

Platelet Dynamics in the Sinusoid of the Liver after Ischemia-Reperfusion

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Received: May 3, 2012

Revised: June 18, 2012

Accepted: June 29, 2012

Published online: August 21, 2012

Tamura T, Kondo T, Fukunaga K, Ohkohchi N. Platelet Dynamics in the Sinusoid of the Liver after Ischemia-Reperfusion. *Journal of Gastroenterology and Hepatology Research* 2012; 1(7): 114-118
Available from: URL: <http://www.ghrnet.org/index./joghr/>

INTRODUCTION

Clamping of the portal triad, i.e., the Pringle maneuver, is an often carried out in liver surgery and the influences of the interruption of hepatic blood flow have been widely reported^[1,2]. However a prolonged clamping time may result in warm ischemic injury, which may complicate the postoperative course^[2]. It has been recognized that the hepatic microcirculation represents a target of ischemia-reperfusion (I/R) induced hepatic injury^[3]. The hepatic microcirculation in I/R is strongly associated with deterioration of components of sinusoid, i.e., sinusoidal endothelial cell (SEC), Kupffer cells (KCs), neutrophils and platelets. It was reported that there are two distinct period of liver injury after warm I/R^[4-7]. The first period of inflammation is characterized by activation of KCs, which generate reactive oxygen species and aggravate the injury occurring up to 120 min after reperfusion^[8-10]. In the second period of I/R, neutrophils accumulate in the post-ischemic liver and induce to hepatocyte injury, which appears longer than 120 min after reperfusion^[6,11].

Clavien demonstrated the target of I/R injury is the SEC^[12]. SEC death occurs rapidly upon oxygenated reperfusion, and the extent of cell death has been shown to be a critical factor in various animal models^[13,14]. Platelets induce SEC apoptosis and significantly contribute to I/R injury^[15]. Furthermore, SEC-platelet interaction contributes to disturbance of the microcirculation^[16]. Few studies have examined the hepatic sinusoid focusing on the period within 120 min after reperfusion. We reported that the adherent neutrophils did not significantly increase immediately after reperfusion, and a remarkable increment of adherent neutrophils was observed 120 min after reperfusion^[17]. Recently, some studies have focused on the role of platelets in earlier period of hepatic I/R^[18,19]. We have provided evidence that in the early period of I/R, platelets adhere to KCs, thereby leading to neutrophils accumulation, disturbances in sinusoidal perfusion, and ultimately liver failure^[18]. Activated platelets produce proinflammatory mediators, i.e., chemokines and cytokines^[16,20]. The platelets likely act in synergy with neutrophils and KCs^[21]. In this

ABSTRACT

Hepatic ischemia-reperfusion (I/R) activates Kupffer cells (KCs), which produce various inflammatory cytokines, causing adhesion of neutrophils to sinusoidal endothelium and microcirculatory disturbance. These phenomena are well known as mechanisms of hepatic I/R injury. Recently, in the early period of I/R, i.e., within 120 min after reperfusion, it was proved that accumulation of platelets to sinusoid is strongly associated with mechanism of the I/R. In this editorial, we describe the mechanism of I/R injury focusing on platelets and KCs in the early period of I/R. We successfully observed the dynamics of platelets and KCs in the hepatic sinusoid time dependently using an intravital microscopy (IVM) system in rat. Platelets were isolated from the whole blood of syngeneic rats and labeled with rhodamine-6G. KCs were labeled using the *liposome entrapment method*. The observations revealed that platelets attached to KCs and sinusoidal endothelial cells (SECs) during the early period of I/R. The number of platelets adhering to both KCs and SECs increased in proportion to the duration of reperfusion. These interactions may be causally related to the downstream effects of I/R and are important to characterize. The existence of platelets as well as KCs is indispensable for the I/R injury.

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Key words: Platelet; Kupffer cell; Hepatic ischemia reperfusion; Intravital microscopy

editorial, we focused on the role of platelets and KCs in the early period of I/R, i.e., within 120 min after reperfusion.

