Angiogenesis and proliferation of bile duct enhances ischemic tolerance in rats with cirrhosis

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Abstract: Background/aims: Primary biliary cirrhosis (PBC), an autoimmune disease of the liver, is marked by slow progressive destruction of bile ducts. These patients with PBC often undergo orthotopic liver transplantation (OLT). Ischemic bile duct lesion (IBDL) is a major source of morbidity and even mortality after OLT. Cirrhosis of the liver has a higher tolerance to ischemia than a normal liver, but the mechanism remains unknown. Angiogenesis and proliferation of bile duct often responses in bile duct ischemia, which may enhance ischemic tolerance in patients with cirrhosis. Methodology: To test the hypothesis, a rat model with cirrhosis was established. Biochemical indexes of ischemic severity were measured including total bilirubin (TBIL) and direct bilirubin (DBIL). Immunohistochemical assay was performed for Ki67 (a biomarker for the proliferation of bile duct) and CD34 (a biomarker of angiogenesis). Results: The levels were lower for TBIL and DBIL in the bile duct from rat model with cirrhosis than that from a normal rat after ischemic surgery (P < 0.05). The levels were higher for Ki67 and CD34 from a rat model with cirrhosis than that from a normal rat after ischemic surgery (P < 0.05). Conclusions: The results suggest that a liver with cirrhosis has a better ischemic tolerance than a normal liver. Angiogenesis and proliferation of bile duct enhances ischemic tolerance in rats with cirrhosis. More research on the pathogenesis of IBDLs is needed for developing more specific preventive or therapeutic strategies.

Keywords: Angiogenesis, bile duct, cirrhosis, ischemia, proliferation

Introduction

Primary biliary cirrhosis (PBC), involving the bile duct after orthotopic liver transplantation (OLT), have been a main problem. Biliary disorders occur approximately 15% in the patients undergoing OLT [1]. There are various biliary complications after liver transplantation. Due to the unique characters of blood supply for the intrahepatic bile ducts, they often cause biliary ischemic injury. While the blood supply of the liver is depended on artery and portal vein, the bile duct only receives its blood supply from artery, which will cause that bile ducts are extremely sensitive to ischemic injury. In liver transplantation, liver resection and hepatic arterial chemotherapy, and biliary ischemic complications are important factors for the failure of the treatment.

Previous studies have found that the liver metastases, receiving transcatheter arterial chemoembolization (TACE), are easier to form tumors, which often occur in the normal liver and hepatocellular carcinoma [2]. Yu et al. also found that normal liver treated with TACE treatment is more susceptible to ischemic injury compared with the liver with biliary cirrhosis [3]. Isozaki et al. found that cirrhosis of the liver has a higher tolerance to ischemia than a normal liver when blocking the hepatic artery [4].

All the information suggests that cirrhosis may have a higher tolerance for liver ischemia. Beausser et al. found early ischemic changes appear in rat with prolonged ischemia including biliary proliferation and cholestasis disappearance [5]. Morphological studies showed that proliferation of bile duct and angiogenesis are the main characteristic changes of the liver [6]. We propose thatbiliary proliferation and angiogenesis may be a compensatory performance for cirrhosis by increasing its tolerance to ischemia. Our aim is to verify whether cirrhosis has a high tolerance to ischemia by the proliferation of bile duct and angiogenesis, which are compensatory functions for the damage of cirrhosis.
Materials and methods

Reagents

CK19 monoclonal antibody was from Sigma (St. Louis, MO, USA). CD34 monoclonal antibody and Ki67 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Establishment of a rat model with biliary cirrhosis

All experimental procedures were approved by Animal Ethic Committee of Nanning Medical University. Sixty-four male SD rats (200-250 g) were purchased from the Experimental Animal Center of Nanjing Medical University and given free water, standard food and keep at room temperature for 24 ± 2°C [7]. A cirrhosis model was induced in 32 male SD rats using 50% of the carbon tetrachloride by subcutaneously injection of olive oil at 0.2 mL/100 g twice weekly. After 12 weeks, pathological examination showed obvious cirrhosis (Figure 2).

Groups

Sixty-four rats were randomly divided into two groups (n = 32): control group (with normal
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Liver); cirrhosis group (the animals with cirrhosis). All rats were underwent double ligation division of the main hepatic artery and double ligation of the extrahepatic peribiliary vascular plexus. This was conducted under cover of bile duct cannulation with 3-mm length and 0.3-mm diameter. Each group was further divided into 0 h, 6 h, 3 d and 14 d subgroups (n = 8).

All the rats were injected subcutaneously with 1% pentobarbital anesthesia at 0.5 mL/100 g. With incision of the abdomen, liver ligament

Figure 3. Bilirubin levels in a rat with bile duct ischemia. A. TBIL levels in a rat with bile duct ischemia. B. DBIL levels in a rat with bile duct ischemia. Data were expressed as mean ± standard deviation (SD). *P < 0.05 via 0 day and #P < 0.05 via a normal liver as a control.

