Deceleration of liver regeneration by knockdown of hepatic stimulator substance gene expression in mice

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Abstract. Hepatic stimulator substance (HSS), an active protein extracted from the liver of a newborn animal or from the residual liver after partial hepatectomy. HSS only stimulated the proliferation of hepatocytes or hepatogenic tumor cells, but had no stimulating effect on other tissue cells or non-hepatogenic tumor cells, indicating that its effect was tissue-specific. In addition, HSS can protect liver cells and reduce the damage of CCl4, galactosamine, thioacetamide and other drugs on liver cells. Although the hepatoprotective function of HSS has been understood to some extent, as a hepatogenic growth factor, the role of HSS in liver regeneration remains unclear and needs further study. To demonstrate if HSS plays a role in the regulation of liver regeneration and its possible mechanisms, we detect liver regeneration related index changes by knockdown of HSS after partial hepatectomy (PH). Results showed that knockdown of HSS lead to decreased liver regeneration, inhibit hepatocyte proliferation and impair mitochondrial. HSS can promote liver regeneration and protect liver cell function, and its protective mechanism may be related to the stabilization of liver cell membrane. In conclusion inhibition of HSS expression by shRNA during liver regeneration obviously delayed the process.

1. Introduction

As an important organ of human body, liver has complex and diverse biological functions, such as participating in substance metabolism, synthesis, storage, nutrient redistribution, and detoxification through biotransformation and bile secretion. In recent years, the incidence of liver cirrhosis and liver cancer caused by chemical factors and biological factors (such as hepatitis virus) has increased year by year. Once the liver is damaged or diseased, it will seriously affect the body function and even threaten life. After partial liver resection, how to effectively stimulate the regenerative potential of residual hepatocytes is the key to the survival of postoperative patients.

Nowadays, the research on the regulation of liver regeneration is deepening to the cellular level and molecular level, and the regulation of growth factor/cytokine on liver regeneration is the focus of scientists. HSS is a thermally stable polypeptide substance. It is acid-resistant, alkali-resistant, and can be destroyed by trypsin and chymotrypsin, suggesting that it is a protein, while neuraminidase does not affect HSS activity, suggesting that it is not a glycoprotein. HSS may be an endogenous growth stimulating factor. The biological function of HSS is reflected in that it can specifically stimulate the proliferation of hepatogenic tumor cells in vitro [1], stimulate the proliferation of residual liver after subtotal hepatectomy [2], and enhance hepatocyte repair in acute liver failure models [3]. HSS can also reduce the damage of CCl4, galactosamine, thioacetamide, H2O2, calcium overload and other harmful substances on liver cells [4-6]. This protective effect is generally believed to be mainly related to the promotion of DNA synthesis and hepatocyte regeneration by HSS, but the more detailed mechanism remains to be further studied [7].

2. Materials and Methods

2.1 Animals and partial hepatectomy (PHx)

C57/BL6J mice, SPF grade, male, weighing 20 to 25 grams. Provided by Department of Zoology, Academy of Military Medical Sciences.C57 male mice were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.01 ml/g) after weighing. After successful anesthesia, the limbs were supine and immobilized on a surgical board. Shave the skin at the conventional incision. According to the classical method of Higgins et al. [1], the weight was taken (the mass of the left lobe and middle lobe of the liver removed accounted for about 70% of the mass of the original liver), the abdominal cavity was closed, and the incision was sutured by layer.

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All the models were made from 8:00 am to 12:00 am. Aseptic operation was performed.

2.2 Calculation of relative liver-weight and recovery

The mouse relative liver weight was estimated based on the following formula: % =100% × Ma/Mb; where Ma is the weight of liver at killing, and Mb is the mouse body weight.

2.3 Liver function testing

After opening the abdominal cavity of the mice, the intestinal organs and other organs were removed with tweezers to expose the inferior vena cava, blood was taken with a syringe, and then the blood was placed in the serum tube at room temperature for more than 1 h, centrifuged, 1800 × g, 4℃, 10 min. Remove supernatant and store at -80℃ in refrigerator. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) were assayed using an autoanalyser in the Clinical ChemistryLaboratory of CapitalMedical University, Beijing.

2.4 shRNA Construction

Target sites for mice HSS mRNA (accession number: NM_023040 were selected using RNAi design sites (Genepharma, China, Shanghai). The target sites were as follows: HSS shRNA (434-454), (Table 1). Plasmids were administered via portal vein immediately after PHx.

