

Effect of Endothelin A Receptor Antagonist on Hepatic Hemodynamics in Cirrhotic Rats. Implications for Endothelin-1 in Portal Hypertension.

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Objective: The effect of an endothelin (ET) A receptor antagonist on hepatic hemodynamics in cirrhotic rats was examined.

Methods: Portal pressure and hepatic tissue blood flow in cirrhotic rats were measured. Plasma ET-1 levels were determined by radioimmunoassay. BQ-123 was infused to these rats at a rate of 10 nmol/min. The sinusoids were observed by scanning electron microscopy. The localization of ET-1 and ETA receptors was examined using the indirect immunoperoxidase method.

Results: In cirrhotic rats, the portal pressure significantly increased to 16.6 ± 1.5 cm H₂O, and the hepatic tissue blood flow markedly decreased. Plasma ET-1 levels in cirrhotic rats were higher than those in normal rats. When BQ-123 was infused, the portal pressure was significantly reduced by more than 2 cm H₂O, compared with the control group ($p < 0.05$). Hepatic tissue blood flow was maintained at the level before infusion. In liver cirrhosis, the sinusoids were covered with continuous endothelial cells, and the number of sinusoidal endothelial fenestrae extremely decreased. ET-1 was remarkably enhanced in sinusoidal endothelial cells within the regenerating nodules, and the reaction products of ETA receptors were mainly recognized in hepatic stellate cells.

Conclusions: The augmented action of ET-1 via the ETA receptor may be involved in the mechanism of portal hypertension in liver cirrhosis.

Key words: portal hypertension, endothelin-1, endothelin A receptor, hepatic microcirculation, hepatic stellate cells

INTRODUCTION

In liver cirrhosis, there is an overexpression of humoral vasoconstrictors, such as norepinephrine, endothelin (ET) and angiotensin-II, leading to an increase in intrahepatic vascular resistance and tone [1]. ET, which is a peptide composed of 21 amino acids, is a powerful endogenous vasoconstrictor produced by endothelial cells [2]. ET has three kinds of isoform, ET-1, 2, and 3 [3]. ET-1 is a potent vasoconstrictive endothelin, and is involved in a variety of physiological functions in vascular tissues. The liver is one of the principal sites of the synthesis and the action of ET-1. ET-1 is considered to play an important role in the pathogenesis of portal hypertension, since its plasma level is increased and overexpressed in patients with liver cirrhosis [4, 5]. The physiological functions of ET-1 are mediated by two ET receptors, i.e., ETA and ETB receptors. ETA receptors bind ET-1 with an affinity more than 100 times higher than ET-3, and ETB receptors bind ETs with a similarly high affinity [6, 7]. ET-1 is produced and released from the sinusoidal endothelial cells, and exerts paracrine effects on the ETA receptors of the vascular smooth muscles and the hepatic stellate cells (HSC), thereby inducing their contraction [8, 9]. ET-1 also has autocrine or intracrine effects on the sinusoidal endothelial cells themselves

via the ETB receptors, releasing vascular relaxing factors such as nitric oxide and prostacyclin [10, 11].

Our previous studies demonstrated that the action of ET-1 via the ETA receptors rather than ETB receptors plays an important role in the regulation of the portal vein pressure as well as the motion of sinusoidal endothelial fenestrae (SEF) in normal rats [12], and that BQ-123, an ETA receptor antagonist reduces the portal vein pressure in an experimental model of portal hypertension caused by acute administration of ethanol [13]. Thus, this study examines the effect of BQ-123 on hepatic hemodynamics in cirrhotic rats to elucidate the microcirculatory mechanism of ET-1 via the ETA receptors in portal hypertension.

