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Splenectomy after partial hepatectomy accelerates liver regeneration in mice by promoting tight junction formation via polarity protein Par 3-aPKC

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ABSTRACT

Aims: Several experimental studies have demonstrated that removal of the spleen accelerates liver regeneration after partial hepatectomy. While the mechanism of splenectomy promotes liver regeneration by the improvement of the formation of tight junction and the establishment of hepatocyte polarity is still unknown.

Main methods: We analyzed the cytokines, genes and proteins expression between 70% partial hepatectomy mice (PHx) and simultaneous 70% partial hepatectomy and splenectomy mice (PHs) at predetermined timed points.

Key findings: Compared with the PHx group mice, splenectomy accelerated hepatocyte proliferation in PHs group. The expression of Zonula occludens-1 (ZO-1) indicated that splenectomy promotes the formation of tight junction during liver regeneration. TNF-α, IL-6, HGF, TSP-1 and TGF-β1 were essential factors for the formation of tight junction and the establishment of hepatocytes polarity in liver regeneration. After splenectomy, Partitioning defective 3 homolog (Par 3) and atypical protein kinase C (aPKC) regulate hepatocyte localization and junctional structures in regeneration liver.

Significance: Our data suggest that the time course expression of TNF-α, IL-6, HGF, TSP-1, and TGF-β1 and the change of platelets take part in liver regeneration. Combination with splenectomy accelerates liver regeneration by improvement of the tight junction formation which may help to establish hepatocyte polarity via Par 3-aPKC. This may provide a clue for us that splenectomy could accelerate liver regeneration after partial hepatectomy of hepatocellular carcinoma and living donor liver transplantation.

1. Introduction

Liver resection is the only curative treatment option for several neoplastic entities of the liver [1]. Living donor liver transplantation is one of the most promising treatments for end-stage liver disease patients. However, postoperative failure of liver regeneration and concomitant liver dysfunction remains an important issue after partial hepatectomy [2]. Although mechanisms of liver regeneration after partial hepatectomy have been explored intensively in vivo, no therapeutic tools are available to accelerate liver regeneration in the clinical setting. Some studies have demonstrated that removal of the spleen accelerates liver regeneration after partial hepatectomy [3,4], indicating that the spleen plays a certain role in liver regeneration. However, whether the spleen involves in the restoration of the original liver mass, structure, and function including the formation of tight junction and establishment of hepatocyte polarity in liver regeneration remains unknown. Investigation of this issue is expected to be helpful in finding a way to accelerate liver regeneration.

It is already known that the correct function of hepatocytes requires the establishment and maintenance of hepatocyte polarity. In polarized hepatocytes, the tight junctions create the border between apical and lateral poles [5,6]. Partitioning defective 3 homolog (Par 3) protein is essential for asymmetric cell division and polarized growth, whereas CDC42 mediates the establishment of cell polarity. PKCζ (genes Prkcz), a member of the atypical protein kinase C (aPKC) subfamily [7], mediates a variety of physiological events, such as cell polarity establishment and cell motility [8]. Thus, we pay attention to whether the polarity protein Par 3 and PKCζ take part in the restoration of the tight junction in liver regeneration.

Here, using a 70% partial hepatectomy mouse model, we investigated the mechanism how splenectomy may be implicated in the process of the tight junction formation and the establishment of

Abbreviation: ZO-1, Zonula occludens-1; Par 3, Partitioning defective 3 homolog; aPKC, atypical protein kinase C; PHx, 70% partial hepatectomy; PHs, simultaneous 70% partial hepatectomy and splenectomy

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hepatocyte polarity in liver regeneration.

2. Materials and methods

2.1. Animal studies

Male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were maintained in a specific pathogen-free facility, fed ad libitum on a standard pellet diet and pure water at the animal experimental center of the second Xiangya Hospital of Central South University. The animals were randomly distributed into two groups: animals that received 70% partial hepatectomy only (PHx group) and animals that received simultaneous 70% partial hepatectomy and splenectomy (PHs group). Five animals were assigned to each of the six time points (0, 24, 48, 72, 168, and 336 h after operation) in each group (total of 30 animals per group). The liver specimens were harvested after sacrifice. Postoperative bodyweight and liver weight were measured before sacrifice. The experimental protocol was approved by the Central South University Animal Care and Use Committee.

2.2. Surgical procedures

Eight-week-old male C57BL/6 mice weighing 20–25 g were anesthetized via a sodium pentobarbital (50 mg/kg of body weight) intraperitoneal injection. In the PHx group, the median and left lateral hepatic lobes, which comprise 70% of the liver weight, were resected, while the caudate and right lobes were left intact, as described previously [9]. In the PHs group, simultaneous splenectomy was carried out; the spleen specimen was removed after ligation of the splenic artery and vein using 4-0 silk.