Table 1. Nonsilencing and HSS shRNA Sequences Used in the In Vivo Experiment

shRNA	Sequence
HSS shRNA	GCCAGAACAACAACAGGATAT
	ATATCCTGTTGTTGTTCTGGC
NC shRNA	GTTCTCCGAACGTGTCACGT
	ACGTGACACGTTCGGAGAAC

2.5 Immunohistochemistry

To quantity the numbers of proliferating cells, proliferating cell nuclear antigen (PCNA) was detected by immunohistochemistry using anti-PCNA (Cell Signaling Technology, Beverly MA, USA). In brief, after deparaffinizition and rehydrated, the tissue sections were prewarmed with 0.1% trypsin solution at 37°C for 40 min, followed by DNA denaturation with 2N HCl at 37°C for 30 min. Then, the sections were incubated with anti-PCNA antibody (1:100) and at 37°C for 1 hr. Microscopy was performed using Leica microscope (DM5000B, Wetzlar Germany) to observe hepatocyte nuclear staining.

2.6 Electron microscope

After opening the abdominal cavity, the precooled 2.5% glutaraldehyde fixing solution was quickly added in situ, the tissue was taken with a sharp double-sided blade, cut into one-millimeter cubes, and quickly placed in the glutaraldehyde fixing solution at 4 ° C for 2 hours. 1% osmic acid fixative solution (pH7.3 ~ 7.4) was fixed for 1 h. The liver ultra-structural morphology was observed using Hitachi H-800; Hitachi, Tokyo, Japan.

2.7 Data Analysis

All experimental data were expressed as means±SD. SPSS 13 statistical software was used to analyze the experimental results. student's test was used for comparison between two groups, and ANOVA was used for comparison between multiple groups. $P < 0.05$ was considered a significant difference.

3. Results

3.1 Serum HSS upregulation in liver regeneration after PH

Serum HSS content were evaluated by elisa. It was shown that serum HSS levels increased inmice after PH. Figure 1 reports serum HSS content evaluated in sham and PH mice sacrificed at different times after PH. To determine whether HSS shRNA could silence the HSS gene expression for a long time in vivo, we detect level of serum HSS expression at different time after PH. Figure 1 reports reports serum HSS content were reduced after PH.

3.2 Knockdown of HSS leads to decreased liver regeneration

Fig. 2 show that whereas in control mice liver weight returns to the original size prior to hepatectomy, in HSS RNAi mice liver regeneration inhibited (at day 7).

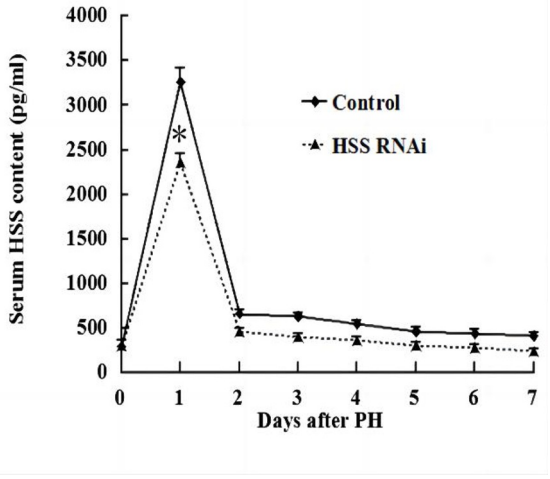

Figure 1. Serum HSS content after PH. HSS expression in blood at different time points (0, 1, 2, 3, 4, 5, 6, 7 d) after PH was assayed by Elisa analysis.

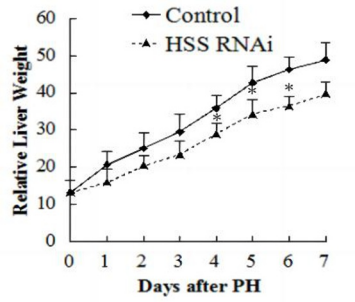

Figure 2. Knockdown of HSS leads to decreased liver regeneration in mice. Representative livers weight of control and HSS RNAi mice at different time points after PH.

3.3 Knockdown of HSS leads to aggravate liver dysfunction in mice in liver regeneration

Fig. 3 show that serum ALT adn ASTcontent evaluated in both control and HSS RNAi mice sacrificed at different times after PH. In control mice, compared to 'time 0' value, an increase of HSS level was soon registeredafter PH; its maximal expression was reached by the first day, subsequently declining to the 'time 0' level for the rest of the period of observation. Fig. 3 reports knockdown of HSS aggravate liver dysfunction.

