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Bile endothelin-1 as a tool for diagnosing ischemia-reperfusion injury

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ABSTRACT

We investigated whether the extent of graft ischemia reperfusion (I/R) injury can be evaluated by assessing the level of bile and serum endothelin-1 (ET-1). In groups 1 to 3 (n=5 in each group), the livers of male Wistar rats were surgically isolated from the surrounding attachments and then perfused in-situ with heparinized cold saline (4℃) under a port-systemic shunt. The livers were then reperfused after a pre-determined cold ischemia time (0, 30, and 60 min in groups 1, 2, and 3, respectively). The ET-1 levels in either the bile or the serum obtained from the suprahepatic vena cava (SHVC) and abdominal aorta, were assessed from -1 hour to +72 hours after reperfusion in each group. In group 3, the ET-1 level at the SHVC increased to 19.9±2.88 and 19.43±3.14 pg/ml at 1.5 and 2 hr after reperfusion, respectively, which were significantly higher than those of group 1 (13.6±2.73, 13.05±0.58, $p<0.05$) and 2 (11.67±2.21, 12.2±2.22, $p<0.05$). The bile ET-1 concentration in group 3 decreased at 1 hr after reperfusion (14.22±6.26 pg/ml), but increased in group 1 (108.1±49.3). The bile ET-1 concentration did not recover to its pre-ischemic level until 72 hr after reperfusion in group 3, but showed no apparent decrease throughout the experiment in groups 1 and 2. In immunohistochemistry, ET-1 expression on the central and portal vein was stronger in group 3 than in group 1, whereas the expression on the bile duct was weaker in group 3 than that in group 1. In conclusion, ET-1 was directly released from the damaged endothelial cells into the SHVC depending on the extent of the I/R injury, whereas the ET-1 excretion into the bile was inversely suppressed depending on the amount of I/R injury. These changes of ET-1 excretion might thus be useful in diagnosing hepatic I/R injury. *Ryukyu Med. J., 20(1)1~6, 2001*

Key words: ischemia-reperfusion injury, endothelin-1, liver

INTRODUCTION

An accurate evaluation of graft ischemia reperfusion (I/R) injury is important in the postoperative management of liver transplantation. I/R injury is an important factor which can dominate the early postoperative prognosis and is considered to be a cause of early graft failure which is called primary non function[1,2]. The prompt and accurate evaluation of the graft liver immediately after reperfusion should thus help improve the patient survival in liver transplantation. Endothelin, which was first reported by Yanagisawa, is a potent vasoconstrictor peptide produced by vascular endothelial cells[3], and is considered to play a major role in ischemia reperfusion (I/R) injury[4,5]. Three isoforms of this peptide, endothelin-1 (ET-1), endothelin-2 and endothelin-3 have been described.[6] ET-1 is known to be secreted from the sinusoidal lining cells of the liver and is excreted through the bile tract in large amounts[7,8]. Since bile, a direct product of the graft liver, is produced immediately after graft reperfusion, it might thus be reasonable to use a bile analysis for evaluating graft I/R injury. In fact, we have previously reported that an increased excretion of bile ET-1 was useful in diagnosing the development of acute rejection[9]. Regarding the physiological status, the lung and kidney also play roles in clearing circulating ET[10,11,12], with the lung removing more than 50 % of the circulating ET in a single passage[10]. These patterns of excreting ET, however, become significantly altered in various pathologic conditions. For example, the plasma ET-1 concentrations markedly increase in patients with liver failure and hepatorenal syndrome, due to the reduced clearance capacity of the liver[13]. In this context, the alteration of these patterns in ET excretion might be useful in diagnosing a variety of pathologic statuses in which the liver, lung and kidney are involved. In the present study, we thus evaluated whether hepatic I/R injury also alters the pattern in ET-1 excretion.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, weighing 250 to 350 g, were used for the experiment. Four weeks prior to the experiment, the rat's spleen was translocated into a left subcostal subcutis to create a porto-systemic shunt. This shunt allows for a reduction of intestinal congestion during the ischemic period[14, 15]. The rats were fasted for 12 hr prior to the experiment with free access to water. All procedures were performed under sterile conditions.

