Article

The Involvement of Heat Shock Proteins in Murine Liver Regeneration

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Partial hepatectomy (PHx) in mammals is a very common experimental model to investigate the process of liver regeneration. The surgery itself could give birth to a series of stresses, such as the temporary raise of body temperature and the ischaemia-reperfusion injury. Heat shock proteins (HSPs) were a family of stress-inducible proteins involved in maintaining cell homeostasis and regulating the immune system. In our study, we intended to investigate the expression and role of HSPs in liver regeneration. Using RT-PCR and Western blotting, we determined the expression in regenerating liver of HSP27, HSP60, HSP70 and HSP90 in mRNA level and protein level, respectively, with mice treated with sham operation as controls. We also used quercertin as an inhibitior of HSPs to explore their effects on liver regeneration. We found that hepatic expression of HSPs increased at the early phase of liver regeneration and declined to the constitutively low level later. Moreover, quercetin pretreatment delayed the progress of liver regeneration in mice *via* inhibition of HSPs. The results indicated that HSPs played an important role in liver regeneration. *Cellular & Molecular Immunology*. 2007;4(1):53-57.

Key Words: partial hepatectomy, liver regeneration, heat shock protein

Introduction

Heat shock proteins (HSPs) were discovered in 1962 when Ferruccio Ritossa noted that temperature shock induced an unusual profile of gene expression in the polytene chromosomes of salivary glands in *Drosophila melanogaster* larva (1). Until recently, HSPs have mostly been regarded as intracellular molecules that mediate a range of essential housekeeping and cytoprotective function (2). However, interest in their role as intercellular signaling molecules has b een fuelled by the observations that these molecules can be released and present in the extracellular environment under physiological conditions. It has been reported that HSPs can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions (3, 4). As ligands of Toll-like receptors, they can also elicit cytokine production by a range of cell types (5, 6). Furthermore, the induction of self HSP immune reactivity can attenuate autoimmunity and delay transplant rejection, and HSPs derived from tumours and pathogens can elicit specific, protective immunity (7). These novel functions suggest HSPs could be immunoregulatory agents with potent and widely-applicable therapeutic uses.

Tissue regeneration is one of the most common processes in response to physical, chemical, or biological injuries, however, in solid organs, only liver can grow to its natural size and perform its normal function after partial hepatectomy (8, 9). Regeneration of the liver is a patho-physiological process elaborately regulated by the innate immune system in the liver. Previous studies have shown that Kupffer cells have the potential to exert both stimulatory and inhibitory influences on hepatocyte proliferation by producing many kinds of cytokines, anti-proliferative and pro-proliferative (10). Except for Kupffer cell's initiating regeneration at the very early stage by secreting interleukin-6 (IL-6) (11, 12) and tumor necrosis factor-α (TNF-α) (13-15), nearly all of other innate immune cells play an inhibitory role in the process of liver regeneration. The negative role of NK cells in liver regeneration has been revealed since last decade (16, 17). Recently, studies on Jα281$^{-/-}$ mice also demonstrated that NKT cells act as negative regulators in liver regeneration (18). Anyway, the final consequences of liver regeneration rely on the balance of growth-promoting capacity and growth-inhibitory capacity.

Although it is clear that liver regeneration is under the control of innate immune system, little is known about how immune system sense the loss or injuries of liver and in turn

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Table 1. Primers and annealing temperature

Gene	Forward	Reverse	Size	Annealing temperature
HSP90	5'-GAACATTGTGAAGAAGTGCC-3'	5'-CATATACACCACCTCGAAGC-3'	320 bp	55°C
HSP70	5'-CCGCCTACTTCAACGACTC-3'	5'-TCT TGA ACT CCT CCA CGAAG-3'	300 bp	60°C
HSP60	5'-ACTGAGCAGCTAGACATCACCA-3'	5'-CATAGCCAACCTCTGAGGAACT-3'	388 bp	62.2°C
HSP27	5'-TCTTCGATCAAGCTTTCG-3'	5'-TCCAGACTGTTCAGACTTCC-3'	533 bp	53°C

exert their stimulatory or regulatory effects. Extensive work has suggested that HSPs could be potent activators of the innate immune system. Therefore, we hope to make in-depth exploration in the variation and the roles of HSPs in a murine model of liver regeneration, which could provide us with useful information to understand the up-stream signaling pathways of immune system in liver regeneration.