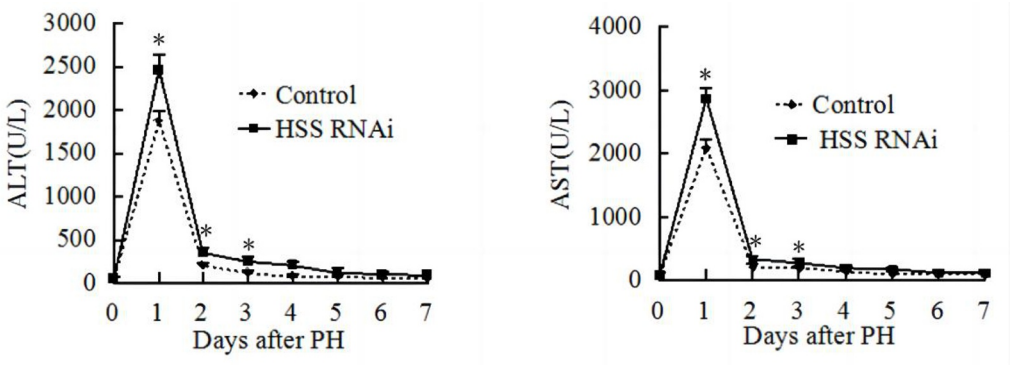

Figure 3. Knockdown of HSS leads to aggravate liver dysfunction in mice. Mouse serum samples were collected different time points (0, 1, 2, 3, 4, 5, 6, 7 d) after PH. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) were assaye in the control and HSS RNAi mice.

3.4 Knockdown of HSS leads to inhibit hepatocyte proliferation in liver regeneration

Changes in liver proliferation kinetics are shown in Fig. 4. We continued to unfold the molecular mechanisms responsible for the regeneration. We evaluated hepatocyte proliferation kinetics using injections of PCNA at several time points (days 2, 3) post-PHx. The detail proliferation kinetics of hepatocytes at different times after PHx are shown in Fig. 4. In both types of mice there is a typical initial increase (2 and 3 days post-PHx) as assessed by PCNA immunohistochemistry. Compared to scramble mice, the HSS RNAi mice exhibited a slightly slower increase in in cell proliferation at 2, 3 days post-PHx.

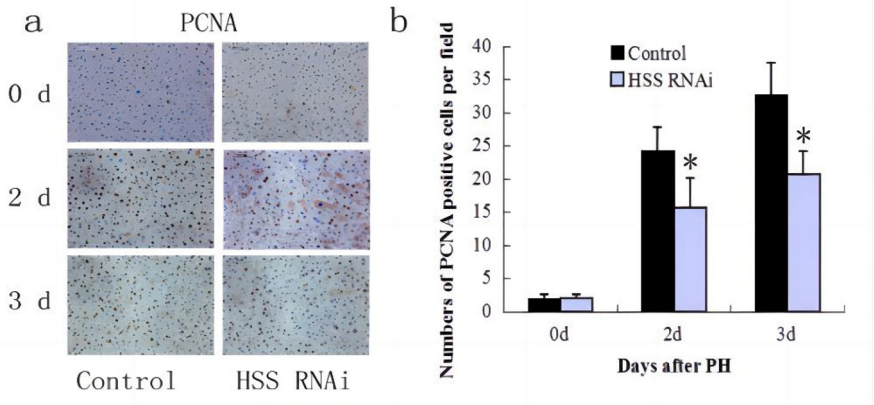

Figure 4. Knockdown of HSS leads to inhibit hepatocyte proliferation in liver regeneration. Representative liver sections of the control and HSS RNAi mice killed at day 2, 3 after PHx immunostained for PCNA(a). Quantification of the PCNA- positive cells (b) was done at the indicated time points for 5 randomly selected fields.

3.5 Knockdown of HSS leads to impair mitochondrial biogenesis

Changes in liver mitochondrial are shown in Fig. 5. In normal liver tissue, the hepatocytes had a complete structure, a large number of mitochondria, a clear boundary of mitochondrial membrane, a large number of cristae and a normal volume. On the first day after PH injection of the negative control plasmid group, lipid droplets increased, mitochondrial number decreased, severe mitochondrial swelling occurred, mitochondria significantly enlarged and became round, the matrix became weak, or even completely vacuolated,

mitochondrial double membrane was visible, and ridge was obviously shortened or even disappeared. 1 day after PH injection of HSS RNAi plasmid group, hepatocytes necrosis and apoptosis increased, primary lysosome increased, glycogen loss, lipid droplets in hepatocytes increased, and concomitoids appeared, mitochondrial damage was more severe, electron density was higher, mitochondrial double-layer membrane boundary was blurred, and even outer membrane rupture occurred, ridge was significantly shortened or even disappeared. In control plasmid group, the number of mitochondria increased, the density was normal, the double membrane was clear, the ridge was visible, and the endoplasmic reticulum expanded and wrapped mitochondria, indicating that protein synthesis was vigorous and the smooth endoplasmic reticulum increased. After 5 days of PH injection of HSS RNAi plasmid, the number of mitochondria was reduced, the density was still high, and the ridge was not clear.