Figure 4. Biochemical parameters of a rat with bile duct ischemia. A. ALP activities in a rat with bile duct ischemia. B. ALT activities in a rat with bile duct ischemia. C. AST activities in a rat with bile duct ischemia. Data were expressed as mean ± standard deviation (SD). *P < 0.05 via 0 day and #P < 0.05 via a normal liver.
were separated as much as possible, in order to cut off the collateral circulation of liver, and the common bile duct and hepatic artery were separated. Different surgical procedures were taken in different groups. In a sham group, the abdomen was closed after complete hemostasis. In the group with ischemia of bile duct, the hepatic artery and a vascular plexus around the extrahepatic bile ducts was ligated according to a previous report [8]. In the bile duct, “V” shaped incision was made and PE10 catheter (long 5 mm, the inner diameter of 0.28 mm) was placed at both ends of the ligated bile duct (Figure 1). The method blocked blood supply for the extrahepatic bile duct rounding perivascular plexus while maintaining the patency of the bile duct. Thus, in ischemia group, intrahepatic artery was completely lost.

Biochemical assay of liver tissue

Blood and liver tissue were taken from all animals after surgery 0 h, 6 h, 3 d and 14 d. Blood samples were centrifuged and supernatants were collected. After that, total bilirubin (TBIL), direct bilirubin (DBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using a DX800 automatic biochemical analyzer (Beckman Coulter, Beckman, CA, USA).

Pathological and immunohistochemical assay

Liver tissues were cut as 4-µm tissue sections, fixed in 4% formalin, stained with HE, Masson and immunohistochemical staining (diaminobenzidine, brown) [5]. Slices were stained with different antibodies, normal serum was used as a negative control, and the brown showed the positive cells [9]. Each slice was observed at least no overlapping vision. CD34 was stained to calculate the microvessel density (MVD) and percentage of duct cells was marked by Ki67 and CK19 expression [10, 11].

Statistical method

SPSS16.0 statistical software was used for data processing. Quantitative data were expressed as mean ± standard deviation (SD) between groups using two-way ANOVA. It is considered as a significant difference if \( P < 0.05 \).

Result

Evaluation of a rat model with cirrhosis

A rat model with cirrhosis was established using carbon tetrachloride after 12-week induction. Obvious nodular cirrhosis was visible (Figure 2A) and pseudolobule formation was observed under an optical microscope (Figure 5).
The results showed that a rat model with cirrhosis was successfully established.

**Biochemistry characters of the rats with bile duct ischemia**

Bilirubin is a tetrapyrrole and derived from hemoglobin degradation. In the serum, bilirubin is measured as both TBIL and DBIL [12]. TBIL is associated with unconjugated and conjugated bilirubin. DBTL is associated with conjugated bilirubin, including the conjugated bilirubin and bilirubin covalently bound to albumin. In a normal rat with bile duct ischemia, TBIL levels increased significantly after 3-day surgery. After 14-day surgery, the levels continued to rise (Figure 3A). In a cirrhosis rat with bile duct ischemia, TBIL levels increased significantly after 0.25-day surgery. The levels began to decline after 3-day surgery. After 14-day surgery, the levels declined to the levels before the surgery (Figure 3B). After 3-day surgery, TBIL and DBIL levels were higher in a normal rat with bile duct ischemia than those in a cirrhosis rat with bile duct ischemia. The results suggest that a cirrhosis rat had a better ischemic tolerance than a normal rat since serum DBIL and TBIL can reflect the of ischemic severity [13].

ALP is a hydrolase enzyme, which is responsible for removing phosphate groups from various molecules, such as nucleotides, proteins and alkaloids so on. In a normal rat with bile duct ischemia, ALP levels increased significantly after 3-day surgery. After 14-day surgery, the levels began to decline. In a cirrhosis rat with bile duct ischemia, ALP levels increased significantly after 0.25-day surgery. The levels began to decline and reached the levels as before the surgery after 3-day surgery. After 14-day surgery, the levels were the same as that before surgery (Figure 4A). Comparatively, ALP levels were lower in a cirrhosis rat that those in a normal rat after 14-day surgery while increases of ALP raise the risk of hepatobiliary disease [14]. The result suggested that hepatobiliary disease became serious in a normal liver with bile duct ischemia compared with a cirrhosis liver.
ALT is a transaminase enzyme and most common in the liver. ALT is often measured as a part of a diagnostic evaluation for hepatocellular injury. Similarly, AST is commonly used to detect liver damage. In a normal rat with bile duct ischemia, ALT and AST levels increased significantly after 0.25-day surgery. After 3-day surgery, the levels decreased significantly. After 14-day surgery, the levels continued to decline to a lower level (Figure 4B). In a cirrhosis rat with bile duct ischemia, ALT levels increased significantly after 0.25-day surgery. The levels began to decline after 3-day surgery. After 14-day surgery, the levels declined to the levels as before the surgery (Figure 4C). Comparatively, ALT and AST levels were lower in a cirrhosis rat after 14-day surgery while increases of ALT and AST raise severity of hepatobiliary disease [15]. Thus, the results suggested that hepatobiliary disease became serious in a normal liver with bile duct ischemia compared with a cirrhosis liver.

