

Effects of hepatocyte growth-promoting factor and panax notoginseng saponins on intrasplenic hepatocellular autotransplantation¹

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KEY WORDS liver regeneration; heterotopic transplantation; hepatocyte growth factor; ginseng

ABSTRACT

AIM: To increase the weight of liver tissue mass present in spleen and to shorten the regeneration period of transplanted hepatocytes by stimulating DNA synthesis and protection against ischemic-reperfusion injury. **METHODS:** Hepatocyte growth-promoting factor (PHGF) and panax notoginseng saponins (PNGS) were used after intrasplenic hepatocellular autologous transplantation (IHAT) with 70 % partial hepatectomy. Histological examinations were carried out under both light and electron microscopy and content of ALT in hepatized spleen homogenate was investigated 2 weeks after transplantation. Furthermore, ^{99m}Tc-diethyl-iminodiacetic acid (^{99m}Tc-HIDA) splenic scintiphotography was carried out and proliferation index of transplanted hepatocytes was detected by flow cytometry at the 12th week after operation. **RESULTS:** (1) Hepatocellular degeneration was slightly less in group B [intrasplenic hepatocyte autologous transplantation (IHAT) + PNGS 25 mg/kg, im, qd] vs the control group (group C, IHAT without drugs) at the 2nd week after transplantation, and the ALT content of group B (928 U/g ± 268 U/g) was higher than that of group C (639 U/g ± 138 U/g, *P* < 0.01). (2) At the 12th week, hepatocellular regeneration in group A (IHAT + PHGF 5 mg/kg, im, qd) was obviously better than that in group C, and the ALT content (2325 U/g ± 401 U/g), the radioactivity accumulation of ^{99m}Tc-HIDA (58 Bq ± 18 Bq), and proliferation index (3.8 % ±

0.4 %) of group A were all higher than those of control (*P* < 0.05). **CONCLUSION:** PHGF has effects in increasing the weight of liver tissue grown in spleen and shortening the regeneration period of the transplanted hepatocytes, while PNGS has certain effects on protecting the hepatocytes against ischemic-reperfusion injury in the early stage of transplantation.

INTRODUCTION

Intrasplenic hepatocyte transplantation offers an attractive alternative to orthotopic liver transplantation in the treatment of end-stage liver disease, acute liver function failure, and inborn errors of liver metabolism. One of the major limitations has been the insufficient survival of an adequate mass of transplanted cells to permanently correct defects in liver function. Understanding the regenerative behavior of transplanted hepatocytes is of great importance for developing and improving such novel therapeutic strategies as hepatocellular transplantation and *ex vivo* gene therapy. For this purpose, we designed the following experiment in which two new mitogenic stimuli, hepatocyte growth-promoting factor (PHGF) and panax notoginseng saponins (PNGS), were used. In this study the proliferative response of transplanted hepatocytes was examined in relation to the administration of PHGF and PNGS.

MATERIALS AND METHODS

Animals and groups Outbred male or female Sprague-Dawley rats weighing about 250 - 350 g were purchased from Experimental Animal Centre of Sun Yat-Sen University of Medical Sciences (Grade II). All animals were performed with 70 % partial hepatectomy according to the technique originally described by Higgins and Anderson. They were randomly divided into four

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groups as following:

Group A ($n = 28$): Intrasplenic hepatocyte autologous transplantation (IHAT) and PHGF was used for 4 weeks postoperatively (5 mg/kg, im, qd);

Group B ($n = 28$): IHAT and PNGS was used for 2 weeks postoperatively (25 mg/kg, im, qd);

Group C ($n = 28$): IHAT and no special drugs were used after operation;

Group D ($n = 19$): 70 % partial hepatectomy without IHAT or application of any drugs.

PHGF was purchased from Yangjiang Pharmaceutical Factory of China. It was obtained from fetal liver of pig formulated as powder for injection, to be stored in an environment under -10°C . The effective dose in this study was decided on the basis of the study by Zhang *et al*⁽¹⁾. The injection of PNGS was bought from Wuzhou Pharmaceutical Factory of China. It was purified from a traditional Chinese medicine and identified to contain 7 saponins. The dose of PNGS in our study was in accordance with other reports⁽²⁾.

