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Cholangiocytes of Cirrhotic Rats are More Tolerant to Ischemia than Normal Rats: A Role for Abnormal Hepatic Arteriovenous Communications?

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Background. **To assess whether cholangiocytes of rats with liver cirrhosis are more tolerant to ischemic changes than normal rats, and whether this is due to arteriovenous fistulas.**

Methods. **Ninety-eight Sprague-Dawley rats were divided into the normal group ($n = 30$) and the cirrhosis group ($n = 68$), and then each group was divided into controls and those with bile duct ischemia. At 0 h, 6 h, 3 d, and 14 d after the induction of bile duct ischemia, the liver of each rat was removed and stained with toluidine blue to compare cholangiocyte morphology. Cholangiocyte apoptosis was evaluated by a deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay. Expression of VEGF, HIF1, NF-kB(p65) was assessed by quantitative analysis of the products of reverse-transcriptase polymerase chain reaction. Resin casts were used to reproduce the intrahepatic vasculature of cirrhotic rats, and the presence of communications between the portal vein and hepatic artery was assessed with stereomicroscopy.**

Results. **Rats with liver cirrhosis were more tolerant than normal rats 6 h after bile duct ischemia ($P < 0.05$); at 3 and 14 d after the ischemic insult, there was no significant difference between the cirrhotic rats and normal rats. Levels of expression of VEGF, HIF1, and NF-kB(p65) genes, either in normal rats or cirrhotic rats, were significantly elevated compared with those in the control group (*, **$P < 0.05$), but a lower extent changes appeared in the cirrhotic rats (***$P < 0.05$). Several communications could be observed between the portal vein and hepatic artery.**

Conclusion. **Cholangiocytes of cirrhotic rats appear to be more tolerant to ischemia of bile duct than noncirrhotic rats. This may be due to the protective role of arterioportal fistulas.**

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Key Words: **arterioportal fistula; liver cirrhosis; cholangiocytes; ischemia; rat model.**

INTRODUCTION

Biliary complications of orthotopic liver transplantation, hepatic artery embolization, or hepatic artery chemoembolization comprise the most important factors that affect operative success [1, 2], with important anatomical relationships to ischemia of the bile ducts [3]. It has been demonstrated that patients with liver cirrhosis are more tolerant to ischemic changes than patients with preoperatively normal livers [4, 5]. This difference in response to ischemia prompts us to recognize whether the cholangiocytes of cirrhotic liver are more tolerant to ischemia of bile duct than normal liver. We cannot ignore that patients with hepatocellular carcinoma can develop arterioportal fistulas, as demonstrated by color Doppler ultrasonography, DSA, and CT [6, 7], which play a protective role after hepatic artery embolization [8]. However, it is not clear whether the abnormal existence of communicating branches between the arterial and venous systems is the reason for this difference. This study aimed to verify whether cholangiocytes in cirrhotic rats are more tolerant to ischemia than normal rats, and whether this was due to arteriovenous connections as a potential mechanism to explain these findings.

MATERIALS AND METHODS

Animals

The experiments were conducted in accordance with guidelines approved by the Chinese Association of Laboratory Animal Care, and protocols were approved by the Animal Ethics Committee of Nanjing Medical University. Ninety-eight adult male Sprague-Dawley (SD) rats (weight, 180 ± 20 g; SLAC Experimental Animal Co., Shanghai,

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China) were used in these experiments. The rats were housed in the Laboratory Animal Center of Nanjing Medical University with a 12-h light/dark cycle, with food and water available *ad libitum*.

Experimental Design and Surgical Procedure

The Sprague-Dawley rats were divided into the normal group ($n = 30$) and the cirrhosis group ($n = 68$). Cirrhosis was induced in the 68 rats in the latter group by the administration of phenobarbitone (35 mg/dL) diluted in tap water. This was the only source of drinking water for 14 d before the initiation of the experiment; the rats were also injected subcutaneously with carbon tetrafluoride (CCl_4; 1:1 in olive oil) at a dose of 0.2 mL/100 g body weight (BW) twice-weekly. Ten weeks after induction, the pathology showed the presence of manifest cirrhosis ($n = 41$). Thirty normal rats were used to establish normal bile duct ischemia model, and 30 rats with liver cirrhosis were used for the establishment of the bile duct ischemia.

Established Animal Model of Bile Duct Ischemia

Sprague-Dawley male rats were anesthetized using 1% sodium pentobarbital (0.5 mL/100g, intraperitonerally). After skin preparation and disinfection, a midline incision was created into the abdominal cavity, and the ligaments around the liver were separated as much as possible. Then, the bile duct was isolated and retracted by a silk thread. The hepatic artery was then ligated and transected. A 'V'-shaped incision was made in the bile duct wall, and a PE10 tube (approximate length, 5 mm) was placed into the bile duct. At both ends of this tube, the bile duct were ligated to block the blood supply from the extrahepatic peribiliary plexus (Fig. 1). After complete hemostasis was achieved, the abdomen was closed.