**Adaptive bile duct proliferative response in bile duct ischemia**

Ki67 is the proliferative cell marker of bile duct [16]. Ki67 was examined using immunohistochemistry to represent proliferation of bile duct cell. Figure 5A and 5B showed immunohistochemistry analysis of Ki67 expression in bile duct cells from normal rats with bile duct ischemia after 0-d, 3-d and 14-d surgery; Figure 5C and 5D showed immunohistochemistry analysis of Ki67 expression in bile duct cells from cirrhosis rats with bile duct ischemia after 0-d, 3-d and 14-d surgery. All the results showed that same trend: the Ki67 levels were higher in a cirrhosis rat than those in a normal rat. Thus, all the results suggested that cirrhosis promoted the proliferation of bile duct compared with a normal rat.

CK19 belongs to a family of keratins and normally expressed in the lining of the hepatobiliary tracts. CK19 is activated during human liver regeneration [17]. CK19 was examined using immunohistochemistry to represent proliferation of bile duct cell. Figure 6A-C showed immunohistochemistry analysis of CK19 expression in bile duct cells from normal rats with bile duct ischemia after 0-d, 3-d and 14-d surgery; Figure 6D-F showed immunohistochemistry analysis of CK19 expression in bile duct cells from cirrhosis rats with bile duct ischemia after 0-d, 3-d and 14-d surgery. All the results showed that same trend: the CK19 lev-
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els were higher in a cirrhosis rat than those in a normal rat. Thus, all the results suggested that cirrhosis promoted the proliferation of bile duct compared with a normal rat.

Adaptive angiogenesis response in bile duct ischemia

CD34 is an endothelial cell marker to study vascular proliferation of bile duct [18] and often used to study the proliferation of the peribiliary vascular plexus (PEP) [19]. Furthermore, angiogenesis in bile duct can be measured based on the expression of CD34 because the microvessel density can be estimated by CD34 [20]. CD34 was examined using immunohistochemistry to represent proliferation of bile duct cell. Figure 7A-C showed immunohistochemistry analysis of CD34 expression in bile duct cells from normal rats with bile duct ischemia after 0-day, 3-day and 14-day surgery; Figure 7D-F showed immunohistochemistry analysis of CD34 expression in bile duct cells from cirrhosis rats with bile duct ischemia after 0-day, 3-day and 14-day surgery. All the results showed that same trend: the CD34 levels were higher in a cirrhosis rat than those in a normal rat. Thus, all the results suggested that cirrhosis liver with ischemia promoted the angiogenesis in bile duct compared with a normal rat.

Biloma

In all animals, a total of three macro-biloma cases were found in normal rats with bile duct ischemia, among them both in bile duct after 14-d surgery. Macro-biloma was observed under a microscope in a normal rat with bile duct ischemia after 2-d surgery (Figure 8B). A 5.5-cm size of biloma was observed in a normal rat with bile duct ischemia after 14-d surgery (Figure 8A).

Discussion

Arterial blood supply is the main way for blood supply of bile duct and provides more than 50% oxygen for the liver. Except the hepatic artery, peribiliary vascular plexus (PBP) is another way for the blood supply of bile duct [5]. PBP stems from the hepatic arteries and flows into the hepatic sinusoids, which nourishes the biliary tree and plays a fundamental role in supporting oxygen for bile duct. Bile duct cells lack antioxidant enzymes, such as catalase and superoxide dismutase while bile duct cells expose to detergent bile. Thus, bile duct cells are more sensitive to hypoxic-ischemic injury. Ischemic injury is the main biliary complications after liver transplant.

Isozaki et al. found that surgical patients undergoing liver surgery via hepatic artery occlusion, the necrosis of liver parenchyma in the patients with cirrhosis is lighter than the patients without cirrhosis. The levels of AST increased, suggesting that the patients with cirrhosis has higher ischemia tolerance than the patients without cirrhosis [4]. Sakamoto et al. reported that formation rate of intrahepatic biloma was 9.6% in the group with metastatic gall tumor, and is significantly higher than the 3.3% in the...
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HCC group after Transarterial Chemoembolization (TACE) treatment of liver cancer [2]. Metastases occur in normal liver, more likely in the liver bile duct with biliary ischemic injury. Yu et al. analyzed 12 cases of ischemic injury associated bile duct, bile duct injury occurs in normal liver were significantly higher than the liver cirrhosis [3].