Surgical Procedure

The rats were anesthetized with ether and laparotomized after injection of heparin sodium (50 IU/animal). The liver was then anatomically skeletonized and total hepatic ischemia was induced by clamping the hepatic artery, portal vein, suprahepatic vena cava (SHVC) and infrahepatic vena cava (IHVC). Immediately after establishing the total hepatic ischemia, the portal vein and IHVC were cannulated with polyethylene tube (PE-10: ID 0.28mm, OD 0.61mm, Imamura, Tokyo, Japan) and the liver was perfused through the portal vein with 20ml of heparinized cold saline (2.5IU/ml) in order to wash out all the blood from the liver. The tubes were removed after perfusion and 2 to 3 sutures with 8-0 monofilament suture were applied on each vessel to repair the tube insertion hole. The suprahepatic and infrahepatic vena cava were declamped immediately after repairing these vessels, without reperfusing the liver. All of these procedures were performed within 15 min. After 0, 30 and 60 min (groups 1, 2 and 3, respectively, n=5 each) of total hepatic ischemia, the reperfusion of the ischemic liver was achieved by removing the clamps on both the portal vein and hepatic artery. Group 1 received a sham operation which included the skeletonization of the liver from the surrounding tissue.

Sample Analysis

Five rats were killed from each group to obtain samples of bile, liver and serum at −1, 1, 2, 4, 6, 12, 24, 48 and 72 hr after reperfusion. The bile was collected by cannulating a PE-10 tube into bile duct, starting 1 hr prior to killing. The serum was also collected at 1.5 hr after reperfusion. The serum was obtained from the SHVC and abdominal aorta. The ET-1 levels in either the bile or the serum obtained from the SHVC and abdominal aorta, were determined from −1 to +72 hr after reperfusion in each group. Bile was collected for 1 hr and the flow rate was expressed as ml/hr/100g rat body weight. The ET-1 output in the bile was calculated based on the bile flow rate and the ET-1 concentration (bile flow rate (ml/hr/100g. animal) ×ET-1 concentration (pg/ml)). The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were also measured in each group to evaluate the liver function. Liver samples were either snap frozen for immunohistochemical staining or fixed in 10% formalin for routine hematoxylin-eosin (H-E) staining.

Radioimmunoassay for ET-1

Blood samples from the SHVC and aorta were used to determine the ET-1 level. These blood samples were centrifuged at 2,500 revolution per minute for 15 min at 4 ℃ and were stored at −80 ℃ until the ET-1 assay. Bile samples were also stored at −80 ℃ until determination. The serum and the bile ET-1 levels were determined by a highly sensitive and specific radioimmunoassay according to the method of Ando *et al*[16]. In brief, each 0.1 ml sample or standard was incubated with 0.1 ml assay buffer and 0.1 ml rabbit anti-hET-antiserum (obtained from Sumitomo Bio-Science, Osaka, Japan). Incubation was carried out at 4 ℃ for 24 hr, followed later by the addition of 0.05 ml ^{125}I-hET (Amersham, Buckinghamshire, UK) and further incubation at 4 ℃ for 48 hr. The bound and free radioactivity was separated by the second antibody method, and then samples were centrifuged at 3000 rpm under 40 ℃ for 30 min. The radioactivity in the precipitate was counted using a gamma counter.

Immunohistochemical Staining

Immunohistochemistry was performed on tissue samples obtained from sacrificed animals. Briefly, 5mm-thich frozen sections in the optimum cutting temperature (OCT) compound were fixed in acetone, followed by a reaction with the rabbit anti-ET-1 primary antibody (FUNAKOSHI, Tokyo, Japan) and the peroxidase-conjugated anti-rabbit-IgG secondary antibody (Amersham, Buckinghamshire, UK). The slides were further developed with diaminobenzidine (DAB) solution, and counter-stained with methyl green. Negative controls were stained using the same procedure, except for the incubation with anti-ET-1 primary antibody.