MATERIALS AND METHODS

Preparation of the Rat Liver

Male Wistar rats weighing 200–250 g were used. They were given a standard laboratory pellet rat diet and tap water ad libitum. Cirrhotic liver was prepared by subcutaneous injection of 50% carbon tetrachloride solution (0.3 ml per 100 g of body weight) twice a week for 3 months. The experimental protocol was approved by the animal care and use committee of Tokai University School of Medicine. All animals were given humane care in compliance with the institutional guidelines. The rats were anesthetized using sodium

pentobarbital (30 mg/kg, i.p.) and fixed in a supine position. After an abdominal median incision was performed, a polyethylene catheter (PE10) was inserted into the mesenteric vein branch.

BQ-123, an ETA receptor antagonist was purchased from Sigma-Aldrich Co., Japan. BQ-123 was infused to six cirrhotic rats at a rate of 10 nmol/min for 10 min, and then physiological saline was infused at the same rate for 50 min via the mesenteric vein catheter [12, 13]. For the control, the same volume of physiological saline was infused to six cirrhotic rats at the same rate for 60 min. A polyethylene catheter (PE50) was inserted into the tail artery, and the arterial blood pressure was monitored using an electronic blood pressure transducer (AP-641G, Nihon Kohden, Tokyo, Japan) during the experiments. A 23-gauge needle was directly inserted into the main trunk of the portal vein. The portal vein pressure was continuously monitored using a hydromanometer and measured every 10 min for up to 60 min after the infusion of BQ-123. The blood flow in the hepatic median lobe was determined using a laser Doppler blood flow meter (PeriFlux-PF3, Perimed, Stockholm, Sweden) before and every 10 min after the infusion of BQ-123 over 60 min [14]. The values are expressed as percent changes from those obtained before infusion.

Scanning Electron Microscopy

The cirrhotic rat livers were washed out by physiological saline 60 min after infusion and fixed in 1.2% glutaraldehyde by a perfusion-fixation technique. Normal rats were used as a control. After fixation, liver tissues were cut into approximately 1 x 3 x 5 mm³ in size, and post-fixed in 2% osmium tetroxide at 4°C for 3 hrs. After dehydration by serial ethanol, the tissue blocks were fractured with a pair of forceps in absolute ethanol and prepared using a critical point dryer (HCP-2, Hitachi, Tokyo, Japan). After gold-coating using an ion sputter (JFC-1100E, JEOL Tokyo, Japan), the fractured surfaces of liver tissues were examined using a scanning electron microscope (JSM840A, JEOL, Tokyo, Japan) with a 15 KV acceleration voltage.

Measurement of serum ET-1 levels

Five normal rats and five cirrhotic rats were used to determine plasma ET-1 levels. Blood samples were collected from the femoral vein catheter. After centrifugation of the samples, plasma levels of ET-1 were measured using radioimmunoassay [15].

Immunohistochemical Staining for ET-1 and ETA-R

Cirrhotic rats were washed out with physiological saline, and they were fixed in 4% paraformaldehyde at 4°C overnight. Normal rats were used as a control. Cryostat sections of the liver tissues were pretreated with 0.3% hydrogen peroxidase solution to block the endogenous peroxidase activity and 5% normal goat serum for 10 min. The sections were incubated with anti-ET-1 monoclonal antibody (Yamasa Shoyu Co., Choshi, Japan) [16] diluted 1 : 50 in PBS at room temperature for 1 hr in a humidified chamber. After rinsing with PBS, they were incubated with HRP-labeled anti-mouse immunoglobulin G at room temperature for

1 hr.

For immunostaining of the ETA receptors, the sections were incubated with anti-ETA receptor antibody (Immuno-Biological Laboratories Co, Fujioka, Japan) (17) diluted 1 : 50 in PBS for 1 hr. After rinsing with PBS, they were incubated with HRP-labeled anti-rabbit immunoglobulin G at room temperature for 1 hr. The sections were followed by a reaction with 0.02% diaminobenzidine (DAB) solution containing hydrogen peroxide for 10 min. The specimens were then counterstained with methyl green.

