Experimental and Molecular Pathology 86 (2009) 101–107

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

The variation of AkT/TSC1–TSC1/mTOR signal pathway in hepatocytes after partial hepatectomy in rats

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ARTICLE INFO

Article history:
Received 13 August 2008
and in revised form 2 January 2009
Available online 5 February 2009

Keywords:
TSC
mTOR
Signal pathway
Partial hepatectomy
Liver regeneration

ABSTRACT

Objective: The aim of this study was to investigate the role and regulatory mechanisms of Akt/TSC1–TSC2/mTOR signal pathway on the hepatocyte growth and proliferation after partial hepatectomy in rats.
Methods: We used the animal model of 70% hepatectomy, separated and cultivated hepatocytes. According to the different time points after partial hepatectomy, it could be grouped into 0 h, 2 h, 6 h, 24 h and 72 h. According to the different kinds of specific inhibitor in the nutritive medium after the separation of hepatocytes, it could be grouped into Triciribine (TR), Rapamycin (RA) and Control (CO). We investigated ^{3}H-Leucine incorporation into protein, the cross section areas of hepatocytes, and detected cell cycle through FCM. The expressions of phosphorylated protein TSC2 and mTOR were observed.
Results: (1) The content of phosphorylated protein TSC2 in group CO began to increase at 2 h and got to the peak at 6 h but declined at 24 h. The content of phosphorylated protein TSC2 in group RA had the same variation with that of phosphorylated protein TSC2 in group CO. (2) At the time point of 0 h, 2 h, 6 h and 24 h after operation, the incorporation efficiency of ^{3}H-Leucine in groups RA and TR was different from that in group CO in statistics ($P<0.01$). (3) It could be seen that the cross section areas of hepatocytes in groups RA and TR were different from that in group CO in statistics at 2 h and 6 h after operation ($P<0.05$). (4) Comparing with the other two inhibitor groups (TR and RA), the number of cells during the period of G_0/G_1 in group CO became fewer, while the number of cells during the period of S and G_2/M grew obviously (referring to Fig. 8). After operation, each time point was different from the inhibitor groups obviously ($P<0.05$ or $P<0.01$). The peak declined greatly at 24 h and 72 h after operation.
Conclusions: These data strongly suggest the effects of Akt/TSC1–TSC2/mTOR signal pathway on hepatocyte growth, protein synthesis and cell cycle, and prove its contribution to liver regeneration.

Introduction

Under normal condition, only 0.0012%–0.01% of hepatocytes of adult animals carry out division growth. However, when the liver is damaged by intoxicant or resected in surgery, the cells will display strong regenerative potentiality. It is very complicated to regenerate hepatocytes. It is believed by most researches that liver proliferation is one of the most important mechanisms for liver regeneration. However, the contribution of cell growth during liver regeneration has not been revealed completely. Liver growth and proliferation are two concepts which are related to and distinct from each other. Cell growth refers to the augmentation of size or volume, while cell proliferation refers to the multiplication of numbers caused by cell division. They play very important roles in organ growth, tissue formation and tumor growth. Cell growth and proliferation are closely related to each other. A cell can carry out division and proliferation only when it has grown to some degree, or it will become smaller and smaller.

Recent studies show that Akt/TSC1–TSC2/mTOR is a conservative signal pathway on evolution, which exists extensively in fruit fly, fish and mammal. It plays an important role in protein synthesis, cell growth, cell proliferation and tumor development (Schmelzle and Hall, 2000). Two important signal molecules of this pathway, TSC2 and mTOR, play central regulation part (Manning and Cantley, 2003; Inoki et al., 2005). mTOR possesses the activity of protein activating enzyme and belongs to serine (threonine) protein kinase (Keith and Schreiber, 1995). Stimulated by growth signals, mTOR is controlled by Akt. mTOR can phosphorylate many kinds of target protein, including p70S6K 4EBP1 and 4EBP2. mTOR not only takes part in the regulation of protein synthesis and genetic transcription, but also has close relationship with cell cycle (Thomas and Hall, 1997). At present the contribution of Akt/TSC1–TSC2/mTOR signal pathway during liver regeneration after partial hepatectomy is still unclear.

Through the classic model of partial hepatectomy in rats established by Higgins, this research will separate and cultivate hepatocytes at different time points and observe the phosphorylated protein TSC2 and mTOR in hepatocytes after partial hepatectomy in order to discuss the influence of Akt/TSC1–TSC2/mTOR signal

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0014-4800/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.yexmp.2009.01.013

pathway on hepatocyte growth, protein synthesis and cell cycle and prove its contribution to liver regeneration.

Materials and methods

Materials

DMEM was provided by GIBCO Co., Ltd. Fetal bovine serum was obtained from Shanghai Biological Product Limited Company (China). Collagenase IV and polylysine were purchased from Sigma Co., Ltd., and Percoll from Pharmacia Co., Ltd. (US). Type IV collagenase was provided by Sigma Co., Ltd. and DNase I was purchased from Roche Co., Ltd. Triciribine (Akt specific inhibitor) was purchased from Calbiochem Co., Ltd. M-PER® protein extract reagent box and chemical emission reagent box were from PIERCE Co., Ltd. (US). Rapamycin and Phospho-mTOR (Ser2448) antibody were from Cell Signaling Technology Co., Ltd. p-tuberin (Thr 1462)-R was provided by Santa Cruz Biotechnology Co., Ltd.

