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Interaction between Kupffer cells and platelets in the early period of hepatic ischemia–reperfusion injury—An *in vivo* study

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ARTICLE INFO

Article history:
Received 10 August 2011
Received in revised form
25 November 2011
Accepted 6 December 2011
Available online 13 March 2012

Keywords:
Intravital microscopy (IVM)
Hepatic ischemia reperfusion
Kupffer cell
Electron microscope
Adherent platelets

ABSTRACT

Background: Hepatic ischemia–reperfusion (I/R) leads to activation of Kupffer cells (KCs). The activated KCs cause platelet and leukocyte adhesion to the sinusoidal endothelium. Previously, we reported that platelet–endothelium interactions occur earlier than leukocyte responses. The aim of this study was to evaluate the interaction between platelets and KCs in the hepatic microcirculation after I/R.

Materials and methods: Sprague-Dawley rats were divided into three groups: the no-ischemia group (control group; $n = 6$); the 20-min ischemia group (I/R group; $n = 6$); and the 20-min ischemia + anti-rat platelet serum group (APS group; $n = 6$). KCs were labeled using the liposome entrapment method. The number of adherent platelets was observed for up to 120 min after reperfusion by intravital microscopy. To investigate the effects of platelets on I/R injury, rats were injected intravenously with rabbit APS for platelet depletion.

Results: In the I/R group, the number of adherent platelets increased significantly after I/R. More than 50% of the adherent platelets adhered to KCs. Electron microscopy indicated that the platelets attached to the KCs after hepatic ischemia. The histologic findings indicated liver damage and apoptosis of hepatocytes in zone 1. In the I/R group, but not in the control and APS groups, serum ALT increased immediately after reperfusion.

Conclusions: We succeeded in visualizing the dynamics of both KCs and platelets in the hepatic sinusoids. Liver ischemia induced the adhesion of platelets to KCs in the early period, which could play a key role in reperfusion injury of the liver.

1. Introduction

Clamping of the portal triad (i.e., the Pringle maneuver) is a standard procedure in hepatic resection, and the effects of the interruption of hepatic blood flow have been widely reported [1,2]. However, if the duration of the Pringle maneuver is prolonged, complications induced by warm ischemia sometimes occur postoperatively [3]. At the time of ischemia–reperfusion (I/R), the hepatic microcirculation is the main target of hepatic injury [4]. The early period of

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doi:10.1016/j.jss.2011.12.010

inflammation after hepatic I/R is mainly characterized by activation of Kupffer cells (KCs), which generate reactive oxygen species and aggravate early hepatic injury [5–7].

Platelet–endothelial cell interaction contributes to I/R injury of the liver, as well as to liver regeneration [8–10]. Recently, some studies have focused on the role of platelets in hepatic I/R [11]. Activated platelets produce proinflammatory mediators (i.e., chemokines and cytokines [8,12]). The platelets likely act in synergy with leukocytes and KCs [13]. We reported that platelet–endothelium interactions occur earlier than leukocyte responses after I/R and that adhesion of platelets requires the presence of activated KCs [14]. In Kupffer cell-depleted rats, the structure of the sinusoidal endothelium was well maintained after I/R, and I/R injury was suppressed [14]. From the results of our previous study, we hypothesized that the interaction between platelets and KCs is indispensable for the early period of I/R injury. Owing to the difficulty of labeling KCs, until now *in vivo* visualization of the KCs in the sinusoids has not been possible. Recently, however, fluorescein labeling of KCs was enabled by the liposome entrapment method [15]. In this study, we focused on the role of platelets in the early period of I/R and observed KCs in the liver *in vivo* using this method. Our aim was to evaluate the interaction between platelets and KCs in the liver hepatic microcirculation after the early period of I/R with intravital microscopy (IVM).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 250 to 300 g, were obtained from CLEA Japan (CLEA Corporation, 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 Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

2.2. Experimental groups

Total warm hepatic ischemia was induced for 20 min by clamping the portal triad. The rats were divided into three groups: (1) no-ischemia group (control group; $n = 6$); (1) 20-min ischemia group (I/R group; $n = 6$); and (3) 20-min ischemia + anti-rat platelet serum group (APS group; $n = 6$). Platelet dynamics and hepatic microcirculation were observed just before ischemia and at 30, 60, and 120 min after reperfusion. To investigate the effects of platelets on I/R, the rats were injected intravenously with rabbit anti-rat platelet serum (APS; Inter-Cell Technologies, Hopewell, NJ) 24 h before induction of ischemia [16,17]. The APS group underwent the same procedure as the I/R group (Fig. 1).

2.3. Surgical procedure

After induction of anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), the animals were tracheotomized. To reduce spontaneous breathing, the animals were ventilated mechanically (KN-55; Natsume Seisakusho, Tokyo, Japan). When spontaneous breathing interfered with mechanical ventilation, additional pentobarbital (10 mg/kg) was administered intravenously. The animals were placed in a supine position on a heated pad to maintain a rectal temperature of 37°C. To monitor arterial blood pressure and allow continuous infusion of saline, polyethylene catheters (PE-50, 0.58/0.96-mm internal/external diameter; Becton Dickinson, Sparks, MD) were inserted into the left carotid artery and left jugular vein. After transverse laparotomy, the ligaments around the liver were dissected to mobilize the left lobe. At the same time, the hepatoduodenal ligament was taped in preparation for clamping later. The left lobe was exteriorized on a plate specially designed to minimize movement caused by respiration, and covered with a cover glass. Surgical procedures were performed under sterile conditions. After 60 min of continuous physiologic saline infusion, baseline IVM was performed. Hepatic ischemia was then induced by portal triad clamping (i.e., clamping of the hepatic artery, portal vein, and bile duct) by means of a microclip (Aesculap, Tuttlingen, Germany) for 20 min. IVM was performed at 30, 60, and 120 min after reperfusion. Blood samples were taken for the analysis of enzyme activities in serum at the same times as IVM. At the end of the experiments, liver tissue was taken for histologic examination. Finally, the experimental animal was euthanized by total blood collection *via* a catheter.

2.4. Liposome entrapment method (Fluorescence labeling of KCs)

There are various methods for preparing liposomes [18,19]. In this study, we applied the method of Watanabe *et al.* [15]. Fluorescently labeled phosphatidylcholine (PC) was incorporated into liposomes consisting of PC. The fluorescent pigment used was 2-(12-[7-nitrobenz-2-oxa 1,3-diazol-4-yl] amino) dodecanoyl-1-hexadecanoly-*sn*-glycero-3-phosphocholine (NBD-C_{12}-HPC; Molecular Probes, Eugene, OR). After intra-arterial injection, KCs in the rat liver were stained and were clearly delineated in the fluorescent IVM image. The phagocytic activity of KCs after the administration of liposomes was determined by measurement of the amount of hepatic uptake of intravenously administered fluorescent microspheres. No detrimental influence of the liposomes on the phagocytic activity was observed. Additionally, no histopathologic changes were found in the livers of liposome-treated rats [15]. Sixty min before induction of hepatic ischemia, liposome-encapsulated fluorescent liposomes (4 mL/kg) were administered *via* the carotid artery catheter.

2.5. Platelet preparation

Platelets were isolated from the whole blood of syngeneic rats and labeled with rhodamine-6G (50 μL/mL whole blood: R-4127; Sigma, St. Louis, MO), as described by Massberg *et al.* [20]. Briefly, the collected blood was diluted with buffer after the