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BRIEF REPORT

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Liberation of vasoactive substances and its prevention with thromboxane A_2 synthase inhibitor in pig liver transplantation

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Abstract There are multiple causes of liver graft nonfunction in the early post-transplant period. Since a severe microcirculatory disturbance based on ischemia-reperfusion liver injury is considered to be the main underlying pathophysiology, it is suspected that various vasoactive substances are liberated after reperfusion of the graft. In order to investigate this matter, we conducted an experimental study with pig liver allotransplantation. Two groups of animals received donor grafts with or without thromboxane synthase inhibitor (sodium ozagrel), 1.25 mg/kg body weight intravenously, given at the time of liver harvesting. All of the recipient animals in the treatment group ($n = 10$) survived longer than 7 days whereas three of ten animals in the control group died within 7 days. Serum lactate dehydrogenase (LDH) in the recipient serum at 1 h after reperfusion was significantly lower in the treatment group (915.1 ± 167.3 U/l) than in the control group (1264.4 ± 134.7 U/l). Serum thromboxane B_2 (2261.7 ± 1055.7 pg/ml) and endothelin-1 (6.3 ± 2.2 pg/ml) after reperfusion in the treatment group were significantly lower than those in the control group (4220.0 ± 1711.0 pg/ml and 11.2 ± 3.1 pg/ml, respectively). Although serum angiotensin II after reperfusion tended to be lower in the treatment group than in the controls serum renin activity was less than 3 ng/ml in both groups of animals. There were no differences in the plasma endotoxin levels between the two groups. We conclude that the administration of sodium ozagrel to the donor animals provided better graft function in recipients than no such treatment. We speculate that the inhibition of thromboxane A_2 production suppresses the liberation of other vasoconstrictive substances, preventing microcirculatory disturbance and, thereby, contributing to improved graft function after liver transplantation.

Key words Reperfusion injury, liver, pig · Sodium ozagrel, liver, pig · Thromboxane A_2, liver, pig · Liver transplantation, pig, sodium ozagrel

Introduction

The causes of graft dysfunction in the early postoperative period after liver transplantation are multifactorial [2]. The most devastating complication is primary nonfunction (PNF) of the graft, which requires an urgent retransplantation if the patient's life is to be saved [17]. Although the exact nature of PNF is not entirely understood, there is convincing evidence that the underlying pathophysiology is a profound microcirculatory disturbance with severe ischemic damage to the graft [3]. It is believed that most of this takes place upon reperfusion of the graft after cold preservation, and thus it is collectively termed "ischemia reperfusion injury" [25].

Among the factors that contribute to ischemia reperfusion injury are various vasoactive peptides and/or other related substances that may play a significant role in the development of microcirculatory disturbances [26]. Recent investigations in both experimental and clinical liver transplantation have demonstrated that after reperfusion of the liver graft, endothelin-1 (ET-1) [10, 20] and angiotensin II [23] are significantly elevated in serum. Thromboxane A_2, which is one of the prostaglandin derivatives, is also known to be elevated in the effluent of the hepatic vein [16]. Besides its vasoconstrictive property, thromboxane A_2 interacts with leukocytes, causing increased polymorphonuclear (PMN) leukocyte endothelial interaction [20]. Activation of these sequestrated PMN, as well as of macrophages and Kupffer cells, allows a number of toxic agents, including oxygen free radicals and lysosomal enzymes, to be liberated with resultant endothelial damage [15].

In this study with a pig liver transplant model, the authors describe the role of various vasoactive substances and their interaction in the mechanism of ischemia reperfusion injury.

Materials and methods

For the animal experiments, the "Principles of laboratory animal care" (NIH Publication No. 85-23, revised 1985) were followed, as well as the regulations of the Animal Research Laboratory of Nagoya University School of Medicine.