Statistical Analysis

All the data were stored in a personal computer and analyzed using StatView 5.0 software. The data were expressed as the mean \pm SE. The statistical analysis of portal vein pressure was performed using repeated ANOVA. The unpaired t-test was used to compare plasma ET-1 levels between normal and cirrhotic rats. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Hepatic Hemodynamics

The mean plasma ET-1 level in carbon tetrachloride-induced cirrhotic rats was 5.4 ± 2.9 pg/ml, which was markedly higher than that in normal rats (1.8 ± 1.8 pg/ml) ($n = 5$, $P < 0.05$). The portal vein pressure in cirrhotic rats significantly elevated to 16.6 ± 1.5 cmH₂O, as compared to the pressure in normal rats (9.1 ± 1.2 cmH₂O) ($n = 6$, $P < 0.001$). After BQ-123 was infused through the mesenteric vein catheter, the pressure gradually decreased and showed a significant reduction ($p < 0.01$) by more than 2 cmH₂O, as compared with that in the control group (Fig. 1). Physiological saline-infused cirrhotic rats in the control group did not show a decrease in portal vein pressure from the baseline over the 60-min observation period.

On the other hand, the mean hepatic tissue blood flow in cirrhotic rats significantly decreased to 36.6 ± 9.4 ml/min/100g, as compared to that in normal rats (91.8 ± 8.7 ml/min/100g) ($n = 6$, $P < 0.001$). However, the hepatic tissue blood flow fluctuated during the 60-min observation period in both the BQ-123 infused group and the control, and it was maintained at the value prior to infusion in these groups. There were no significant differences in the hepatic tissue blood flow between the two groups (Fig. 2). The systemic blood pressure was not changed in either group during the experiments.

Sinusoidal Endothelial Cells

In carbon tetrachloride-induced cirrhotic rats, the hepatic microvasculature was quite different from that of normal rats. Scanning electron microscopy showed regenerating nodules surrounded by fibrous septa (Fig. 3). The sinusoids within the nodules were narrow. In normal rats, a number of SEF were evenly distributed in the sinusoidal endothelial cells in both zone 1 and zone 3 of the liver acinus. SEF were recognized as sieve plate-like pores, approximately 100 nm in diameter (Fig. 4a). On the other hand, the sinusoids were covered with the continuous endothelial cells, and the number of SEF extremely decreased in cir-

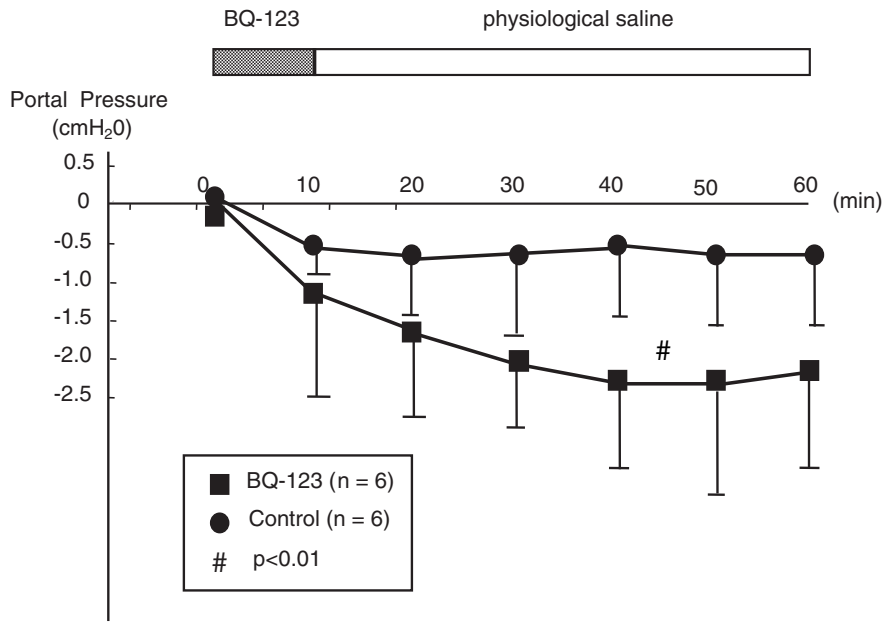


Fig. 1 The effect of infusion of BQ-123 on portal vein pressure in cirrhotic rats. In the BQ-123-infused group, the portal pressure in cirrhotic rats decreased gradually over time, and significantly reduced by more than 2 cmH₂O, when analyzed by repeated ANOVA ($p < 0.01$).