METHOD OF OBSERVING THE MICROCIRCULATION OF THE LIVER AFTER ISCHEMIA-REPERFUSION

Intravital microscopy (IVM) system

Evaluation of platelets and KCs dynamics in the hepatic microcirculation is essential to elucidate the mechanisms of I/R injury^[18]. In general, the existence of platelets and KCs in the hepatic sinusoid has been observed by electron microscopy and immunohistochemical methods in a rodent model^[19,22], because evaluating the precise dynamics of platelets and KCs in the hepatic sinusoids in real time was difficult. There has been some research on attempting to observe the liver sinusoid by IVM^[23-40]. IVM demonstrated that microcirculatory derangement is induced during liver I/R. However, in the conventional method, these data were not based on continuous observations prior to and after induction of ischemia in each animal. In addition, it was very difficult for observation to be performed in identical fields. Therefore, these data were obtained from randomly selected microvessels at each point of observation. Repeated observation of the identical microvessels has not been performed yet. We developed the method in which we can observe identical microvessels before and after the induction of ischemia^[41]. Using a specially designed cover glass with the grid, it was possible that identical acini and venules could be observed long period and allow for determination on duration of hepatic microvascular perfusion. The IVM system may be very effective for investigating the movement of platelets and KCs as well as neutrophils in various kinds of pathophysiology, such as I/R. Using this IVM system, we demonstrated the interaction of platelets with KCs and SECs (Figure 1).

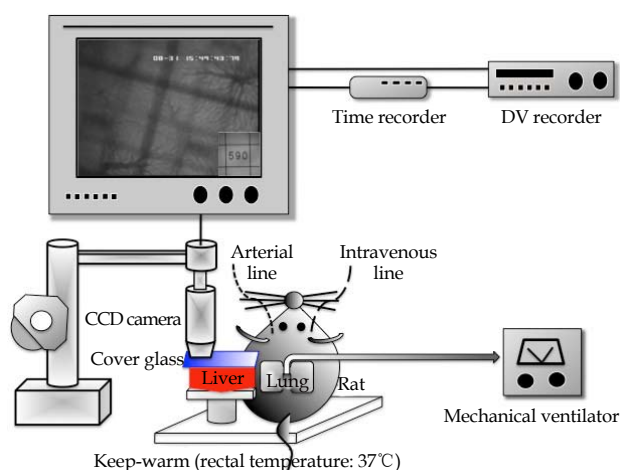


Figure 1 Intravital microscopy (IVM) system. IVM was performed using a modified microscope (BX30 FLA-SP; Olympus, Tokyo, Japan) with a 100-Wmercury lamp attached to a filter block. The hepatic microcirculation was recorded with a CCD camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) and a digital video recorder (GV-D1000 NTSC; Sony, Tokyo, Japan) for offline analysis. Using objective lenses (10×0.3 to 20×0.7 ; Olympus, Tokyo, Japan), a final magnification of $\times 325$ to $\times 650$ was achieved on the video screen. To assess sinusoidal perfusion, sodium fluorescein (2×10^{-3} M/kg, F-6377; Sigma, St. Louis, MO) was injected via the jugular catheter. Rhodamine-6G labeling platelets were infused intraarterially just before ischemia and at 30, 60, and 120 min after reperfusion, and 10 randomly chosen acini were visualized. Quantitative assessment of the microcirculatory parameters was performed offline using WinROOF imaging software (version 5.0; Mitani Co, Tokyo, Japan).

We already clarified the appropriate ischemic time in the experiment

of IVM for 20 min^[41,42]. It was observed that there was a gradual increment of neutrophils adhesion along with an attenuation of the initially severely depressed sinusoidal reperfusion during the 120 min of reperfusion^[41,42]. Therefore, we selected total warm liver ischemia as the Pringle maneuver with the 20 min ischemia time.