Materials and Methods

Mouse treatment

Male C57BL/6 mice (B6 mice), 6-8 weeks old, were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science (Shanghai, China). All mice were maintained under controlled conditions (22°C, 55% humidity, and 12 h day/night rhythm) in compliance with the regulations of animal care of University of Science and Technology of China. Quercetin (Yousi Biotechnology, Shanghai, China) was dissolved in the dimethyl sulfoxide (DMSO) at the concentration of 40 mg/ml. For *in vivo* inhibition of expression of heat shock proteins, mice were intraperitoneally injected with quercetin at dose of 1 g/kg body weight (19) with mice treated with DMSO as controls. Two-third PHx was performed as described previously by Higgins and Andersen (20). Mice treated with sham operation were used as controls. Animals were humanely killed at 2 h, 12 h, 24 h, 48 h, and 4 days after the surgery. After mice were sacrificed, livers were removed and frozen at -70°C.

RT-PCR

Total RNA was isolated from liver tissues using TRIzol (Invitrogen, store at 2~8°C) and reverse transcribed at 25°C for 10 minutes and 37°C for 50 minutes, followed by heat denaturation at 70°C for 15 minutes and cooling at 4°C. The reaction mixture (20 μl) included 4 μg of total RNA, 250 ng random primer, 10 mM dNTP mix, 0.1 M dithiothreitol, 200 U M-MuLv reverse transcriptase (Invitrogen). The PCR mixture (50 μl) included 4 μl DNA template from the reverse transcription reaction, 50 pmol each sense and antisense primer, and 1.25 U DNA polymerase (Taq, Shanghai Shenggong). The PCR was performed with the conditions as shown in Table 1. The PCR products were separated by 1.5% agarose gel electrophoresis

Western blotting

The liver tissue was grinded and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% sodium dexycholate, 1% NP 40, 1 mM EDTA, 1 mM PMSF. After centrifugation for 15 min at 13,000 rpm, supernatant protein was collected, and the concentrations were measured by ultraviolet spectrophotometer. Proteins were separated on

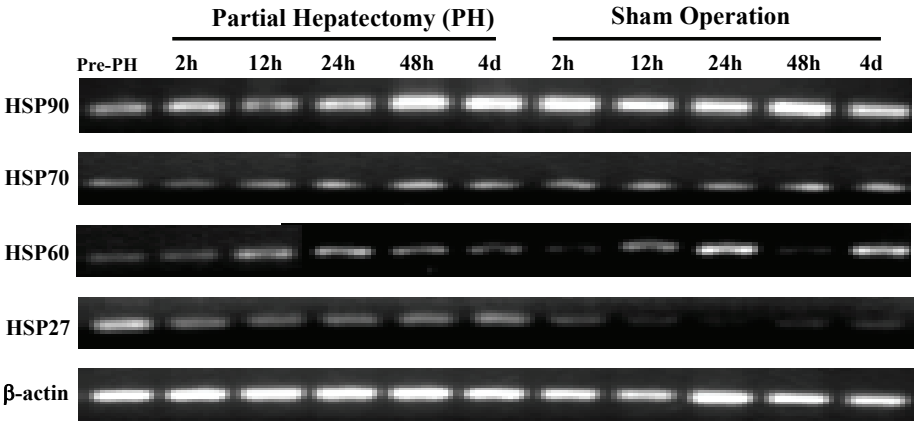

Figure 1. Expression of heat shock proteins mRNA in liver regeneration after partial hepatectomy. C57BL/6 mice were treated with two-third PHx as described previously by Higgins and Andersen, and then humanly killed at 2 h, 12 h, 24 h, 48 h and 4 days after the surgery, with mice only receiving sham operation as controls. The total RNA was then isolated from the liver tissues collected at different time points after the surgery, and the quantities of the mRNA of HSP27, HSP60, HSP70 and HSP90 were detected by RT-PCR analysis.