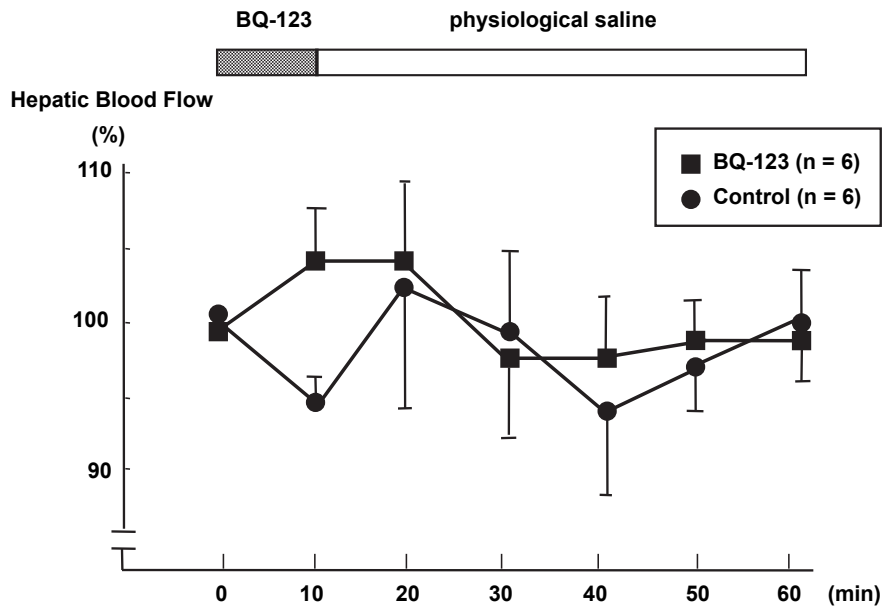


Fig. 2 The effect of infusion of BQ-123 on hepatic tissue blood flow in cirrhotic rats. The hepatic tissue blood flow is maintained at the value before infusion in both groups. There are no significant differences between the two groups.

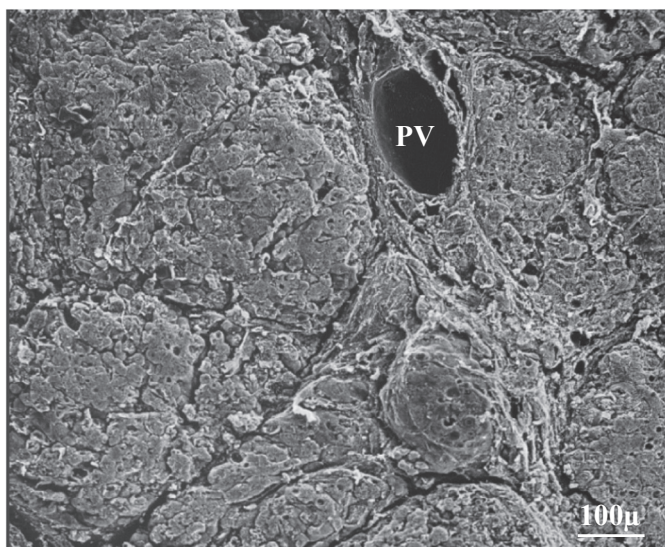


Fig. 3 Scanning electron micrographs of the hepatic microvasculature in cirrhotic rats. The hepatic microvasculature in liver cirrhosis is clearly recognized using scanning electron microscopy. Note that the micronodules are surrounded by the fibrous septa. The sinusoids within regenerating nodules are narrow.

