

Expression of growth hormone receptor and its mRNA in hepatic cirrhosis

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Abstract

AIM: To investigate the expression of growth hormone receptor (GHR) and mRNA of GHR in cirrhotic livers of rats with the intension to find the basis for application of recombinant human growth hormone (rhGH) to patients with liver cirrhosis.

METHODS: Hepatic cirrhosis was induced in Sprague-Dawley rats by administration of thioacetamide intraperitoneally for 9-12 weeks. Collagenase IV was perfused in situ for isolation of hepatocytes. The expression of GHR and its mRNA in cirrhotic livers was studied with radio-ligand binding assay, RT-PCR and digital image analysis.

RESULTS: One class of specific growth hormone-binding site, GHR, was detected in hepatocytes and hepatic tissue of cirrhotic livers. The binding capacity of GHR (R_T , fmol/mg protein) in rat cirrhotic liver tissue (30.8 ± 1.9) was significantly lower than that in normal control (74.9 ± 3.9) at the time point of the ninth week after initiation of induction of cirrhosis ($n=10$, $P<0.05$), and it decreased gradually along with the accumulation of collagen in the process of formation and development of liver cirrhosis ($P<0.05$). The number of binding sites ($\times 10^4/\text{cell}$) of GHR on rat cirrhotic hepatocytes (0.86 ± 0.16) was significantly lower than that (1.28 ± 0.24) in control ($n=10$, $P<0.05$). The binding affinity of GHR among liver tissue, hepatocytes of various groups had no significant difference ($P>0.05$). The expression of GHR mRNA (riOD, pixel) in rat cirrhotic hepatic tissues (23.3 ± 3.1) was also significantly lower than that (29.3 ± 3.4) in normal control ($n=10$, $P<0.05$).

CONCLUSION: The growth hormone receptor was expressed in a reduced level in liver tissue of cirrhotic rats, and lesser expression of growth hormone receptors was found in a later stage of cirrhosis. The reduced expression of growth hormone receptor was partly due to its decreased expression on cirrhotic hepatocytes and the reduced expression of its mRNA in cirrhotic liver tissue.

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INTRODUCTION

Liver cirrhosis is a common pathway of a variety of chronic liver diseases^[1] and is associated with high protein catabolism, low anabolism and negative nitrogen balance^[2] resulting in hypoproteinemia which contributes to ascites, dysfunction of coagulation and suppressed immune responses^[3] as pathophysiological outcomes. In fact, progressive nutrition deficiencies and muscle wasting are universal problems and critical predictors of morbidity, mortality and survival^[4,5] after surgical intervention. Early reports showed that cirrhotic patients requiring emergency abdominal surgery exhibited a higher mortality^[6]. In retrospective studies of liver transplant recipients, protein-calorie malnutrition had been associated with adverse outcomes in patients with end-stage liver diseases^[7,8]. A prospective study showed that cirrhotic patients with hypermetabolism and body mass loss had a much higher mortality rate after liver transplantation than those with normometabolism^[9]. Nutritional support is critical for patients with hepatic cirrhosis. Yet, studies have shown that aggressive nutritional support is essential but not effective enough to prevent protein loss and optimize the care of these patients in severe catabolic illness, including cirrhosis^[10,11].

Recent research has investigated the role of exogenous growth factor therapy in catabolic illness. Recombinant human growth hormone (rhGH) has been used in catabolic states such as after abdominal operation^[12], organ transplantation^[13], major trauma^[14] and severe burns^[15] to enable them to survive an aggressive surgery and gain access to a new life^[16]. After treatment of rhGH, donor site healing rate in children with severe burns enhanced and thus decreased a 14-day hospitalization time^[17-19], Mortality rates in severely burned adults dropped 26 %^[20]. Cell-mediated immunity significantly enhanced, wound infection rates and length of hospitalization in a large group of post surgical patients decreased^[21]. Some clinical trial reported that growth hormone enhanced nitrogen retention in patients with chronic obstructive lung diseases^[22], severe sepsis^[23,24] and wasting status of AIDS^[25,26] and in fasted adult volunteers. Although there are many controversies^[27-31], it has been confirmed that rhGH is an effective drug to accelerate protein anabolism^[32] and plays a central role in metabolic intervention with a significant cost-effect benefit^[33].