Statistical Analysis

All data were expressed as the mean±standard error (SE) of the mean value. A statistical analysis was performed by an analysis of variance (ANOVA) among the obtained values from groups 1 to 3. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Liver function test

In group 3, the serum AST levels increased immediately after reperfusion and peeked at 6 hr after reperfusion (2793±533.9 IU/L), followed by a steep decrease and reaching a normal level (107.2±15.47) at 72 hr after reperfusion. The AST level was significantly higher in group 3 than in group 1 at 2 (1773.3±431.66, 124±26.87), 4 (2597.9 ±910.69, 405.2±25.19)), and 6 hr (2793±533.9, 389.2± 36.09) after reperfusion ($p<0.05$), and also higher in group 3 than in group 2 at 4 (496.5±41.31), and 6 hr (1399± 905.93) ($p<0.05$, Fig. 1). The serum levels of ALT also

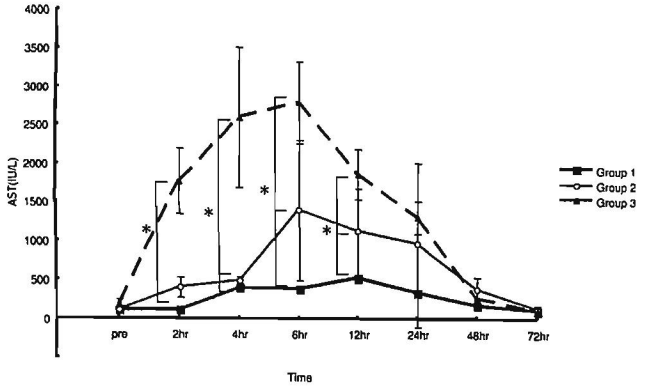

Fig. 1 The serum AST levels after I/R injury (mean±SE). The AST level was significantly higher in group 3 than in group 1 at 2 and 6 hr after reperfusion, and also higher in group 3 than in group 2 at 4 and 6 hr. *P<0.05 (ANOVA)

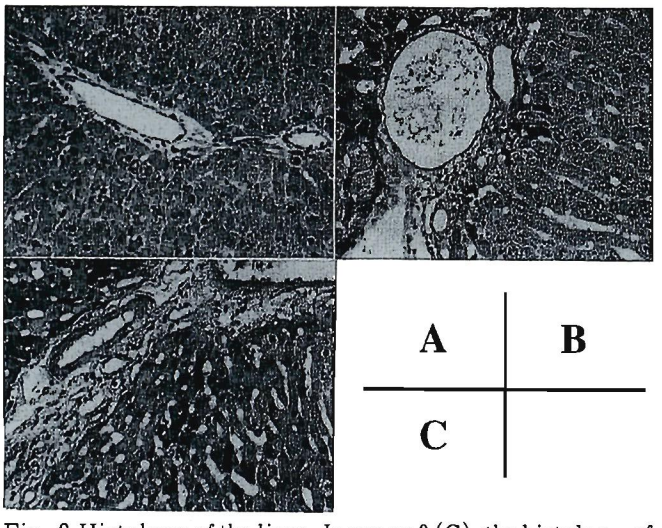

Fig. 2 Histology of the liver. In group 3 (C), the histology of the liver showed neutrophil infiltration, condensation of the cytoplasm of the hepatocytes, pycnosis of the nuclei and a disruption of the hepatic trabeculae. Some hepatocytes were necrotic and fell into karyorrhexis. In group 2 (B), both mild neutrophil infiltration and mild degeneration of hepatocytes were seen. In group 1 (A), the architecture of the hepatic trabeculae were well preserved and the hepatocytes showed a normal morphology.

showed changes similar to those seen in the AST level. The serum ALT level in group 3 peeked at 6 hr (1675±320.34 IU/L) after reperfusion and then recovered to a normal level at 72 hr (116.6±38.79).

Histology of the liver

In group 3, the histology of the liver showed neutrophil infiltration, condensation of the cytoplasm in the hepatocytes, pycnosis of the nuclei and a disruption of the hepatic trabeculae. Some hepatocytes were necrotic and fell into karyorrhexis. In group 2, mild neutrophil infiltration and mild degeneration of hepatocytes were seen. However, no hepatocyte necrosis or karyorrhexis was seen. In group 1,