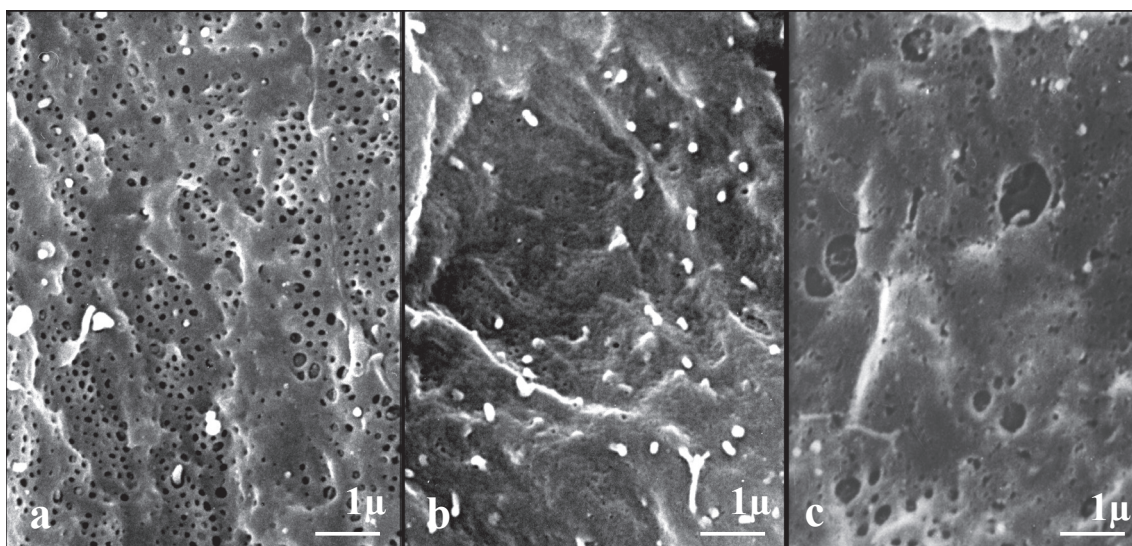


Fig. 4 Scanning electron micrographs of sinusoidal endothelial fenestrae (SEF) in normal, cirrhotic and BQ-123-infused rats.

a. normal rat. b. cirrhotic rat. c. BQ-123-infused cirrhotic rat.

In normal liver, SEF are recognized as sieve plate-like pores, approximately 100 nm in diameter. A number of SEF were evenly distributed in the sinusoidal endothelial cells (a). SEF are covered with continuous endothelial cells and almost disappear in cirrhotic rats (b). In the BQ-123-infused groups, some pores of the SEF were observed in the endothelial cells, but the morphology of sinusoidal endothelial cells appears to be similar to those of cirrhotic rats (c). Bar, 1 µm.

rhotic rats (Fig. 4b). In the BQ-123-infused group, the ultrastructures of sinusoidal endothelial cells appeared to be similar to those of cirrhotic rats. The sinusoidal endothelial cells were almost defenestrated, but some pores of the SEF were observed in the endothelial cells in the BQ-123-infused group (Fig. 4c).

Immunohistochemical Activity of ET-1 and Localization of ETA Receptors

Immunohistochemical examinations revealed that ET-1 was evenly distributed along the sinusoidal walls from zone 1 to zone 3 in normal rats (Fig. 5). On the other hand, the activity of ET-1 was remarkably enhanced in sinusoidal endothelial cells within the regenerating nodules and in the fibrous septa in cirrhotic

rats (Fig. 6). The reaction products of ETA receptors in cirrhotic rats were found not only along the vascular walls of the portal veins but also in the sinusoidal cells, i.e., HSC and part of the endothelial cells (Fig. 7).