The action of growth hormone depends on its binding with growth hormone receptor on cell membrane^[34]. Whether the cirrhotic hepatocytes express growth hormone receptors (GHR), and the relationship between the GHR expression and the staging of liver cirrhosis are not clear. So, we adopted multiple techniques such as radioligand binding assay and RT-PCR to measure the changes of GHR and its mRNA in an experimental liver cirrhosis model at cellular and histological levels for understanding of biological feature of expression of GHR in cirrhotic hepatocytes.

MATERIALS AND METHODS

Induction of liver cirrhosis

Liver cirrhosis in rats was induced by daily intraperitoneal injection of 3 % thioacetamide (TAA, Shanghai, China) 50 mg/kg for 9 to 12 weeks^[35,36]. Two hundreds male Sprague-Dawley rats (200-300 g, Medical Animal Center, Sun Yat-Sen University) were randomized and allotted to two groups, normal group ($n=82$) received saline intraperitoneally everyday; and cirrhotic group ($n=118$) received drugs for induction of hepatic cirrhosis. From each group, 80 rats were available for analysis. Rats were fed with regular chow and water *ad libitum* in cages placed in a room with 12-hour light-dark cycle and constant humidity and temperature (25 °C).

At the 3rd, 6th, 9th, 12th week and the 15th week (3 weeks after withdrawal of TAA) after induction of liver cirrhosis 10 rats from each group were fasted overnight and sacrificed. Liver tissue samples were obtained after hepatic sinusoids were flushed by perfusion of liver with normal saline through the portal vein. Tissue samples from the right major liver lobe were obtained and frozen in liquid nitrogen immediately and then stored at -80 °C until use, or fixed in 10 % neutral buffered formaldehyde solution and dehydrated and embedded in paraffin for regular pathological examination.

Digital image analysis

Liver collagen content was calculated by histomorphometric measurement in 4- μ m sections with Masson's trichrome stain. Under photographic analytic microscope (Carl Zeiss Axiotron, OPTON, Germany), three random areas in each section of liver slide were chosen and captured in RGB format with 24-bit true colors at a resolution of 768 \times 512 in real time by the JVC ky-F30B 3-CCD color video camera with digital conversion connected with a computer system of Kontron IBAS2.5 (OPTON, Germany) for digital image analysis. The total area and the area of fibrosis with positive staining were automatically selected, outlined and evaluated by planimetry. The relative content of collagen (RCC %) was expressed as a percentage of positive staining area in the total area^[37,38].

Hepatocyte isolation

At 9-12 weeks of the study, livers in 10 rats from each group were perfused *in situ* through portal vein with collagenase (type IV, 90 or 120 U/ml)^[39,40]. Hepatocytes were isolated at 4 °C by centrifugation (50 \times g, 5 min or 1 \times g, 10 min). Cells were re-suspended for radio-ligand binding assay in RPMI 1640 medium without serum to a final concentration of 1.0 \times 10⁶ cell/ml with a purity of more than 98 % and a viability of more than 90 % determined by trypan-blue exclusion.

Total RNA isolation

Total RNA of liver tissue was prepared from 10 rats in each group at the 9th week during the induction of cirrhosis by a single-step method^[41]. Integrity and quality of RNA were tested by electrophoresis in 1 % agarose-formaldehyde gels stained with ethidium bromide. The RNA concentration was evaluated

at spectrophotometric absorbance at 260 nm.

Primers for RT-PCR

Primers for the PCR amplification of GHR transcripts were purchased from Sangon Co. (Shanghai, China). The primers used for detection of GHR mRNA by RT-PCR were specific for the intracellular domain of the rat GHR, thus excluding detection of the smaller transcripts encoding GH-binding protein (GHBP)^[42]. The forward primer (GHR4548) was 5' - *ATGTGAGATCCAGACAACG-3' with the first base *A at the 5' end as an unmatched base of the original G and spanned from 876 to 894 located in exon 7 and the reverse one 5' - ATGTCAGGGTCATAACAGC-3' spanned from 1 356 to 1 374 located in exon 10. The PCR product was supposed to be 499 bp in length.