Donor operation

Young female Landrace pigs weighing between 12.5 and 14.5 kg were matched for orthotopic liver transplantation. After being starved overnight, donors were anesthetized with ketamine sulfate (10 mg/kg body weight) and an oxygen-mixed halothane inhalation. They were orotracheally intubated and ventilated with 40 % oxygen mixed with 1 %–2 % halothane. An indwelling catheter was placed in the jugular vein using a cutdown technique; through this catheter a maintenance intravenous solution of lactated Ringer's solution, 100 ml/h, was given. The abdominal cavity was entered through a midline incision. After systemic heparinization intravenously (500 mg/kg body weight), the infrarenal abdominal aorta was cannulated with a 16 Fr plastic catheter in retrograde fashion, and the splenic vein was also cannulated towards the main portal vein. After the infrarenal aorta distal to the cannulation site and thoracic aorta were crossclamped, 250 ml of cold normal saline was infused through both the aorta and the portal vein. The intrathoracic vena cava was transected to allow the blood to escape. The graft was then perfused with 500 ml University of Wisconsin (UW) solution. Topical cooling was also achieved with iced slush. The liver was then removed with its vascular attachment in en bloc fashion. The graft was perfused again with 300 ml of UW solution via the portal vein and 100 ml via the hepatic artery and was stored at 4 °C for 6 h.

Preparation of drugs

Thromboxane synthase inhibitor (sodium ozagrel) was a generous gift from Ono Pharmaceuticals (Osaka, Japan). Sodium ozagrel, 1.25 mg/kg body weight dissolved in 100 ml normal saline, was infused via the jugular vein into half of the donor animals ($n = 10$). Recipients of these livers made up the treatment group. The other donor animals ($n = 10$) did not receive the drug; recipients of these livers constituted the control group. Infusion of the drug continued for 30 min before the heart of the donor animal ceased beating.

Recipient operation

The recipient animals were randomly selected for allograft liver transplantation. They were anesthetized and ventilated in the same manner as in the donor operation. Extracorporeal passive bypass was used while the hepatectomy and liver implantation were performed. Vascular anastomoses of the graft were carried out based on the original techniques by Starzl et al. [22]. Immediately before portal venous reperfusion, the graft was rinsed with 200 ml of cold lactated Ringer's solution. Bile duct reconstruction was performed by cholecystoduodenostomy. Immunosuppression consisted of a single 200-mg intravenous bolus injection of methylpredonisolone intraoperatively and cyclosporin, 3 mg/kg body weight intravenously, immediately after the operation. Cyclosporin, 9 mg/kg body weight, was given orally on postoperative days (POD) 1, 2, and 3. After the operation, all of the animals were extubated and kept in the recovery cage with a maintenance intravenous infusion of 5 % dextrose mixed with normal saline for 24 h. Oral feeding was usually started on the 1st POD, as soon as the animals were able to tolerate it. Otherwise a supplemental intravenous infusion was given.

Monitoring and follow-up of graft function

Serum samples were taken for aspartate transaminase (AST) and lactic dehydrogenase (LDH) at pretransplantation, 1 h after reperfusion, and on POD 1, 3, and 7. Serum samples for thromboxane (Tx) B_2 and 2,3-dinor-6-keto- prostaglandin $PGF_{1\alpha}$ ($PGF_{1\alpha}$) were obtained before transplantation and 1 h after reperfusion of the graft. They were measured by radioimmunoassay using an antibody whose crossreactivity with heterologous prostanoids was less than 0.1 % [19]. Serum ET-1, renin activity, and angiotensin II were also measured at the same time points as TxB_2. Plasma endotoxin was measured with the use of Toxicolor as previously described [14]. Postmortem examinations were performed at the time of death for those animals that survived less than 7 days; otherwise the animals were sacrificed on POD 7 for autopsy examination.

Statistical analysis

Data are expressed as the mean ± standard deviation of the mean. An analysis of variance and the paired Student's t-test were used to determine significance. A P value less than 0.05 was considered significant.

Results

Three of ten animals (30 %) in the control group died 1, 3, and 6 days after liver transplantation, whereas none in the treatment group did. The cause of death in the ani-