DISCUSSION

In the present study, the plasma ET-1 level in cirrhotic rats (5.4 ± 2.9 pg/ml) was three times higher than that in normal rats (1.8 ± 1.8 pg/ml), which is similar to the data in humans reported by Moore *K et al.* [4]. Pinzani M *et al.* reported that the ET-1 expression is markedly enhanced in the cytoplasm of sinusoidal cells in human cirrhotic liver tissue, using in situ hybridization and immunohistochemistry [5]. Our immunohistochemical study revealed that ET-1

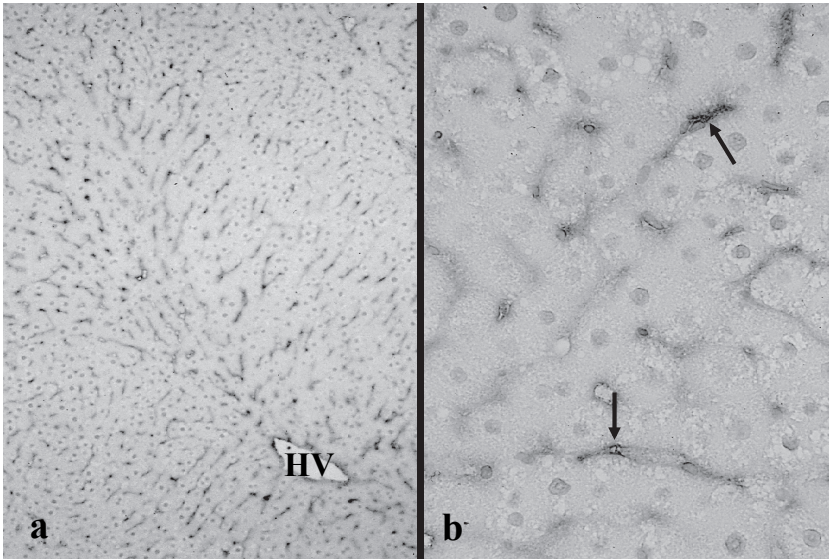


Fig. 5 Immunohistochemistry for ET-1 in liver tissues of normal rats. Counterstained with methyl green. a. x40. b. x400. The reaction products of ET-1 are present along the sinusoidal walls (arrows) and evenly distributed from zone 1 to 3 in normal rats.

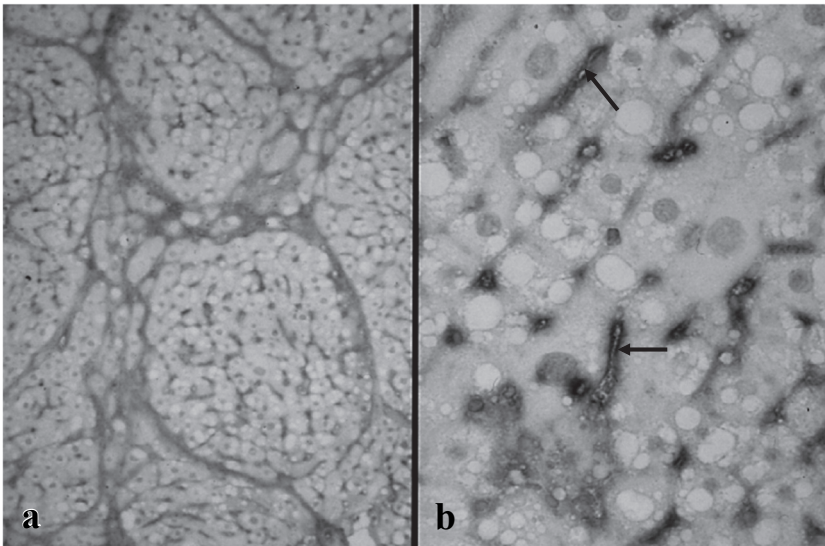


Fig. 6 Immunohistochemistry for ET-1 in liver tissues of cirrhotic rats. Counterstained with methyl green. a. x40. b. x400. In cirrhotic rats, the reaction products of ET-1 markedly increased along the sinusoidal walls (arrows) within the regenerating nodules and in the fibrous septa.