Tripure, chloroform, dimethyl carbinol, DEPC water, ethidium bromide, 1% of ortho ester liquor and 0.1% of ortho ester liquor, 3H amidocaproic acid (Beijing Institute of Atomic Energy, obtained from nuclear medical central experiment of Third Military Medical University), Beckman LS 6500 events-per-unit-time meter, micropipette, ZT-II multicipital cell sample collector (Satellite Medical Equipment Made (ShaoXing) Co., Ltd.), 12 mm × 12 mm cover glass, Eppendorf 5804R refrigerated centrifuge, FACS 420 flow cytometry (BD, US), and a scanning electron microscope were made by AMRAY 1000B, US.

Animal

Male Sprague–Dawley rats (190 ± 10 g, body weight) were used with the approval of the Animal Care Committee of The Third Medical University. The animals were housed in a temperature- and humidity-controlled environment with a 12 h light/dark cycle. The study followed our institution's criteria for care and use of laboratory animals in research, which conform to the National Institutes of Health guidelines.

Operative procedure

The classic model of partial hepatectomy in rats established by Higgins & Aderson was used (Higgins and Anderson, 1931): The rats were fasted for 4 h before operation and anaesthetized with ether. The roots of left and middle lobe of liver were ligated and resected. After operation the rats were allowed free access to food and water in order to observe their survival condition. Forty male Sprague–Dawley rats and five time points, namely 0 h, 2 h, 6 h, 24 h and 72 h, were used in this experiment. Six rats were used in each time point to make sure six rats could survive in each group at each time point. Moreover, another 10 rats were taken for the measure of the hepatectomy rate to get the result of 69 ± 1.5%.

The separation and cultivation of the hepatocytes

It had been improved partly according to the separation methods in rat hepatocytes introduced by Seglen (1976) and Chen et al. (2006). 45% Percoll centrifugal purification was put to get high yields and high active hepatocytes. In brief, 200 homogenate of liver tissue after digestion was filtered and then rinsed by D-Hanks. Cell suspension was set in centrifuge tube of 50 ml and another 50 g on the Eppendorf 5804R refrigerated centrifuge and centrifuged for 5 min. The supernatant was removed, which was repeated twice. Then it was prepared with Percoll centrifugal liquid to get depurated hepatocytes.

The content of hepatocytes was measured through cell counter. The activity of hepatocytes was measured through Trypan blue dye exclusion test and the concentration of cells was turned into (1.0–1.5) × 10^6/ml. Hepatocytes were cultivated in a six-hole cultivated board (hepatocytes were enhanced to stick to cell wall closely through polylysine), in which 10% fetal calf serum DMEM was put. The cells were round under microscope and the refractive power was very good. Then they were put into incubator of 37 °C and 5% CO_2 to be cultivated. The nutritive medium was changed every 8 h in order to get on with cultivation. Two hours after separation, the morphology of hepatocytes was observed within 24 h.

Experimental groups

According to the different kinds of specific inhibitor in the nutritive medium after the separation of hepatocytes, it could be grouped into Triciribine (TR), Rapamycin (RA) and Control (CO). After the separated hepatocytes were cultivated at each time point for 2 h, and according to the concentration recommended by the introduction of Triciribine and Rapamycin, Triciribine was put into group TR to make its final concentration 20 μM and Rapamycin was put into group RA to make its final concentration 10 μM. An equal amount of DMEM nutrient medium was put into group CO.

Western blot

Referring to Chen's method (Chen et al., 2006), in brief, after the cells in every group (about 1 × 10^6) were cultivated for 24 h, the nutritive medium was removed and the cells were rinsed once with PBS. Then the cells were digested by 1 ml pancreatin and blown off, which were centrifuged in 1500 rpm for 10 min. The supernatant was removed, and the cells were re-suspended in 1 ml PBS and centrifuged in 1000 rpm for 10 min once again.

The electrophoresis gel was prepared to undertake SDS-PAGE electrophoresis: after I anti-incubation, a membrane was cleaned by TBS once and water twice and 3–5 min each time. After the cleaning, skimmed milk powder was used to prepare a second anti-incubation. Then the membrane was put into the second anti-incubation. The incubation lasted for 1.5–2.5 h. After the second anti-incubation, the membrane was cleaned by TBS third times and 3–5 min each time. Then it was colored (before coloration it must be cleaned completely).

The colored area and density was measured by image analysis software of Image-pro Plus 5.1.

^{3}H-Leucine incorporation into protein

In brief, after the hepatocytes separated at each time point were cultivated in an incubation box of 37 °C, 5% CO_2 for 2 h, a specific inhibitor was put into different groups accordingly. After being cultivated for 20 h, 10 μl (1 μ Ci) of ^{3}H-Leucine was put into every hole, which was stopped after being cultivated for 4 h. The cells were suction filtered and collected on cellulose acetate membrane by ZT-II

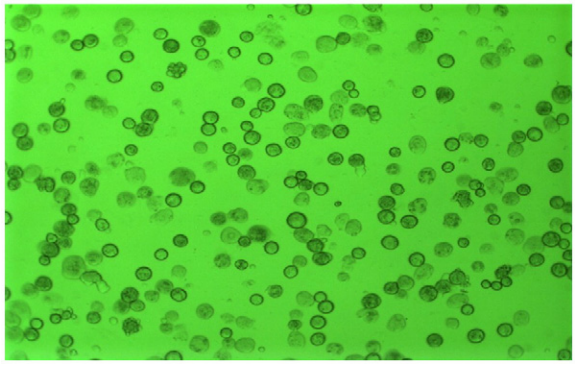

Fig. 1. Part of hepatocytes began to stick to the cell walls after being cultivated for 4 h (×200).