Fluorescence Labeling of Kupffer cell and Platelet

As drug carrier and delivery system, liposome has been significantly paid much attention in biological and medical study field^[43,44]. There are various methods for preparing liposomes to label the KCs^[43-45]. In this study, we applied a method reported by Watanabe *et al.*^[45]. Labeled phosphatidylcholine (PC) with fluorescence was incorporated into liposomes. The fluorescent pigment used was 2-[12-(7-nitrobenz-2-oxa 1,3-diazol-4-yl) amino] dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD-C12-HPC; Molecular Probes, Eugene, OR). In the preliminary study, after injection of the liposome through the carotid artery catheter, KCs in the rat liver were stained and clearly delineated in the fluorescent IVM image (Figure 2A). No detrimental influence of the liposomes on the phagocytic activity was observed by measurement of the amount of hepatic uptake of intravenously administered fluorescent microspheres^[45]. Additionally, no histopathological changes were found in the livers of liposome-administrated rats^[45]. Sixty min before induction of hepatic ischemia, liposome encapsulated fluorescent liposomes (4 mL/kg) were administered via the carotid artery catheter.

Platelets were isolated from the whole blood of syngeneic rats and labeled with rhodamine-6G (50 μ L/mL whole blood: R-4127; Sigma, St. Louis, MO), as described by Massberg *et al.*^[46]. Briefly, the collected blood was diluted with buffer after the addition of prostaglandin E1 and rhodamine-6G. After 2 cycles of centrifugation, fluorescent platelets were resuspended in PBS. Rhodamine-6G-labeled platelets were infused intraarterially just before ischemia and at 30, 60, and 120 min after reperfusion. The labeled platelets were clearly recognized by the fluorescent IVM image (Figure 2B).

By using these methods, we succeeded in observing the dynamics of platelets and KCs in the hepatic sinusoids on a real-time^[47].

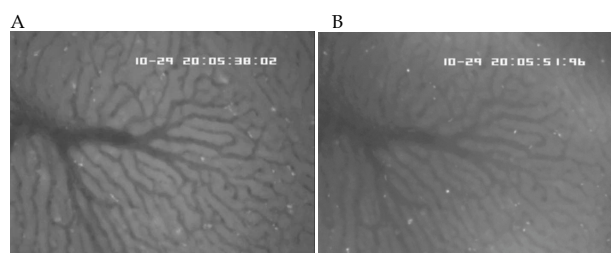


Figure 2 Video images. A: Kupffer cells (KCs); B: platelets in acini 30 min after reperfusion. The field is approximately 0.2 mm^2 (A, B).

EVALUATION OF PLATELETS IN THE HEPATIC SINUSOID AFTER ISCHEMIA-REPERFUSION

Platelets dynamics after hepatic ischemia-reperfusion

We reported that platelet-endothelium interactions occur earlier than leukocyte responses after I/R and that adhesion of platelets to SECs requires the presence of KCs^[18]. From the results of the previous study, we supposed that the interaction between platelets and KCs is indispensable for the I/R injury within 120 min after reperfusion.

After hepatic I/R, the number of adherent platelets to KCs and SECs significantly increased along with the reperfusion time (Figure 3). Indeed, the number of adherent platelets was significantly increased at 30 min after reperfusion compared with that before ischemia. On

the other hand, in the rats without ischemic procedure, the number of adherent platelets remained within twice which was significantly lower than with I/R procedure. In both groups, more than 50% of the platelets adhered to KCs by IVM.