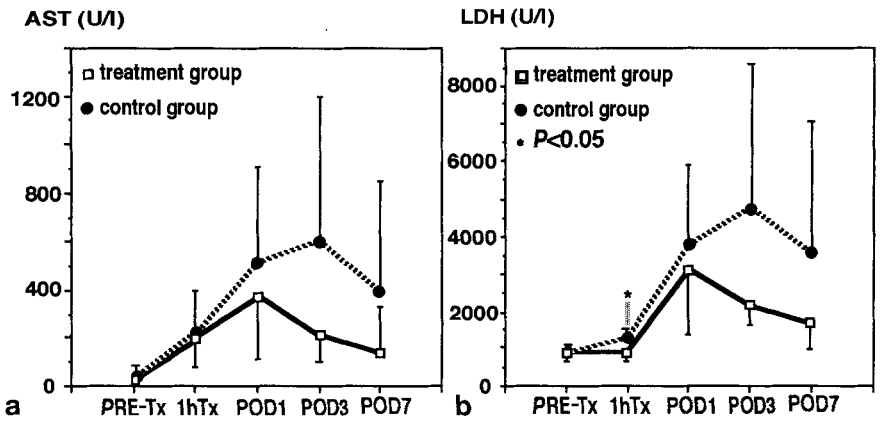

Fig. 1 a,b Changes in the mean: **a** serum AST and **b** LDH levels at different time points [pretransplantation *(Pre-Tx)*, 1 h after portal reperfusion *(1 h Tx)*, and on postoperative day (POD) 1, 3, and 7] in the untreated (control) group and in the sodium ozagrel (treatment) group. Significantly lower LDH levels were noted in the treatment group than in the control group at 1 h Tx ($P < 0.05$)

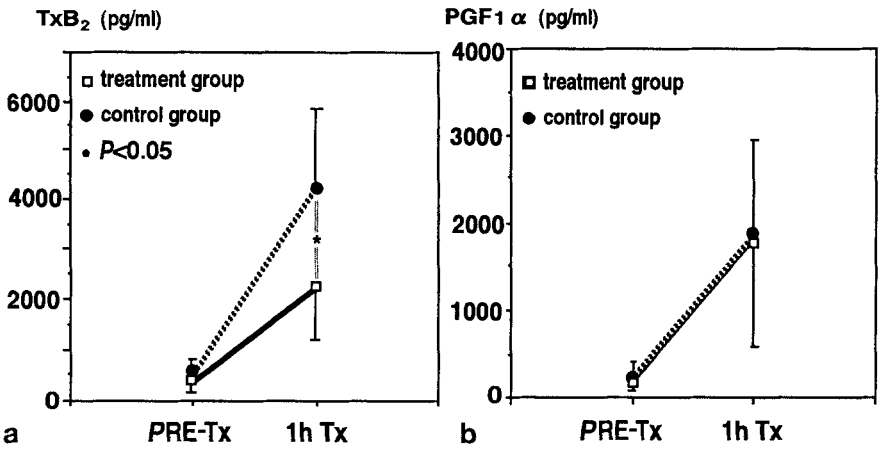

Fig. 2 a,b Changes in the mean: **a** serum thromboxane B_2 *(TxB_2)* and **b** 6-keto-$PGF_{1\alpha}$ *($PGF_{1\alpha}$)* at different time points [pretransplantation *(Pre-Tx)* and 1 h after portal reperfusion *(1 h Tx)*] in the untreated (control) group and in the sodium ozagrel (treatment) group. A significantly lower TxB_2 level was noted a 1 h Tx in the treatment group than in the control group ($P < 0.05$)

mal that died on the 6th post-transplant day was massive bleeding from the stomach ulcer. Postmortem studies of the two other animals showed patent vascular and biliary anastomses and normal appearance of the cardiorespiratory system. Histological examination of these grafts showed diffuse, patchy, hepatocyte necrosis with varying degrees of sinusoidal narrowing and occasional interstitial hemorrhage. Neither of them showed evidence of cellular rejection. Thus, the cause of graft failure in these two animals was said to be PNF since there were no obvious mechanical or immunological causes other than those related to cold preservation and reperfusion of the grafts. In none of the other animals that survived for 7 days could any significant abnormality be found during the postmortem studies. However, histological examinations revealed that approximately one-third of them had varying degrees of acute cellular rejection, with or without minimal to mild ischemic changes in the hepatocytes.