RT-PCR

RT-PCR was performed using the MasterAmp™ RT-PCR kit (Epicentre, Madison, USA) for detection of GHR mRNA^[43]. The procedure of RT-PCR was similar to that of literature^[44]. Negative controls in RT-PCR included reactions in the absence of RNA sample or primers or RT reaction to detect possible contamination of genomic DNA in RNA samples. The experiment was considered ineffective if there was any incidental band in lane of negative control. 10 μ l of each PCR products was subjected to electrophoresis for about 1 hour on 1 % agarose gel using electrophoresis apparatus (Bio-Rad, UK) and visualized by staining with ethidium bromide. The bands were observed using a ultraviolet gel device (UVI, UK) and captured into its computer system for digital image analysis. The level of GHR mRNA in each sample was expressed as: iOD (pixel)=the average optical density of each band \times its area (pixel).

Membrane microsome preparation

The microsomal liver membrane fraction was prepared by differential centrifugation of liver homogenate according to Papotti^[45]. About 1 g tissue from rat livers was chopped and homogenized with an electric homogenizer (Vorsicht, Malaysia) in ice-cold (4 °C) phosphate buffer (PBS) containing 0.25 M sucrose. The mixture was centrifuged at 1 500 \times g for 15 minutes. The supernatant was collected and centrifuged at 30 000 \times g for 30 minutes. The pellet was re-suspended in 3M MgCl₂ for 5 minutes to remove endogenous GH binding^[46] and centrifuged, the pellet was suspended in PBS. Protein concentration of the solution was determined by Lowry method.

GH binding assay

In radioreceptor assay, 100 μ l (approximately 20 000 cpm) of ¹²⁵I-hGH (NEN Inc, Boston, USA) with a specific activity of about 108 μ Ci/ μ g, 100 μ l of unlabeled hGH in PBS and 100 μ l of liver membrane microsomes or liver cells were mixed and incubated at 4 °C over night. Parallel incubations were performed with various amounts of hGH (0-3nM divided into 7-9 concentration gradients) in duplicate. An excess of hGH was used to determine nonspecific binding. At the end of the incubation, bound and free hormones were separated by filtration (0.18 μ m mesh filters, Shanghai, China)^[47]. The pellets on membrane were subjected to a gamma counter (SN γ 682, Rihuan, Shanghai, China). GH binding affinity constant (K_d, nM) and binding capacity (R_T, fmol/mg protein) or numbers of ¹²⁵I-GH binding sites of hepatocytes (\times 10⁴/cell) were calculated by Scatchard analysis using the LIGAND program^[48,49].

Statistical analysis

Results were expressed as the mean \pm SEM ($\bar{x}\pm s$) and analyzed by ANOVA and least significant difference (LSD) method with an acceptance of significance as $P<0.05$.

RESULTS

Changes of lobular structure and RCC in the development of liver cirrhosis

In control group, no evident pathological change of lobular structure was found in liver tissue over 15 weeks. In cirrhotic model group, 4 stages of pathological changes were manifested during the development of liver cirrhosis: (1) scattered necrosis and cellular degeneration at the 3rd week; (2) fibrous proliferation at the 6th week; (3) pseudo-lobule formation at the 9th week; (4) massive nodule formation at the 12th week and (5) partial reversion of cirrhosis at the 15th week.

In control group, there was no significant change of RCC ($P>0.05$) among the different time points during the period of 15 weeks (Table 1). While compared with that of the control group at the same time point, RCC kept no significant change at the 3rd week ($P>0.05$) and increased significantly at the 6th, 9th, 12th and 15th week respectively ($P<0.05$) in the cirrhotic model group. When compared among the different time points in the cirrhotic model group, RCC increased gradually at the 3rd, 6th, 9th week and reached to its turning point at the 12th week ($P<0.05$), then went down, but still above normal level at the 15th week.