Evaluation of Cholangiocyte Necrosis

All rats used to establish the animal model, whether from the normal or cirrhotic groups, were subsequently killed at 0 h, 6 h, 3 d, and 14 d after the induction of bile duct ischemia ($n = 5$ in each group). Liver blocks (1–2 cm^2) were taken from each animal and fixed in 4% buffered formalin. We evaluated cholangiocyte necrosis by toluidine blue staining of the paraffin-embedded liver sections (three slides evaluated/animal, 5 μm thick), which were examined by light microscopy (OLYMPUS CKX41; Tokyo, Japan). At least 10 different portal areas were evaluated for necrosis. One hundred cells per slide were counted in a coded fashion in 10 non-overlapping fields [9].

In Situ Apoptosis Detection

Cholangiocyte apoptosis was evaluated by the TUNEL analysis [10, 11]. The liver sections were stained by hematoxylin/eosin, and examined for apoptosis under a light microscope. TUNEL analysis was performed using a commercially available kit (KeyGen Biotech. Co. Ltd., Nanjing, China).

RNA Isolation and Reverse Transcriptase PCR

Liver blocks removed form each animal at different time points, whether from the normal or cirrhotic groups, were immediately frozen in liquid nitrogen. Total RNA was isolated by Trizol extraction, which is a common procedure in microarray experiments [12]. Reverse-transcriptase reaction was performed with first strand cDNA synthesis kit (ReverTra Ace, TOYOBO,Osaka, Japan), polymerase chain reaction was performed using β-actin gene as an internal control to standardize comparison. Primer sequences of genes were as follows: VEGF, 307bp 5′- CCCTCGT GGAACTGGATTCGC-3′ (forward) and 5′- TCCCATTGT CTTCCCTGTCGG-3′ (reverse); HIF1, 76bp 5′- TAAGAAAGAGCCCG ATGCCC-3′ (forward) and 5′-GCTGCCGAAGTCCAGT GATA-3′ (reverse); NF-kB(p65), 98bp 5′- CCTG GAGCAAGCCATTAGCC-3′ (forward) and 5′-CGGACCGCATTCAAGTCATAG-3′ (reverse); β-actin, 250bp 5′- CACGATGGAGGGGCCGGACTCATC-3′ (forward) and 5′-TAAAGACCTCTATGCCAACACAGT-3′ (reverse).

Hepatic Microvascular Corrosion Casting and Stereomicroscopy

Five Sprague-Dawley rats with liver cirrhosis (350–450 g, male) were used for this study. They were anesthetized by barbiturates, and 30 min after systemic heparinization, the abdominal aorta and subhepatic inferior vena cava were opened and allowed to bleed; the thoracic aorta and inferior vena cava located above the liver were both ligated closed. A catheter was introduced into the abdominal aorta and physiologic saline solution warmed to 37°C was perfused *via* this catheter until the effluent from the inferior vena cava was saline that was completely clear of blood. The portal vein and hepatic artery were manually injected with approximately 15 and 3 mL, respectively, of a low-viscosity liquid epoxy resin (Sanmu Group Co., Ltd., Jiangsu, China) until no more could be injected. The liver was removed and placed in an incubator at 40°C for 24 h. The liver was then macerated for 3–5 d in a solution of 50% H_2SO_4. Samples were rinsed in distilled water and dried at room temperature. Each sample was observed under a stereomicroscope (Zeiss, Lumar.V12; Jena, Germany) to assess whether there were any abnormal communicating arteriovenous communications.

Statistical Analysis

Paired Student's t-test was used to determine the significance in the differences between two group means. One-way ANOVA was used to assess significant differences among treatment groups. Data represent the mean ± SE. Differences were considered to be statistically significant at the level of $P < 0.05$.

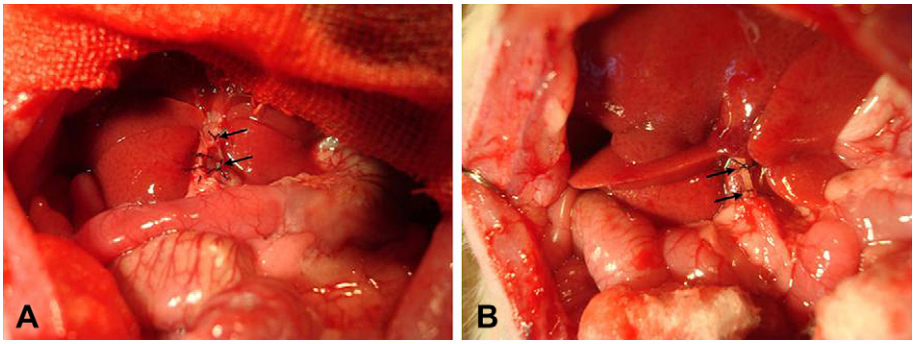

FIG. 1. An animal model of bile duct ischemia was established by ligating the hepatic artery (arrow A), blocking the blood supply of the extrahepatic peribiliary vascular plexus (arrow B).