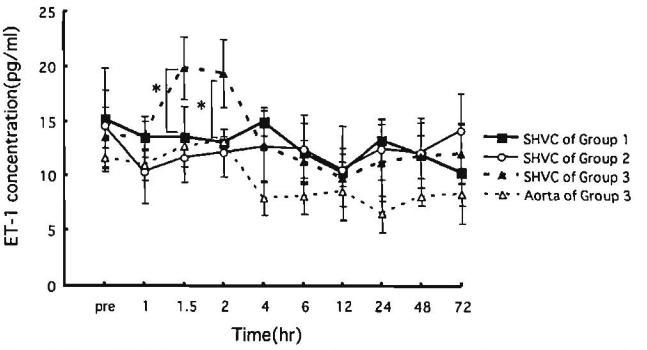

Fig. 3 The ET-1 levels in serum obtained from the suprahepatic vena cava and abdominal aorta after I/R injury (mean ±SE). In group 3, the ET-1 level in the SHVC increased at 1.5 and 2 hr after reperfusion, which were significantly higher than group 1 and 2. The ET-1 levels in the aorta were always lower than those in the SHVC throughout the experiment without any differences between the groups. *P<0.05 (ANOVA)

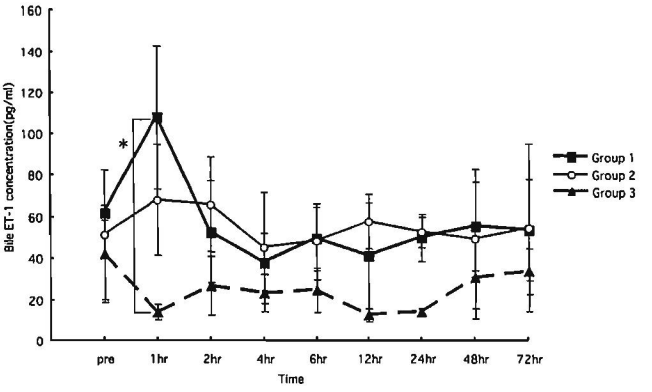

Fig. 4 Bile ET-1 concentration in group 3 decreased at 1 hr after reperfusion compared to the pre-ischemic level, and returned to its pre-ischemic level at 72 hr. In contrast, ET-1 concentration in group 1 temporarilly increased at 1 hr after sham (mean±SE). *P<0.05 (ANOVA)

the architecture of the hepatic trabeculae were well preserved and the hepatocytes showed a normal morphology. In addition, no endotheliitis in the portal vein was seen in any groups (Fig. 2).

Bile and serum ET-1

In group 3, the ET-1 level in the SHVC increased to 19.9±2.88 and 19.43±3.14 pg/ml at 1.5 and 2 hr after reperfusion, respectively, which were significantly higher than those of group 1 (13.6±2.73, 13.05±0.58, $p<0.05$) and 2 (11.67±2.21, 12.2±2.22, $p<0.05$). The ET-1 level returned to its pre-ischemic level at 4 hr after reperfusion in group 3. The ET-1 levels in the aorta were always lower than those in the SHVC throughout the experiment without any differences between the groups, reflecting an absorption of ET-1 by the lung (Fig. 3).

Bile ET-1 concentration in group 3 decreased at 1 hr after reperfusion compared to the pre-ischemic level, and returned to its pre-ischemic level at 72 hr (Fig. 4). In

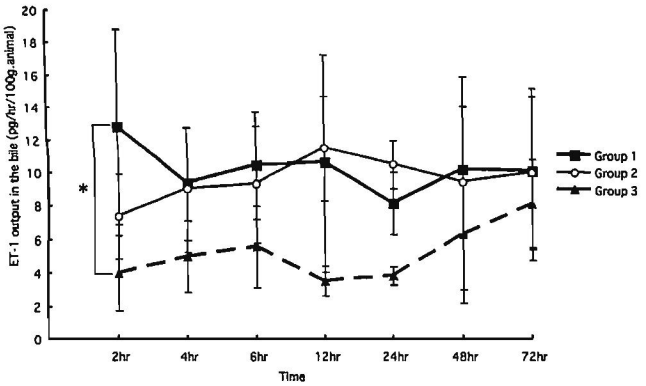

Fig. 5 The ET-1 output in the bile was also significantly lower in group 3 than in groupl at 2 hr. The ET-1 output in group 3 always demonstrated the lowest values among the 3 groups at every time point evaluated (mean±SE). *P<0.05 (ANOVA)