Table 1 Dynamic alteration of RCC, R_T and K_d in cirrhotic liver tissues

Time	RCC (%)		R_T (fmol/mg protein)		K_d (nM)	
	Control Group	Model Group	Control Group	Model Group	Control Group	Model Group
3 rd week	1.29±0.23	1.56±0.35	75.8±5.1	55.2±4.5 ^b	0.62±0.03	0.61±0.07
6 th week	1.19±0.21	10.8±2.5 ^{ab}	77.3±4.3	42.6±2.1 ^{ab}	0.64±0.05	0.58±0.06
9 th week	1.16±0.18	20.2±3.6 ^{ab}	74.9±3.9	30.8±1.9 ^{ab}	0.61±0.09	0.60±0.04
12 th week	1.27±0.25	28.0±4.3 ^{ab}	73.2±5.4	17.5±2.5 ^{ab}	0.60±0.08	0.59±0.07
15 th week	1.13±0.24	15.2±2.6 ^{ab}	71.5±4.9	20.8±1.6 ^b	0.58±0.06	0.61±0.09

^a $P<0.05$ vs a previous time point, ^b $P<0.05$ vs control group. RCC: relative content of collagen (%) indicating severity of liver fibrosis; R_T : total binding capacity of receptors (fmol/mg protein) corresponding to the quantity of receptors; K_d : dissociation constant in equilibrium (nM) which is equal to the affinity of receptors.

GH binding in the development of liver cirrhosis

GH binding assay was carried out using liver membrane microsomes from rats at different time points in various groups and specific binding for ¹²⁵I-hGH was detected in all samples. The Scatchard analysis of GH binding capacity (R_T) and affinity (K_d) on each sample was performed and a single class of specific GH-binding sites on liver cell membranes, that is GHR, was revealed in both normal and cirrhotic liver tissue samples by linear Scatchard plots (Figure 1).

No significant change of R_T and K_d was found in liver tissue samples during the period of 15 weeks in control group ($P>0.05$) (Table 1 and Figure 1). While compared with that of the control group at the same time point, R_T decreased significantly ($P<0.05$) at the 3rd, 6th, 9th, 12th and 15th week although no change of K_d was observed in cirrhotic model group. When compared among the different time points in the cirrhotic model group, R_T decreased gradually at the 3rd, 6th, 9th week and reached to its lowest point ($P<0.05$) at the 12th week, then increased but still under normal level at the 15th week.

The correlation analysis manifested a significant negative linear correlation between R_T and RCC in rat liver cirrhotic tissue ($r=-0.82$, $n=50$, $P<0.05$) (Figure 2).

Quantitative analysis of GH binding sites on rats hepatocytes

There were linear Scatchard plots of normal and cirrhotic hepatocytes, which indicated the presence of a single class of

specific GH-binding sites, GHR, on hepatocyte membranes. The quantity of GH-binding sites ($\times 10^4$ /cell) on hepatocytes of cirrhotic model group (0.86 ± 0.16) was significantly less than that (1.28 ± 0.24) in normal control group ($n=10$, $P<0.05$) while the affinity (K_d , nM) of hepatocytes from model group (0.56 ± 0.08) and control group (0.61 ± 0.11) was similar ($n=10$, $P>0.05$).

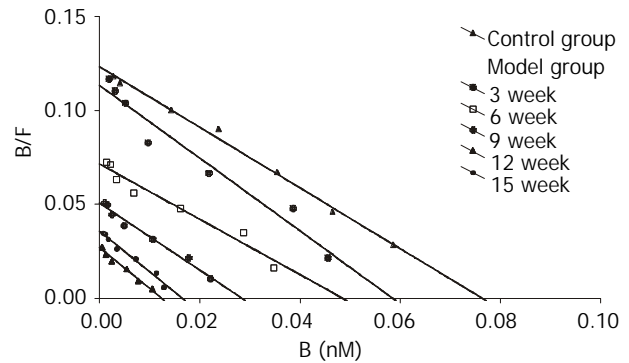


Figure 1 Scatchard analysis of GH-binding in rat liver tissue. Microsomes of liver tissues were incubated with ¹²⁵I-labeled hGH and increased concentrations of unlabeled hGH. The bound/free radioactivity ratio (Y axis) was plotted as a rectilinear function of bound hormone (X axis). GH binding capacities (B_{max} =intercept on X axis, $R_T=B_{max}$ /protein concentration) and affinities (K_d =negative reciprocal of slope) were calculated from the competition assays by Scatchard analysis.