The mean serum AST levels were 23.1 ± 5.7, 193.5 ± 117.9, 381.4 ± 271.5, 206.6 ± 99.1, and 139.4 ± 194.4 IU/l in the treatment group, whereas they were 25.3 ± 10.8, 213.6 ± 83.7, 517.2 ± 393.2, 609.3 ± 589.9, and 396.6 ± 450.2 IU/l in the control group at pretransplantation, 1 h after reperfusion, and POD 1, 3, and 7, respectively. The mean AST level in the treatment group tended to be lower than that in the control group at POD 1 ($P = 0.09$, Fig. 1 a). The mean serum LDH levels were 1070.6 ± 181.3, 915.1 ± 167.3, 3156.4 ± 1787.5, 2175.3 ± 1212.2, and 1659.0 ± 648.0 IU/l in the treatment group, whereas they were 913.6 ± 162.7, 1264.4 ± 134.7, 3784.6 ± 2023.3, 4814.3 ± 3755.1, and 3574.1 ± 3452.6 IU/l for the control group at the respective time points. The mean LDH level in the treatment group was significantly lower than that in the control group at 1 h after reperfusion ($P < 0.05$, Fig. 1 b). The mean serum TxB_2 levels were 330.3 ± 165.5 and 2261.7 ± 1055.7 pg/ml for the treatment group and 466.3 ± 268.9 and 4220.0 ± 1711,0 pg/ml for the control group at pretransplantation and at 1 h after transplantation, respectively. The mean TxB_2 level after reperfusion in the treatment group was significantly lower than that in the control group ($P < 0.05$, Fig. 2 a). The mean serum $PGF_{1\alpha}$ levels were 181.7 ± 106.9 and 1808.6 ± 1222.1 pg/ml for the treatment group and 192.8 ± 152.8 and 1811.4 ± 1145.3 pg/ml for the control group at each

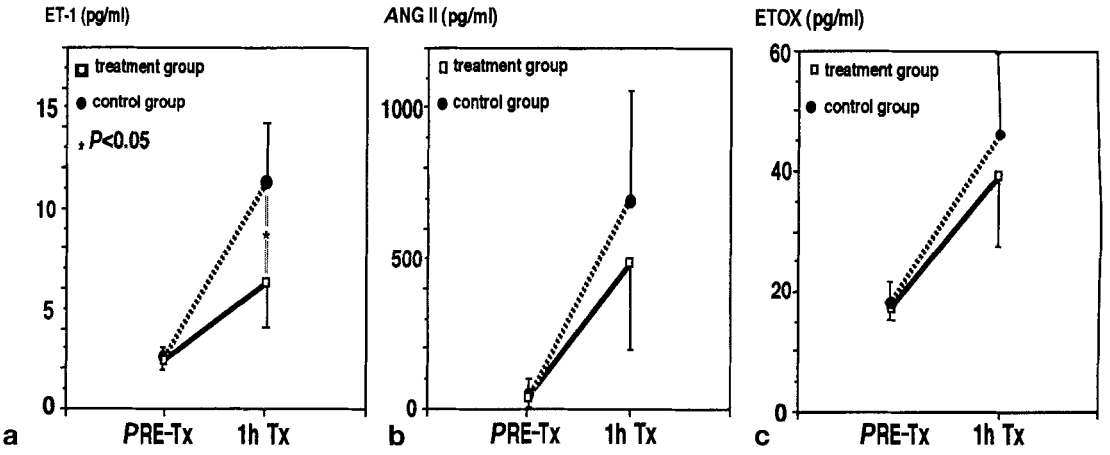

Fig. 3a–c Changes in the mean: **a** serum endothelin-1 *(ET-1)*, **b** angiotensin II *(Ang II)*, and **c** plasma endotoxin *(ETOX)* at different time points [pretransplantation *(Pre-Tx)* and 1 h after portal reperfusion *(1 h Tx)*] in the untreated (control) group and in the sodium ozagrel (treatment) group. A significantly lower ET-1 level was noted at 1 h Tx in the treatment group than in the control group ($P < 0.05$), whereas there was no difference in Ang II or ETOX between the two groups

time point, respectively. There was no difference in the $PGF_{1\alpha}$ level between the two groups (Fig. 2b). The mean serum ET-1 levels were 2.4 ± 0.2 and 6.3 ± 2.3 pg/ml in the treatment group and 2.5 ± 0.3 and 11.2 ± 3.1 pg/ml in the control group at the respective time points. The mean ET-1 level after reperfusion in the treatment group was significantly lower than that in the control group ($P < 0.05$, Fig. 3a). The mean serum angiotensin II levels were 17.5 ± 5.0 and 482.6 ± 285.9 pg/ml for the treatment group and 16.3 ± 2.8 and 680.0 ± 376.2 pg/ml for the control group at the respective time points. The mean angiotensin II level after reperfusion in the treatment group tended to be lower than that in the control group ($P = 0.1$, Fig. 3b). Renin activity was below 3.0 pg/ml at each of the time points before and after transplantation in both groups of animals. Plasma endotoxin levels were 17.3 ± 3.5 and 36.7 ± 10.5 pg/ml in the treatment group and they were 18.8 ± 5.2 and 45.7 ± 12.2 pg/ml in the control group at each time point. There was no difference between the groups (Fig. 3c).