To investigate the mechanism for ischemic tolerance in a cirrhosis liver, we established a rat model with bile duct ischemia. The binding of the hepatic artery blocked the arterial traffic among extrahepatic bile duct, intrahepatic bile duct and the collateral ligament. The levels were lower in the bile duct from a cirrhosis rat model than that from a normal rat after ischemic surgery for the biochemical indexes of ischemic severity, such as total bilirubin (TBIL) and direct bilirubin (DBIL) (P < 0.05) (Figure 3). The results suggest that a cirrhosis liver has a better ischemic tolerance than a normal liver after ischemic surgery. In our study, three cases of macro-biloma occurred in the normal liver with bile duct ischemia and no one in the cirrhosis with bile duct ischemia. In a liver with cirrhosis after ischemia surgery, serum bilirubin increased immediately, but later returned to the levels as before the surgery. Comparatively, the levels of serum bilirubin increased significantly in a normal liver with bile duct ischemia after two-week surgery (Figure 3). Our study supports the liver with cirrhosis has higher tolerance to ischemia than the normal liver without cirrhosis.

Our research shows that cirrhosis promotes the proliferation of bile duct (Figures 5 and 6) and may play a compensatory function. Thus, biliary damage caused by ischemia seems lighter than that from a normal liver with bile duct ischemia. Meanwhile, ischemic injury is reduced after bile duct proliferation. In a normal liver with bile duct ischemia, serious injury can be observed because there is no compensatory proliferation of bile duct. In our experiment, the proliferation marker of the bile duct, Ki67 and CK19, showed that a liver with cirrhosis has more active proliferation than a normal liver with bile duct ischemia (Figures 5 and 6).

There are more abnormal arteriovenous traffics in a liver with cirrhosis [6, 21], which may offer enough blood supply and reduce ischemic injury. Our research shows that an angiogenesis occur by the expression of CD34 in bile duct from a liver with cirrhosis when we block the blood supply (Figure 7). PBP nourishes the bile duct because hepatic artery and portal vein exist in collateral cycle. Chen et al. reported the occurrence of hepatocellular carcinoma has several times higher than a liver with cirrhosis after TAE treatment, which causes biloma because TAE treatment leads to the obstruction of periductal microvascular. However, biliary blood still cannot compensate the loss and bile duct injury becomes serious [22], which suggests angiogenesis around bile duct plays an important role in the blood supply of liver. Gaudio et al. found that biliary cirrhosis is associated with bile duct proliferation in a cirrhosis model. The proliferation can meet the needs of nutrition and function of bile duct. Meanwhile, PBP has also undergone a proliferation around the bile duct [6]. Therefore, cirrhosis is characterized by the proliferation of bile duct and angiogenesis, which may be functional response to hypoxia. Our study also found that bile duct and PBP proliferated greatly in a liver with cirrhosis. Comparatively, bile duct injury is serious in a normal liver with bile duct ischemia. The proliferation of bile duct and PBP appears weak even after two-week surgery.

We found that the proliferated PEP communicates with each other in a liver with cirrhosis. The bile flow is in an opposite direction with the blood flow in PBP around bile duct. The bound for the blood supply of the bile duct blocks the metabolism of bile acid. After the ischemic surgery, serum bilirubin increases in a short period, bile can be secreted in a good way, which also confirms the intrahepatic venous collaterals play a protective role after biliary ischemia. Beausnier also found bile duct proliferation duct plays an important role in bile secretion and absorption [5]. From above information, cirrhosis of liver bile ducts and blood vessels has protective function in arterial ischemia. New arteriovenous traffic can be created and enough oxygen can be obtained via the proliferation of bile duct. In contrast, bile duct damage becomes serious in a normal liver with cirrhosis without the compensations such as the proliferation of bile duct and angiogenesis.

In sum, the levels were lower in the bile duct from a cirrhosis rat model than that from a normal rat after ischemic surgery for the biochemical indexes of ischemic severity, such as total
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bilirubin (TBIL) and direct bilirubin (DBIL) (P < 0.05). The levels were higher for Ki67 (a biomarker for the proliferation of bile duct) and CD34 (a biomarker of angiogenesis) in the bile duct from a cirrhosis rat model than that from a normal rat after an ischemic surgery (P < 0.05). The proliferation of bile duct and angiogenesis has compensatory function in a liver with cirrhosis after ischemia, which can reduce bile duct injury and enhance ischemic tolerance. More research on the pathogenesis of ischemic bile duct lesions (IBDLS) is needed for developing more specific preventive or therapeutic strategies.

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Disclosure of conflict of interest

None.

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