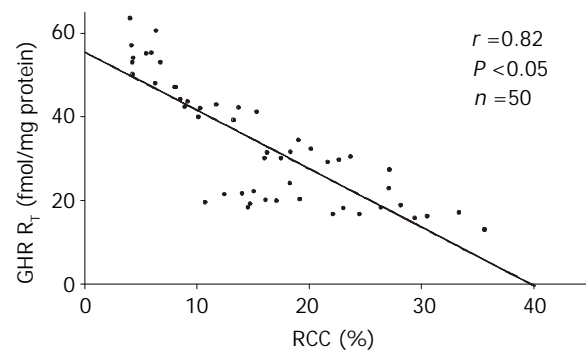


Figure 2 Correlation between R_T (quantity of GHR) and RCC (degree of cirrhosis) in rat cirrhotic liver samples.

GHR mRNA detected by RT-PCR

A specific band of RT-PCR products (499 bp in length) was detected in liver tissue samples from both control group and cirrhotic model group, indicating that GHR mRNA was expressed in both groups. The level of GHR mRNA (IOD, pixel) in cirrhotic liver (23.3 ± 3.1) was lower than that (29.3 ± 3.4) in normal liver ($n=10$, $P<0.05$) (Figure 3).

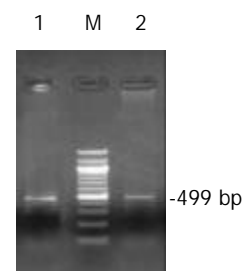


Figure 3 Comparison of GHR mRNA expression in liver tissue in normal control group and cirrhotic model group (the result of RT-PCR). Lane M: 100 bp DNA ladder; Lane 1: Normal control group; Lane 2: Cirrhotic model group.

DISCUSSION

Much has been done regarding the expression of GHR and signal transduction^[34, 47, 50, 51], but the expression of GHR in some pathological states such as cirrhotic hepatocytes, malignant cells is not clear. Chang^[52] reported that ¹²⁵I-rhGH binding activity in 6 cases of hepatocellular carcinoma and adjacent cirrhotic liver tissue could not be detected and believed that the GHR in cirrhotic hepatic tissue disappeared although the study only examined one aspect of the GHR, GH binding. Another study^[53] showed that specific binding of ¹²⁵I-hGH in liver tissue from liver transplant of 17 cases with end-stage liver diseases was lower than that in normal control, but only in 3 cirrhotic livers Scatchard analysis was performed for calculation of GH binding capacity and affinity. In this setting of tissue-based GH binding assay, it was not convincing enough to clear up the controversy about the expression of GHR on cirrhotic liver cells. So, GH binding assay at cellular level was used to analyze the expression of GHR on hepatocytes or in liver tissue of rats with cirrhosis. We have demonstrated that the growth hormone receptor was expressed on cirrhotic hepatocytes and lesser expression of GHR was found in a later stage of cirrhosis.

In present study, ¹²⁵I-hGH binding activity of cirrhotic hepatic tissue was studied with radioligand binding assay, and a single class of growth hormone specific binding sites with normal affinity was detected in the cirrhotic hepatic tissue, which indicated the expression of GHR in cirrhotic hepatic tissue. These results were compatible with those of clinical specimens from liver transplants^[53].

The relationship between the GHR expression in cirrhotic tissue and the stage of liver cirrhosis has not been reported. In our study, ¹²⁵I-hGH binding capacity (*i.e.* the quantity of GHR) decreased significantly even in the early stage of cirrhosis, and gradually decreased during the development of liver fibrosis and dropped to the lowest level in late stage of cirrhosis on week 12 after induction of hepatic cirrhosis, then increased but still below the normal level after withdrawal of TAA. This implicated that the quantity of GHR in liver tissue altered dynamically without any significant change of GH binding affinity over the development or recovery of liver cirrhosis. In other words, GHR expression was suppressed in the process of formation of liver cirrhosis. The more severe the cirrhosis was, the more significant the down regulation of GHR expression would be.