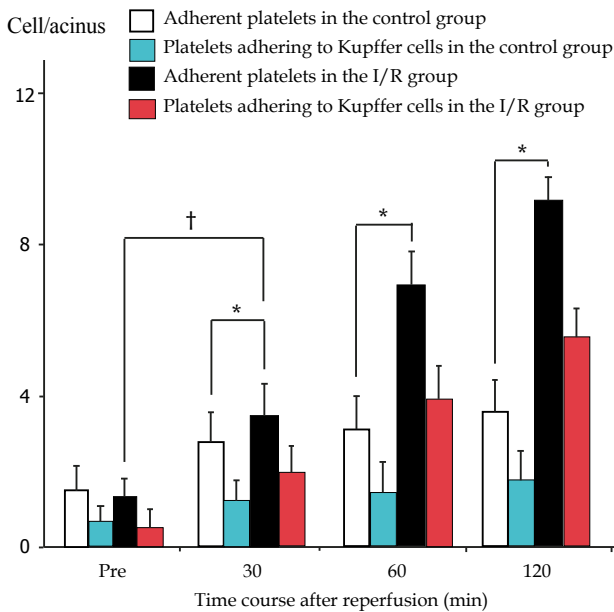


Figure 3 Platelet dynamics in sinusoids. In the I/R group, the number of adherent platelets was significantly more increased than in the control group at 30 min after reperfusion and concomitant with the duration of reperfusion. The control group (; no ischemia group: n=6), the 20-min ischemia group (; I/R group: n=6). Data are shown as mean±SD. There were six animals in each groups. *P<0.05 versus Control groups. †P<0.05 versus before ischemia. From Tamura T *et al*^[47].

In the electron microscopical findings, the platelets were adherent to the endothelial cells of the hepatic sinusoids, and they were adherent to the KCs in some parts (Figure 4). Platelets were adherent to KCs and SECs in the early period of I/R.

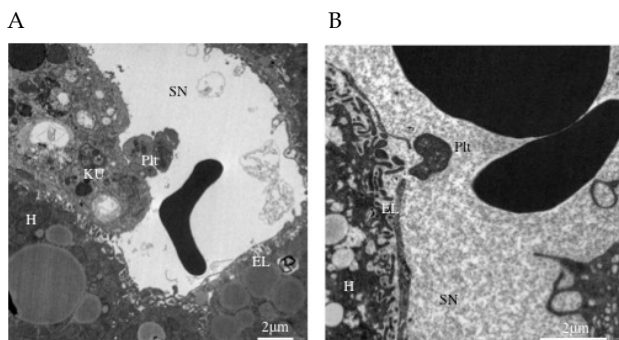


Figure 4 Transmission electron microscopy findings after 30 min of I/R in the I/R group. A: The platelets are directly adhered to the KCs; B: The platelets are directly adhered to the sinusoidal endothelium. From Tamura T *et al*^[47]. Plt: platelet; KCu: kupffer cell; EL: endothelial cell; SN: sinusoid; H: hepatocyte.

The role of platelets in hepatic I/R was evaluated using rabbit anti-rat platelet serum (APS) for deletion of platelets^[48,49]. In APS-treated rats, hepatic dysfunction of I/R, i.e., serum ALT and apoptosis of hepatocytes, was significantly ameliorated^[47]. Therefore, hepatic I/R process requires the presence of platelets.

Platelets dynamics after ischemia-reperfusion under condition of eliminating Kupffer cells

KCs represent the largest population of resident macrophages in the body and activated KCs release various kinds of mediators in hepatic I/R injury^[50]. Deletion of KCs is previously reported that it was associated with reduced I/R injury and improved outcomes for liver transplantation^[51,52].

We examined the role of KCs in hepatic I/R under the condition of eliminating KCs using dichloromethylene disphosphonate (Cl2MDP) liposome. In non-treated rats (KC existing group), the number of adherent platelets was significantly increased at 30 min of reperfusion compared with before ischemia and it increased in proportion to the duration of reperfusion up to 120 min (Figure 5). In Cl2MDP-treated rats (KC deletion group), platelet adhesion was significantly suppressed compared with the KC existing group.

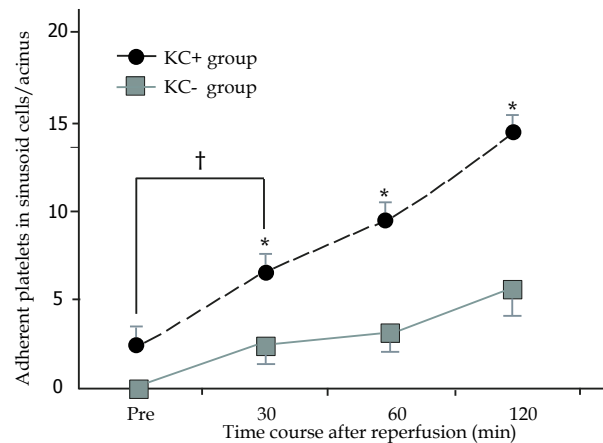


Figure 5 In the KC+ group (KC existing group), the number of adherent platelets was significantly increased at 30 min after I/R and with duration of reperfusion. *P<0.05 versus KC- group (KC deletion group), †P<0.05 versus before ischemia. (mean±SD; n=6). From Nakano Y *et al*^[18].