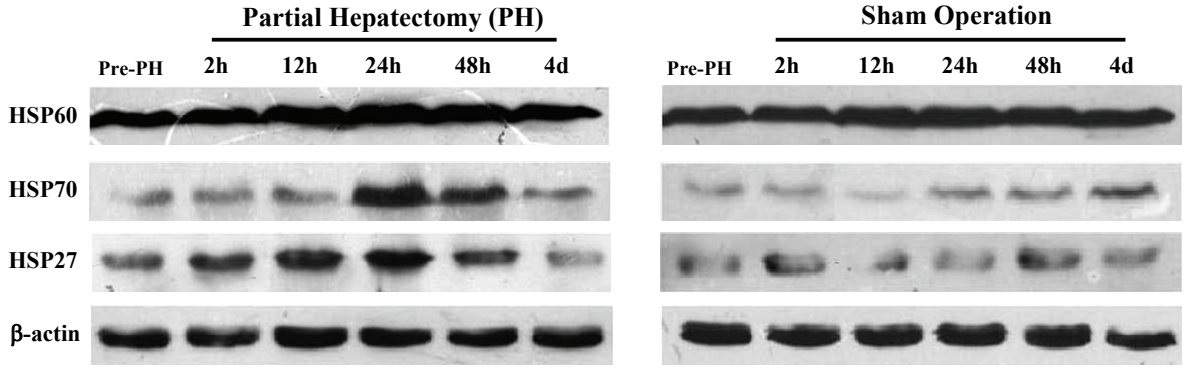

Figure 2. Synthesis of heat shock proteins in liver regeneration induced after partial hepatectomy. C57BL/6 mice were treated with two-third PHx described previously by Higgins and Andersen, and then humanly killed at 2 h, 12 h, 24 h, 48 h and 4 days after the surgery, with mice only receiving sham operation as controls. The total protein was then isolated from the liver tissue collected at different time points after the surgery, and the quantities of protein of HSP60, HSP70 and HSP27 were detected by Western blotting analysis.

14% Tris-HCl SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 hour at room temperature in TBST (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk powder, and incubated overnight at 4°C with the corresponding antibody as follow: rabbit anti-β-actin antibody (1:1,000), rabbit anti-HSP60 antibody (1:400), goat anti-HSP70 antibody (1:400), goat anti-HSP27 antibody (1:400). These blots were washed in TBST five times, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, and then washed in TBST three times and TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) twice (5 minutes per time). Specific bands were detected using the enhanced chemiluminescence reagent on autoradiographic film. For quantitation of protein levels, the amount of protein loaded on the gel was optimized, and multiple exposures were performed to ensure that the signals were within the linear response range of the film. Anti-HSP60 antibody was purchased from Boshide Biotechnology (China). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis

Differences between the groups were analyzed by Student's *t* test and $p < 0.05$ was taken to imply statistical significance.

Results

The expression of HSP mRNA and protein synthesis in liver regeneration after PHx

In this study, we aimed to investigate the changes and effects of HSPs on liver regeneration in mice. At first, we detected the mRNA expression of HSPs in liver regeneration. C57BL/6 mice were treated with two-third PHx described previously by Higgins and Andersen (20), and then humanly killed at 2 h, 12 h, 24 h, 48 h and 4 days after the surgery, with mice only receiving sham operation as controls. The total RNA was then isolated from the liver tissues collected at different time points after the surgery, and the quantities of the mRNA of HSP27, HSP60, HSP70 and HSP90 were detected by RT-PCR analysis. As shown in Figure 1, HSP90 mRNA increased immediately at 2 h after the PHx. Both HSP70 and HSP60 mRNA showed notable raise at 12 h after the PHx. There were no significant changes in HSP27 mRNA and in the sham operation groups.