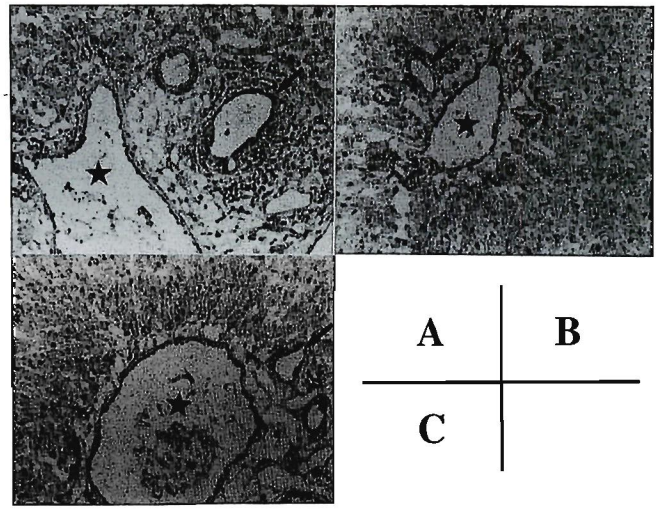

Fig. 6 Immunohistochemical staining for ET-1 in the liver specimen. The expression of ET-1 on the endothelium of portal vein (★) were stronger in group 3 (C) than in group 1(A). In contrast, the expression of ET-1 on the bile duct (→) was weaker in group 3 than in group 1. In group 2(B), the staining intensity in the portal vein and bile duct ranged between those of group 3 and group 1.

contrast, ET-1 concentration in group 1 temporally increased at 1 hr after sham operation. The ET-1 level in group 3 was significantly lower compare to group 1 at 1 hr (14.22 ±6.26 pg/ml, 108.1±49.33) after reperfusion. The bile ET-1 levels in group 3 remained lower compared to group 1 and 2 throughout the experiment.

The ET-1 output in the bile was also significantly lower in group 3 than in groupl at 2 hr (1.90±0.39 pg/hr/100g. animal, 12.87±5.16, respectively). The ET-1 output in group 3 was the lowest among the 3 groups throughout the experiment at every time point evaluated, however showed an increasing trend after 24 hr (Fig. 5).

Immunohistochemistry for ET-1

Immunohistochemical staining for ET-1 was performed to evaluate the expression of ET-1 in the injured liver at 4 hr after reperfusion. The expression of ET-1 on the endothelium of portal vein were stronger in group 3 than in group 1. In contrast, the expression of ET-1 on the bile duct was weaker in group 3 than in group 1. In group 2, both the portal and central vein and bile duct were stained. The staining intensity in the portal vein and bile duct was between those of group 3 and group 1 (Fig. 6).

DISCUSSION

The involvement of ET-1 in hepatic I/R injury or in liver transplantation has been studied exclusively by investigating the serum levels[9, 17]. In those studies, ET-1 was thought to be secreted from the sinusoidal endothelial cells, thus resulting in a narrowing of the sinusoids, constriction of the portal and hepatic veins, and finally a reduction of the hepatic blood flow[17]. Nakamura *et al* further reported that ET-1 is released from the liver after I/R injury and participates in systemic and local changes of hemodynamics that subsequently affect survival[19]. ET-1 might thus be closely involved in the pathophysiology of hepatic I/R injury immediately after the reperfusion. However, in experimental liver transplantation, these elevated serum ET-1 levels returned to normal levels in the relatively early phase after hepatic reperfusion, thus indicating that the ET-1 is promptly excreted from the circulation[20]. In this context, it is important to determine the organs from which ET-1 is produced and excreted after hepatic I/R injury in-vivo. We thus evaluated the changes in the ET-1 levels at the multiple sites, using a clinically relevant model of orthotopic liver transplantation (OLT).