In the KC existing group, about 80% of adherent platelets were located in zones 1 and 2 after 120 min of reperfusion (Figure 6A). The distribution of platelets correlated with the location of KCs. Conversely, in the KC deletion group, the number of adherent platelets in zones 1 and 2 was significantly decreased compared with the KC existing group (Figure 6B).

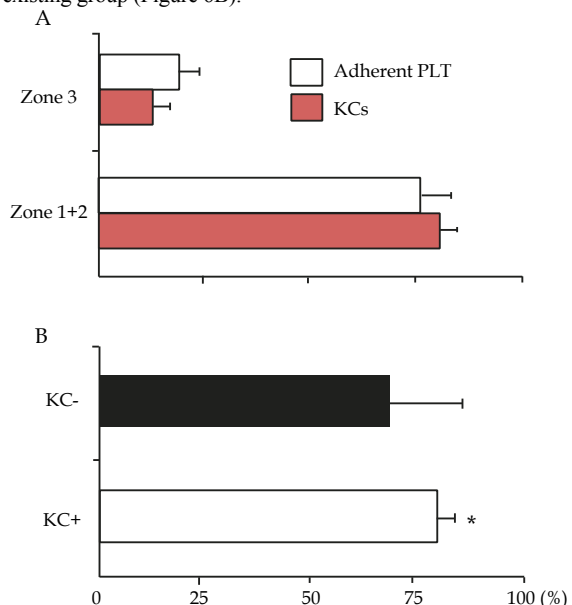


Figure 6 Distribution of adherent platelets and KCs. A: In the KC+ group (KC existing group), about 80% of adherent platelets are located in zones 1 and 2

and 2 after 120 min of reperfusion (mean±SD, n=6). Distribution of platelets correlated with location of KCs; B: In the KC- group (KC deletion group), the number of adherent platelets in zones 1 and 2 was significantly decreased (*P < 0.05, mean±SD, n=6). From Nakano Y *et al.*^[18].

In the KC deletion group, hepatic dysfunction of I/R, i.e., serum ALT and endothelial cell destruction, was ameliorated^[18]. We previously reported that half of the adherent platelets located were attached to KCs, and these were abundant in hepatic zone 1^[47]. The I/R induced the adhesion of platelets to KCs, and this interaction plays a key role during the early period of I/R. Therefore, hepatic I/R process also requires the presence of KCs.

CONCLUSION

We investigated platelets dynamics relating with KCs in liver sinusoid after I/R by using the IVM system. The platelets were directly adherent to the KCs in the hepatic sinusoids. Considering the time course of I/R, hepatic I/R injury might be triggered by platelets adhesion to KCs. We propose the concept that the interaction of platelets and KCs is meaningful for hepatic I/R injury in the early period (Figure 7). It is necessary to decrease interaction between platelets and KCs for treatment of I/R injury.

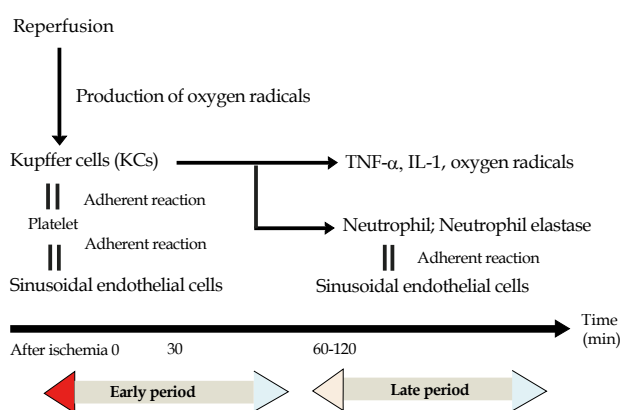


Figure 7 A concept of ischemia-reperfusion. Kupffer cells and platelets-dependent injury of I/R.

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