We next explored the variation of HSPs at the level of protein in the process of liver regeneration. As mentioned above, mice were sacrificed at 2 h, 12 h, 24 h, 48 h and 4 days after the PHx. Livers were then collected and used in Western blotting analysis. As shown in Figure 2, liver expression of HSPs at protein level raised after the surgery, compared to the sham operation groups. In details, HSP27 increased immediately at 2 h after the PHx, and reached its peak value at 24 h after the surgery; while HSP70 had no remarkable change until 24 h after the surgery. The decline of HSP27 went with the raise of HSP70, and the expression of both HSP27 and HSP70 declined to the normal level at day 4 after the PHx. Moreover, HSP60 underwent self-degradation after partial hepatectomy and generated large quatities of HSP-derived peptides (data not shown). The coincidence of the variation of HSPs and the progress of liver regeneration might imply an essential role of those proteins in liver regeneration.

Inhibition of HSP synthesis with quercetin pretreatment delayed liver regeneration in mice

In order to further investigate the detailed role of HSPs in liver regeneration, we treated mice with quercetin, a kind of inhibitor of HSPs, before the PHx. Mice treated with DMSO, the solvent for quercetin, were used as controls. As shown in Figure 3A, quercetin could effectively inhibit the raise of HSP70 in the liver regeneration, slightly inhibit HSP27, and have no obviously effects on the expression of HSP60. Meantime, we found that the ratios of remnant liver mass to body weight in quercetin-inhibited mice were lower than those of the mice only receiving DMSO at 24 h and 48 h after the PHx (Figure 3B), indicating quercetin pretreatment

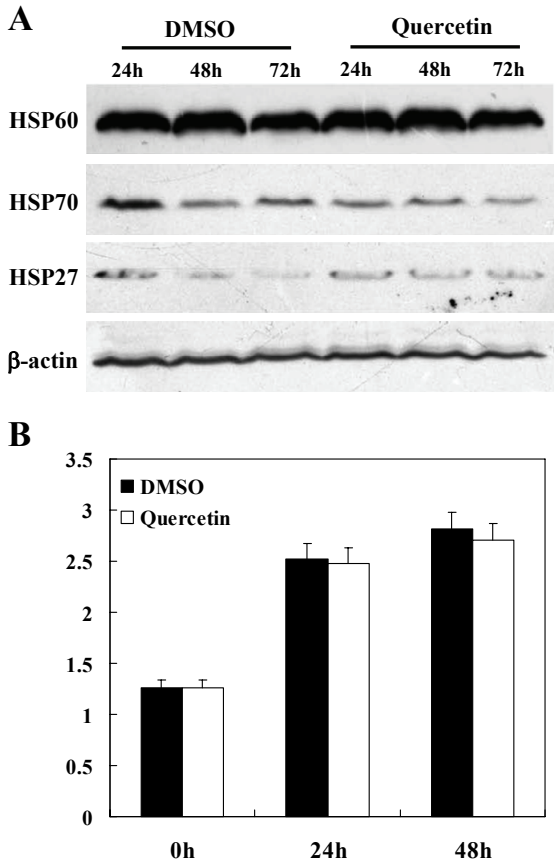

Figure 3. Inhibition of HSP synthesis with quercetin pretreatment delayed liver regeneration in mice. For *in vivo* inhibition of expression of HSPs, C57BL/6 mice were intraperitoneally injected with quercetin at dose of 1 g/kg body weight at 6 h before the partial hepatectomy (PHx) surgery, and with the same dose daily after the surgery. Mice treated with DMSO, the solvent for quercetin, were used as controls. (A) The total protein was isolated from the liver collected at 24 h, 48 h, and 72 h after the PHx, and the liver expressions of HSP27, HSP60 and HSP70 were analyzed by Western blotting. (B) The liver regeneration was assessed by the ratios of remnant liver mass to body weight after the PHx.

delayed the progress of liver regeneration in mice *via* inhibiton of HSP70 and HSP27. These results suggested that the raise of HSPs might play a protective role in liver regeneration, or HSPs serve as a stimulator for initiation of liver regeneration.