In previous studies of hepatic I/R injury, hepatic ischemia was induced only by clamping the hepatic artery and portal vein without washing out the blood from the liver[19,21,22]. In this simple clamp model, endothelial cells are exposed to several factors such as neutrophils[21,22], adhesion molecules[23], platelet activating factors[24] and others during the ischemic period. In clinical liver transplantation, however, such factors are minimized by flushing the blood out of the liver. This simple clamp model therefore can not be claimed to be a clinically relevant model of hepatic I/R injury. A simple clamp of the portal triad without a port-systemic shunt causes other problems such as a severe reduction in the venous return, systemic hypotension, and intestinal congestion, during the hepatic ischemia. These factors are thought to induce even greater hepatic I/R injury due to cytokines in the portal vein and hypotensive organ failure, thus rendering this model even less appropriate as a model to investigate hepatic I/R injury in OLT. From our experience in the rat OLT, subtle differences in the surgical procedures cause a large deviation in the amount of hepatic I/R injury. The rat OLT model may thus be also inappropriate for investigating hepatic

I/R injury. In the present study, we used in-situ hepatic cold ischemia in which the blood was flushed out in-situ under the pre-established port-systemic shunt. Next, the liver was reperfused with the animal's own blood without any complicated anastomosis of the multiple vessels, thus resulting in relatively small deviations in the amount of hepatic I/R injury.

In the present study, ET-1 levels at the SHVC rapidly increased after hepatic reperfusion, while those of aorta remained almost unchanged. In immunohistochemistry at 4 hr after reperfusion, ET-1 was strongly stained at the endothelium of the portal and central vein only in the ischemic groups. These facts directly indicate that ET-1 was released from the endothelium of the intra-hepatic vessels in the liver and released into the SHVC in the ischemic groups. However in the bile, both the ET-1 output and its concentration rapidly decreased only in the ischemic groups, in spite of the increased ET-1 release from the damaged sinusoidal endothelium. These facts might thus indicate that hepatic I/R injury also had a negative effect on the ET-1 excretion mechanism into the bile.

The increased levels of serum ET-1 returned to its pre-ischemic level as early as 4 hr after reperfusion. On the other hand, increased levels of the serum hepatic enzyme continued as late as 48 hr after reperfusion, with apparent liver damage in HE staining at the same period. These facts directly indicated that the serum ET-1 did not reflect the time course in the development of hepatic injury after reperfusion, thus indicating a rapid elimination of circulating ET-1. ET-1 is known to be secreted from the bile duct epithelial cells into the bile[25]. In the present study, the ET-1 output and its concentration was kept lower after severe cold ischemia (60 min) throughout the experiment, and these low levels of bile ET-1 continued even after the recovery of the bile flow rate after severe cold ischemia. If the bile ET-1 derived only from the biliary endothelium, ET-1 secretion might increase after I/R injury due to the damage of the biliary epithelial cells. Bluhm and colleagues[26] first reported ET-1 to be secreted into the bile and its concentration was relative to that in the perfusate using an isolated perfused rat liver model. They also demonstrated that the liver plays a major role in clearing the circulating ET-1 and in excreting concentrated ET-1 into the bile. Accordingly, the ET-1 concentration in the bile was remarkably higher compared to that in the perfusate. These facts indicate that bile excretion works as one of the clearance pathways of ET-1, in addition to the other known pathways like the lung and kidney[10,11,12]. A decrease in bile ET-1 output and concentration in the present study were thus considered to be induced by a disturbance of this mechanism of the ET-1 excretion due to hepatic I/R injury. In our previous reports, the bile ET-1 output increased markedly with the development of acute rejection, after the rat model of allogeneic liver transplantation[9]. In this case the basic liver function, such as bile production, was relatively well preserved, and ET-1 was thought to be released not only from the hepatic endothelial cells, but also from the biliary endothelium, since all are known to be the main targets of hepatic allograft rejection. These findings indicate that the bile ET-1 excretion makes totally different patterns depending on the pathologic status. These characteristic movements of the bile ET-1, combined with the serum ET-1 levels, might thus be utilized in differentiating hepatic I/R injury from acute hepatic rejection.

In conclusion, ET-1 was promptly released from the injured hepatic endothelium into the SHVC, depending on the extent of the I/R injury. In contrast, the bile ET-1 excretion was found to be inversely proportional to the amount of hepatic I/R injury. These characteristic changes in ET-1 excretion might thus be useful in making a more accurate differential diagnosis of hepatic I/R injury.

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