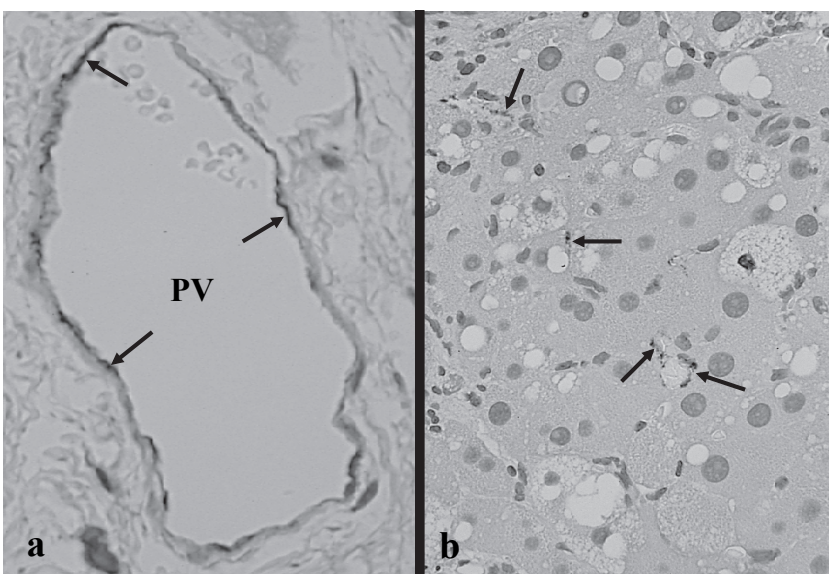


Fig. 7 Immunohistochemistry for ETA receptors in liver tissues of cirrhotic rats. Counterstained with methyl green. x400. The reactions of ETA receptors are recognized not only in the vascular walls of the portal vein (arrows) (a), but also along the sinusoidal wall, possibly HSC (arrows) (b).

was overexpressed in liver tissue of cirrhotic rats, particularly in sinusoidal endothelial cells within the regenerating nodules. It has been reported that hepatic ET receptor density has also increased in experimental cirrhosis [15], and that activated HSC, contributing to the regulation of intrahepatic vascular resistance, showed increased sensitivity to the constrictive action of ET-1 correlated with the degree of experimental fibrosis [18]. These findings indicate that in liver cirrhosis, ET-1 may play a major role in high sinusoidal vascular resistance mediated by the HSC contraction. Thus, ET receptor antagonists are considered useful for the pharmacological therapy of portal hypertension.

ET-1 plays a role in the mechanism of portal hypertension through both ETA and ETB receptors by increasing intrahepatic vascular resistance in the cirrhotic liver [18–20]. In this study, the effect of BQ-123, an ETA receptor antagonist, on the portal vein pressure was examined to elucidate the precise mechanism of ET-1 in liver cirrhosis from the viewpoint of the regulation of sinusoidal circulation. BQ-123 significantly decreased the portal vein pressure by more than 2 cmH₂O, as compared with that in the control group. HSC are perisinusoidal cells featuring tissue pericytes which have been implicated in the regulation of sinusoidal circulation. ET receptors are present in all cell types, but they are more numerous in the HSC than in other sinusoidal cells and hepatocytes [21, 22]. The present immunostaining study revealed that the ETA receptor was localized in HSC in the perisinusoidal space. The results were consistent with those obtained from the previous study using immunohistochemistry and immunogold electron microscopy, which showed the presence of ETA receptors in HSC and to a lesser extent in sinusoidal endothelial cells in the cirrhotic rat liver [23]. It is therefore conceivable that a decrease in portal vein pressure after the BQ-123 infusion may result from the inhibitory effect of BQ-123 on the ET-1 induced contractions of HSC in the sinusoids. The decrease in portal pressure would not be a phenomenon secondary to the inhibitory effect of a BQ-123 on the ET-1-induced constriction of the portal vein. We have to await further studies using vital microscopy to confirm the vascular changes of the sinusoids and the portal veins after infusion of BQ-123 in cirrhotic rats.