2.3. Mouse blood sample collection and biochemical measurements

Blood samples from each mouse were obtained at the specified timed points after operation (0, 24, 48, 72, 168, and 336 h). The samples with the addition of heparin were used for the assessment of platelet count. The samples without the addition of heparin were centrifuged for 10 min at 10000 rpm, and serum was collected. We used 50 μL serum for measuring the serum concentrations of aspartate transaminase (AST), alanine aminotransferase (ALT), direct bilirubin (DBIL), total bilirubin (TBIL) with an IDEXX Vet Test Chemistry Analyzer (IDEXX Laboratories Inc.). The residual serum stored at − 80 °C and used for the follow-up experiment.

2.4. ELISA

Blood serum was obtained at the specified time points after operation (0, 24, 48, 72, 168, and 336 h) from PHx and PHs mice. The concentrations of TNF-α, IL-6, HGF, TSP-1, and TGF-β1 in the serum were measured ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.5. Primary hepatocyte culture and cell viability assay

Primary hepatocytes were prepared from male C57BL/6 mice using the collagenase (Gibco) perfusion method [10]. Cell viability was measured by MTT assay. Primary hepatocytes (1×10^4 cells per each well) were cultured in Collagen-coated 96-well plates for 24 h, 5 μL of serum obtained from mice of the five timed points (0, 24, 48, 72, and 168 h after operation) in each group were added, and cells were then incubated for an additional 48 h. After removing the medium, the cells were stained with 20 μL sterile MTT (5 mg/mL) (Sigma-Aldrich) for 4 h at 37 °C, The resulting formazan crystals were then dissolved by adding 150 μL DMSO (Sigma-Aldrich). The optical density was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). Each experiment was repeated at least three times independently.

2.6. Assessment of 5-bromo-2-deoxyuridine incorporation

Mice were injected intraperitoneally with 100 mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) 2 h prior to euthanasia. After sacrifice, the liver tissue sections (5 μm) were prepared. Tissue sections were incubated with a biotinylated anti-BrdU antibody (1:500 dilution; Sigma-Aldrich). The DNA synthesis index was scored in at least 5 fields at 200 × magnification and reported as mean ± SD. Five mice were used in each group.

2.7. Immunohistochemistry

For histological analyses, the liver sections were incubated with diluted primary antibodies for Ki-67 (1:500 dilution; Abcam). Diaminobenzidine solution was used as chromogen, followed by counterstaining with Mayer's haematoxylin. Negative controls for immunostaining were prepared by omitting the primary antibody. Cells with positive staining were scored in at least 5 fields at 200 × magnification and the data were presented as the mean ± SD. Five mice were used in each group.

2.8. Immunofluorescence

Frozen liver sections (5 μm) were fixed with 4% paraformaldehyde, permeabilized by 0.2% Triton-X 100 (Sigma), and blocked with 2% bovine serum albumin. The sections were incubated overnight sequentially at 4 °C with primary antibodies: ZO-1 antibody (1:200 dilution; Thermo Fisher Scientific), Par 3 (1:100 dilution; Abcam), and PKCζ (1:100 dilution; Abcam) for overnight at 4 °C. The following day, the sections were treated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen) or Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (Invitrogen) diluted at 1:1000. Finally, sections were treated with 0.1% DAPI (Invitrogen) for nuclei staining and observed under a fluorescence microscope.

2.9. Transmission electron microscopy

Liver tissues obtained at various time points after operation (0, 72, 168, and 336 h) from PHx and PHs mice were sectioned into 0.1–0.2 mm^3 small chips and fixed in 2.5% glutaraldehyde. Transmission electron microscopy was used for examining, as described by Wack et al. [11]. Three mice were used in each group.

2.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

A 25-mg piece of liver tissue was homogenized in 1000 μL TRIzol®Reagent (Thermo Scientific). Total RNA was extracted according to the manufacturer's instructions. To measure mRNA expression, real-time PCR assay was performed using the LightCycler® 96 system (Roche). Amplification of the target genes was normalized using the amplification levels of GAPDH as the endogenous control. Data analysis and calculations were performed using the $2^{-\Delta\Delta CT}$ comparative method, as described by the manufacturer. The primers used were summarized in Table 1.

2.11. Western blotting

Whole cell liver lysates were prepared by homogenizing 50 mg of liver tissue in lysis buffer. 40 μg of protein from liver tissue lysates were electrophoretically separated on 10% sodium dodecyl sulfate (SDS)-gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). For western blotting, the membrane was incubated in primary antibodies: Par 3 (1:400 dilution; Thermo Fisher Scientific), ZO-1(1:400 dilution; Abcam) and PKCζ (1:400 dilution; Abcam). β-Actin