GH binding varied among liver samples^[47, 53]. Our results indicated that GH binding capacity in rat cirrhotic liver tissue differed in various stages of liver cirrhosis, and only slight variation (data not listed) of such binding was found in liver tissue with the same time point during the development of cirrhosis. Therefore, histological staging of liver cirrhosis was one of the important factors predisposing the down-regulation of GHR expression^[54].

Fibrotic tissue accumulated in the portal area during the reconstruction of lobule of cirrhotic liver while hepatocytes regenerated continuously after repeated necrosis in the development of liver cirrhosis. The fibrous structure, hepatocytes and interstitial cells with heterogeneous distribution and various combination in cirrhotic tissue should be considered respectively as we analyzed the expression of growth hormone receptor. In the present study, the result of GH-binding capacity (R_T) in cirrhotic liver tissue indicated the GHR expression on membrane microsomes in hepatocytes and interstitial cells and could be affected by non-parenchymal cells rather than fibrotic tissue^[53]. The quantitative measurement of GH binding sites on parenchymal cells from cirrhotic livers had not been reported previously. Therefore Scatchard analysis was resorted to again and a single class of growth hormone

specific binding sites with normal affinity was detected in isolated cirrhotic hepatocytes, suggesting the expression of GHR on cirrhotic hepatocytes disassociated *in vitro*. It was found that the binding sites on cirrhotic hepatocytes was significantly decreased as compared with that on normal hepatocytes, which implied that cirrhotic hepatocytes expressed GHR in a reduced level without any significant difference of binding affinity. So, the decreased expression of GHR in cirrhotic liver tissue should be attributed not only to accumulation of interstitial cells but to the reduced expression of GHR on cirrhotic hepatocytes themselves also.

It is possible that the reduced level of GH binding is partly owing to the reduced population of hepatocytes and increased numbers of interstitial cells in cirrhotic livers. But the mechanisms of the reduced GHR expression on cirrhotic hepatocytes has not been defined yet. The reduced GH binding level was unlikely due to changes in the receptor itself. Occupancy of the receptors by endogenous GH and ligand-induced internalization of GHR may affect the measurement of the binding, since dissociation of GH from its receptor and recycling of GHR is incomplete in several hours^[55]. Therefore, GH binding was determined on microsomes that had been desaturated by exposure to 3M MgCl₂ for 5 minutes^[46]. It was reported that the specific binding of ¹²⁵I-labelled bovine GH to a GHR-enriched low density membrane fraction from regenerating rat liver was reduced to 10-35 % between 12 and 24 h and remained low until 48 h after partial hepatectomy^[46], which indicated the amount of functional GHR on hepatocytes was decreased with repeated degeneration, necrosis and regeneration in the microenvironment of cirrhosis. In this situation several cytokines such as IL-1 β , TNF- α and glucocorticoids seemed to be involved in the likely mechanism of the reduced GHR expression^[40, 56, 57].

It is necessary to investigate the changes of GHR and its mRNA simultaneously in order to understand the biological events at different levels. Shen^[53] reported that the reduced expression of GHR mRNA identified by ribonuclease protection assay in human cirrhotic livers was in the similar order of magnitude as reduction in GH binding. Relative quantity of GHR mRNA in liver tissue of cirrhotic rats was tested with RT-PCR assay and a similar result of our study indicated that the expression of GHR mRNA in cirrhotic liver tissue was lower than that in normal controls. Accordingly, it was suggested that the reduced GH binding may be secondary to reduced GHR gene expression and decreased GHR synthesis.

In summary, GH binding assay with staging analysis of liver cirrhosis was applied and our study showed that the growth hormone receptor was expressed with normal binding affinity on cirrhotic hepatocytes and expression of growth hormone receptors in a later stage of cirrhosis reduced significantly. These results implicated that there was a physiological basis of GHR for GH action in cirrhotic livers, but the sensitivity of cirrhotic hepatocytes to growth hormone might be decreased. Further investigations should be concentrated on the signal transduction of GHR in cirrhotic hepatocytes^[58].

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