Laser-Doppler flowmetry on the liver surface reflects relative changes of the hepatic tissue blood flow, consisting of mainly sinusoidal blood flow and partly portal and hepatic arterial blood flow. The technique is more sensitive to blood flow changes in the hepatic artery as compared with the portal vein [14]. The present study revealed that the systemic blood pressure was not changed in either group during the experiments. The hepatic tissue blood flow was maintained at the value prior to infusion in both the BQ-123 infused group and the control against our expectation. It is speculated that a decrease of approximately 2 cmH₂O in portal vein pressure after infusion of BQ-123 would not influence the hepatic tissue blood flow in liver cirrhosis.

Rockey DC *et al.* reported that ET-1-induced contraction is enhanced in HSC from the cirrhotic rat liver and the intact liver, and that ET-1 causes sustained vasoconstriction [18]. Using intravital microscopy, Zhang

JX *et al.* demonstrated that ET-1 induces specific constriction of hepatic sinusoids, mediated by ET-1 action on the HSC [24]. Thus it has been proposed that increased hepatic production of ET-1 contributes to portal hypertension by mediating intrahepatic HSC contraction and an increase in hepatic sinusoidal tone. We have demonstrated that the action of ET-1 via the ETA receptors rather than the ETB receptors plays an important role in the regulation of the portal pressure as well as the motion of SEF in normal rats [12]. This hypothesis has been confirmed by the present results, showing that an ETA receptor antagonist decreased the portal pressure in cirrhotic rats. These findings have also been supported by a recent study reported by Feng HQ *et al.*, which demonstrates that intravenous infusion of ETA receptor antagonists into normal and cirrhotic mice reduces the portal pressure, whereas ETB receptor antagonists increase the portal pressure [25]. Furthermore, it is reported that ET-1 produces a direct vasoconstriction on the collateral vessels, mediated by ETA, but not ETB receptors in portal hypertensive rats induced by partial portal vein ligation [26].

Hepatic sinusoidal endothelial cells possess fenestrae mostly arranged in sieve plate-like pores, i.e., SEF, which allow free exchange of plasma substances between the sinusoids and the hepatocytes, thus contributing to the regulation of hepatic microcirculation [27, 28]. Our previous study demonstrated that infusion of BQ-123 causes a marked dilation of the SEF in both zone 1 and zone 3 in normal rats [12]. It was speculated that the marked dilation of the SEF after the BQ-123 infusion may result from the inhibitory effect of BQ-123 on the ET-1-induced contractions of HSC. This speculation leads to a hypothesis that SEF may change their pore size in response to morphological alterations in the sinusoids, which is caused by the contraction of HSC mediated by the ET-1 action via the ETA receptor. However, in liver cirrhosis, the sinusoids are surrounded by the continuous basement membrane with the accumulation of type IV collagen and laminin, and they are covered with the defenestrated endothelial cells, which is termed sinusoidal capillarization [29]. In the present study, some pores of the SEF were observed in the endothelial cells in the BQ-123-infused cirrhotic rats, but the ultrastructures of sinusoidal endothelial cells appeared to be similar to those of cirrhotic rats against our expectation. It is unlikely that BQ-123 at the present dose could dilate the pores in sinusoidal endothelial cells covered with the continuous basement membranes in liver cirrhosis.

CONCLUSIONS

In liver cirrhosis, the level of ET-1 increased in plasma as well as liver tissues, leading to the elevation of the portal vein pressure. The augmented action of ET-1 via the ETA receptor may be involved in the mechanism of portal hypertension in liver cirrhosis.

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