Discussion

Liver regeneration is one of the most important physiological processes in human body. It can be divided into three steps: priming pathway, growth-promoting pathway and growth-inhibitory pathway. Abnormality or lack in any step will lead to impaired liver regeneration, which has been considered to be the major cause of fulminant hepatitis. Thus, the studies on the cellular and molecular mechanisms of liver regeneration are of great significance for the improvement of liver regeneration and the treatment of liver injury. Our study aimed at understanding the changes and roles of HSPs in liver regeneration. Through RT-PCR and Western blotting analysis, we found that liver expression of HSPs increased at both mRNA and protein levels during the early phase of liver regeneration. Additionally, using a combination of quercetin inhibition and PHx, we also demonstrated that liver regeneration could be delayed by quercetin *via* inhibition of HSPs. Taken together, these results suggested that the induced expression of HSPs could play a stimulative role in liver regeneration.

One possible mechanism of the positive effect of HSPs on liver regeneration could be due to its capability of stimulating Kupffer cells to produce a series of cytokines. It has been confirmed that Kupffer cells have the potential to exert stimulatory influences on hepatocyte proliferation by producing many kinds of cytokines, like IL-6 (11, 12) and TNF-α (13-15). However, it remains unknown how Kupffer cells sense the loss of liver after the PHx and initiate liver growth. As we have known, Kupffer cells are resident macrophages in the liver, and several kinds of Toll-like receptors are distributed on its surface, in charge of sensing pathogen-associated molecular patterns (PAMP) like lipopolysaccharide (LPS) (21). Recently, many reports have shown that HSP signaling pathway are also used by LPS, and Toll-like receptors, important mediators of innate immunity. As ligands of Toll-like receptors, HSPs are capable of inducing cytokine production by many types of cells including macrophages (5, 6). Therefore, we presume that the raise of HSPs observed at the early phase of liver regeneration may provide a startup signal *via* interactions with Toll-like receptors on the surface of Kupffer cells. Nevertheless, it is necessary to obtain more powerful evidences to certify our hypothesis by investigating liver regeneration after the PHx in Toll-like receptor knockout mice.

In addition to this indirect effect on hepatocytes *via* cytokine production by Kupffer cells, it is expected that HSPs could also exert their protective effects directly on regenerated hepatocytes. It is widely known that HSPs play an important role in maintaining protein homeostasis (21). Stresses involved in the liver regeneration induced by the PHx, such as the temporary raise of body temperature and the ischaemia-reperfusion injury could challenge the protein homeostasis and result in an increased flux of non-native proteins in rapidly proliferative hepatocytes. Thus, the increased expression of HSPs during liver regeneration could react to those physiologic stresses, repair protein damage and in turn help hepatocytes return to their normal growth conditions. On the other hand, previous study suggested that some members of HSPs were responsible for controlling apoptosis. It is reported that HSP70 and HSP27 are antiapoptotic, whereas HSP60 is proapoptotic. The role of HSP90 in controlling apoptosis is ambiguous and depends on the apoptotic stimulus, but the effect is usually antiapoptotic (22). Moreover, recent publications have indicated that the antiapoptotic action of HSPs is due to an inhibitory effect on

caspase activation (23-25). As we figured out in Figure 3, the protein expression of both HSP27 and HSP70 increased significantly after the PHx. We also observed that HSP60 underwent self-degradation after the PHx and generated large quatities of HSP-derived peptides (data not shown). It is conceivable that the up-regulation of antiapoptotic HSPs and degradation of proapoptotic HSPs could promote liver regeneration by preventing hepatocytes from apoptosis *via* direct inhibition of caspase activation.

In summary, the exact underlying mechanisms of the accelerating role of HSPs in liver regeneration are complicated, including both direct and indirect effects. The treatment of extraneous recombinant HSPs may be a potential strategy for treating liver diseases with impaired liver regeneration. In future study, we will focus on these potential mechanisms involved in the roles of HSPs in liver regeneration, which will be instructive for therapy of impaired liver regeneration.

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