that sinusoid diameter was significantly constricted after hepatic I/R. In addition, distribution of ED-2-positive KCs correlated with that of adherent platelets. The present results indicate that constriction of sinusoids and KCs themselves are strongly correlated with the mechanism of platelet adhesion in the early phase of I/R. Although a few studies have offered evidence that large numbers of blood cells stagnant in sinusoids neither cause injury nor affect sinusoidal perfusion[30], microcirculatory perfusion disturbances are induced by alterations in vascular diameter and potentially altered vascular responsiveness, and can lead to local blood flow imbalances.

Recently, platelets have been reported as having no direct effect on the pathogenesis of warm hepatic I/R injury, instead playing roles in tissue repair and liver proliferation[11]. In this study, significant differences were seen between groups in the number of adherent platelets within 30 min of reperfusion, but serum ALT levels did not differ significantly between groups. Serum ALT was significantly aggravated 60 min after reperfusion, and at that time the increment of accumulated leukocytes was recognized. From these results, we hypothesize that adherent platelets alone have little effect on hepatic injury, but lead to increases in adherent leukocytes, which then results in progression of hepatic injury. We thus suggest that evaluation of platelets dynamics in the hepatic microcirculation is essential for determining activation of KCs and prognosis of liver injury.

In the investigation of hepatic ischemia reperfusion, severe ischemia models using
ischemia for longer than 30 min have been used. However, with extreme duration of ischemia, hepatocytes and endothelial cells become necrotic and the vasculature becomes permanently obstructed after reperfusion of ischemic tissue, in the so-called no-reflow phenomenon[31] [32]. In addition, hepatic ischemia with longer than 30min does not restore the hepatic blood flow to the adequate level [33]. Cause of hepatic injury thus cannot be distinguished between accumulated leukocytes and accumulated platelets. For these reasons, 20 min period of hepatic ischemia model was suitable for evaluation of platelet dynamics in this study.

In conclusion, platelet-endothelium interactions occur earlier than leukocyte responses after reperfusion, and adhesion of platelets requires the presence of activated KCs. Evaluating platelet dynamics in the hepatic microcirculation appears essential for activating KCs and determining the prognosis of liver injury.
REFERENCES


Figure Legends

Figure 1: Experimental groups
Normothermic hepatic ischemia was performed for 20 min by clamping the portal triad in subject animals. Animals were divided into two groups: A) KC+ group; and B) KC- group. A total of $1 \times 10^8$ fluorescence-labeled platelets, representing approximately 1% of all circulating platelets in the recipient rat, were injected intravenously before 5 min of IVM.

Figure 2: Platelet dynamics
A) Video images of adherent platelets in acini 120 min after reperfusion. Field is approximately 0.2 mm$^2$. No thrombus is present.
B) In the KC+ group, the number of adherent platelets was significantly increased at 30 min after I/R and with duration of reperfusion. Conversely, in the KC- group, the number of adherent platelets was significantly decreased compared with the KC+ group. $^5$p<0.05 vs. before-ischemia; $^*$p<0.05 vs. KC- group (mean ±SD; n=6).

Figure 3: Distribution of adherent platelets and KCs
A) In the KC+ group, about 80% of adherent platelets are located in Zones 1 and 2 after 120 min of reperfusion (mean±SD, n=6). Distribution of platelets correlated with location of KCs.
B) In the KC- group, the number of adherent platelets in Zones 1 and 2 was significantly decreased (*p<0.05, mean ±SD; n=6).

Figure 4: Alteration of sinusoidal diameter
In the KC+ group, sinusoidal diameter decreased by about 20-30% during the reperfusion period. Conversely, without KCs, sinusoidal diameter remained the same as before ischemia. *p<0.05, **p<0.01 vs. KC+ group (mean ±SD, n=6).

Figure 5: Histological findings
A) The number of accumulated leukocytes was increased in sinusoids in the KC+ group after 120 min of reperfusion on histological examination. Conversely, in the KC- group, accumulated leukocytes were significantly decreased. p<0.05 KC+ vs. KC- group (mean ±SD, n=6).
B) In the KC+ group, TEM of the sinusoid revealed endothelial cell destruction and increased numbers of swollen KCs. In the KC- group, KCs were completely absent and structural integrity of the sinusoidal endothelium was well maintained.