Discussion

Thromboxane B_2 is a stable hydrolysis product of thromboxane A_2, which is a potent vasoactive, proaggregatory, and chemotactic prostanoid produced from arachidonic acid via the cyclo-oxygenase pathway [9]. Sodium ozagrel is a thromboxane A_2 synthase inhibitor that selectively blocks the pathway from prostaglandin (PG) H_2 to thromboxane A_2, leaving the other pathway to PGI_2 production intact [12]. Platelets are considered to be the main site of thromboxane production [4, 11], although it has been shown that there are other possible sites, such as PMN, macrophages, Kupffer cells, and endothelial cells [7, 24]. A recent investigation has shown that the cells of most organs contain thromboxane synthase in varying degrees with the highest content in the platelets, followed by the lung and the liver in decreasing order [13]. Thus, it is easy to see why one might choose to administer sodium ozagrel to the liver in order to selectively control prostaglandin metabolism.

It is known that in experimental liver transplantation with large animals, thromboxane B_2 is increased after reperfusion of the graft, a finding that was confirmed in the present study. Moreover, the serum thromboxane level after reperfusion of the graft was effectively lowered with the administration of sodium ozagrel. An additional finding from the present experiment was that various vasoactive substances, such as ET-1 and angiotensin II, were also released into the serum of the recipients after reperfusion of the grafts. Of importance is the fact that sodium ozagrel administration appears to modify the metabolism of those vasoactive substances.

Elevation of ET-1 has been shown in the serum of transplant recipients after cold preservation of the liver [10, 21]. Goto et al. showed that ET-1 was involved in the pathogenesis of reperfusion injury of the liver in the rat ischemic liver reperfusion model [8]. As shown in this study, elevation of ET-1 seems to be associated with thromboxane A_2 production. Endothelial cells are considered to be the main sites of ET-1 production [18]. A significantly higher concentration of ET-1 can be released from damaged endothelial cells during and after cold preservation and reperfusion of the grafts [21]. The fact that significantly lower ET-1 levels were seen in the animals pretreated with sodium ozagrel can be attributed to the lesser amount of endothelial cell damage in these animals than in those without pretreatment. Furthermore, damaged endothelial cells induce an in-

flammatory response that involves various inflammatory mediators, such as cytokines and thrombin. Indeed, thrombin is known to be one of the most potent mediators of ET-1 secretion [1].

Although not statistically significant, angiotensin II, which is also a potent vasoconstrictive agent, seemed to be influenced by thromboxane production. As the renin activity was not increased in either group of animals, with or without pretreatment, elevation of angiotensin II is considered to be independent of the renin-angiotensin system. A recent investigation by Dauser et al. has shown that angiotensin II is derived from other sources than renin and angiotensin I in intact pigs [5]. They showed that a significant proportion of angiotensin production was derived from de novo production in various extrarenal organs such as myocardium. It is quite conceivable that low renin activity is secondary to a negative feedback mechanism in the renin-angiotensin-aldosterone system, although aldosterone was not measured in the present study.

Another potential vasoactive substance that is known to be increased after graft reperfusion is endotoxin. However, the administration of sodium ozagrel did not influence endotoxin production, which indicates that the latter is independent of thromboxane elevation. Yet, it is still possible that endotoxin interacts with various cytokines and vasoactive substances, as has been suggested [26].

We feel that the results of the present study may help explain the pathophysiological mechanism involved in reperfusion injury of the graft in liver transplantation. It is very likely that thromboxane production plays a significant role in its pathogenesis, particularly as a modulator in the interaction of various vasoactive substances that are liberated after reperfusion of the liver graft. We conclude that thromboxane A_2 synthase inhibitor pretreatment of the donor may improve post-transplant graft outcome in liver transplantation, possibly by effectively controlling the liberation of vasoactive agents such as ET-1 and angiotensin II.

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