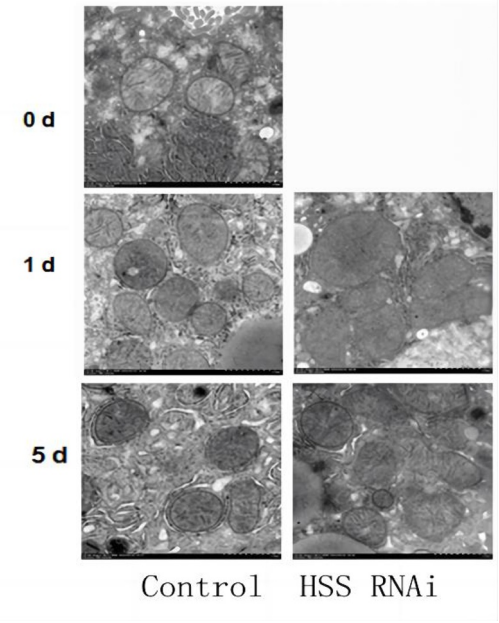

Figure 5. Knockdown of HSS leads to impair mitochondrial. Changes of electron micrographs of mitochondria were checked from the control and HSS RNAi mice.

4. Discussion

In 1975, LaBrecque et al. [8] first confirmed the hepatic stimulatory substance (HSS) that can specifically stimulate DNA synthesis in mammalian hepatocytes. HSS is thermally stable and can be precipitated by 40% ethanol, and its effects are organ-specific but not species-specific. HSS, which is widely present in mammalian embryonic liver, fetal liver and regenerated liver cell serous fluid, is secreted by liver cells themselves and is the only growth factor that has been shown to have liver specificity. In the process of isolation and purification of HSS, human hepatopoietin and other cytokines with liver regeneration stimulating activity were found.

HSS has obvious organ-specific effect, that is, it only stimulates the proliferation of hepatocytes or hepatogenic tumor cells, but does not stimulate the proliferation of other organs or non-hepatogenic tumor cells. In vivo experiments show that HSS can promote liver regeneration in animals with partial hepatectomy, but has no effect on non-liver organs such as bone marrow, spleen and kidney. In vitro experiments also confirmed that HSS can only stimulate the proliferation of normal liver cells or malignant liver cancer cells, among which HTC liver cancer cell line is the most sensitive to HSS stimulation, its DNA synthesis rate can be increased by up to 30 times, and the effect of HSS is dose-dependent, and its biological activity is significantly increased with the increase of purity.

5. Conclusions

As hepatocyte regeneration is an extremely complex process, mechanical injury, ischemia-reperfusion injury and immune reaction are also affected in the process of partial liver resection. The exact mechanism of HSS promoting liver regeneration after liver transplantation remains to be further studied. The process of liver regeneration is that many cytokines play a role at the same time and cross each other, forming a complex proliferation regulatory network. It is not easy to fully reveal this process, and further studies are needed. In summary, HSS can promote liver regeneration and protect liver cell function, and its protective mechanism may be related to the stabilization of liver cell membrane. As hepatocyte regeneration is an extremely complex process, mechanical injury, ischemia-reperfusion injury and immune reaction are also affected in the process of partial liver resection. The exact mechanism of HSS promoting liver regeneration after liver transplantation remains to be further studied.

Acknowledgments

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References

1. Zhang XueJun, Olsavszky Victor, Yin Yuhan, et al. (2019)Angiocrine Hepatocyte Growth Factor Signaling Controls Physiological Organ and Body Size and Dynamic Hepatocyte Proliferation to Prevent Liver Damage during Regeneration.*Am J Pathol* 190: 358-371.
2. Chen Y, Hata T, Rehman F, et al. (2019) Visualization of Hepatocellular Regeneration in Mice After Partial Hepatectomy.*Journal of Surgical Research* 235: 494-500.

3. Elchaninov A, Fatkhudinov T, Makarov A, et al.(2020) Inherent control of hepatocyte proliferation after subtotal liver resection.*Cell Biol Int* 44(1) : 11203.
4. Hoffmann K, Nagel AJ, Tanabe K, et al.(2020) Markers of liver regeneration-the role of growth factors and cytokines: a systematic review.*BMC Surg* 20:31.
5. Theocharis SE, Margeli AP, Agapitos EV, Mykoniatis MG,Kittas CN, Davaris PS. (1998,)Effect of hepatic stimulator substance administration on tissue regeneration due to thioacetamideinduced liver injury in rats. *Scand J Gastroenterol* 33: 656-663.
6. Lü ZB, Li Q, Ye BP, et al. (2004)Protective effects of shark hepatic stimulator substance against acute hepatic injury induced by acetaminophen in mice. *Acta Pharmaceutica Sinica* 39: 17-21.
7. Chuxi Lin, Xueer Wang, Nuyun Liu. (2019)Characterization and evaluation of HGF-loaded plga nanoparticles in a CCl_4 -induced acute liver injury mouse model. *Hindawi Journal of Nanomaterials Volume* 2: 13.
8. Higgins GM, Anderson RM. (1931)Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 12: 186-202.