Isolation of hepatocytes and transplantation procedure Preparation of isolated hepatocytes was performed by the modified mechanical method described by Pfaff *et al*⁽³⁾. The liver tissue was gently minced and agitated at 4°C in RPMI-1640 (pH: 7.35 - 7.45), then filtered through a 200- μm nylon mesh as soon as possible. Apart from dexamethsone (0.25 g/L) and glucogen (25 g/L), PHGF (5 g/L) was added into the suspension in group A and PNGS (25 g/L) was added in group B. The hepatocyte suspension was counted with a hemocytometer after testing its viability by the trypan blue exclusion test. Hepatocellular autotransplantation was then carried out by direct injection of hepatocyte suspension 1 mL containing approximately 5×10^6 hepatocytes into the splenic parenchyma, with temporary occlusion of the vascular system at the splenic hilum and clipping at the site of injection.

Post-transplantation examinations In second week post-transplantation, hepatedized spleen specimen were fixed in 10 % formalin and stained with hematoxylin and eosin (HE), and periodic acid-Schiff (PAS). The routine histological examination was carried out under direct light microscopy. Morphometric analysis was also carried out under electron microscopy. Furthermore, the content of alanine transaminase (ALT, U/g) of hepatedized spleen homogenate was assayed to assess the proportional amount, function state, and the extent of injury of liver tissue contained in spleen⁽⁴⁾. The homogenate was prepared ultrasomically ($\lambda = 22 \mu\text{m}$) by

an instrument made in England (MSE).

In the 12th week post-transplantation, apart from the examinations mentioned above, two new methods were used. The first was $^{99\text{m}}\text{Tc}$ -diethyl-iminodiacetic acid ($^{99\text{m}}\text{Tc}$ -HIDA) splenic scintiphotography to monitor the quantitation and function of transplanted hepatocytes⁽⁵⁾. The second was detection of proliferation index (PI, the percentage of DNA-synthesizing cells in a cell circle) of transplanted hepatocytes with FACSTAR PLUS flow cytometry. It reflected objectively the proliferating ability of transplanted hepatocytes. The modified method for separating hepatocytes from spleen was involved in collagenase perfusion, cell culture under certain conditions, and density gradient centrifugation for lymphocytes.

Statistical analyses All data were presented as $\bar{x} \pm s$, and compared with the *F*-test, except PI which was analyzed by *H*-test.

RESULTS

The number and viability of transplanted hepatocytes

It is shown in Tab 1.

Tab 1. The number and survival rate of transplanted hepatocytes. IHAT: intrasplenic hepatocyte autologous transplantation. $n = 28$. $\bar{x} \pm s$. * $P > 0.05$ vs group C. Group A: IHAT + PHGF 5 mg/kg. Group B: IHAT + PNGS 25 mg/kg. Group C: IHAT.

	Group A	Group B	Group C
$10^{-6} \times$ Hepatocyte number	5.8 ± 2.1^a	5.7 ± 1.9^a	6.1 ± 1.7
Hepatocyte survival rate/%	69 ± 10^a	68 ± 8^a	68 ± 9

Histological findings in the hepatedized spleen In 2-week post-transplantation, light microscopic study revealed that the grafted liver cells suffered from serious degeneration and necrosis in the early stage of transplantation, with presence of retrodifferentiation, and only a small number of residual cells survived. Electron microscopic study showed swollen mitochondria and a considerable decrease in rough endoplasmic reticuli and glycogen particles in the swollen cytoplasm of the surviving hepatocytes. However, the extent of degeneration appeared to be slight in group B (IHAT + PNGS) compared with Group A (IHAT + PHGF) and Group C (IHAT). The degree of PAS staining was also relatively positive in

Group B than in others. In 12th week post-transplantation, the surviving hepatocytes proliferated and reconfigured to form hepatocellular islets and cords. Some cells had almost the same morphology as normal hepatocytes, and took up PAS well. An abundance of binuclear cells was observed and mitosis was easy to be seen. On electron microscopy, there were abundant organelle with considerable increase in mitochondria, rough endoplasmic reticuli, and glycogen particles. The bile canaliculus was observed accidentally between the borders of two hepatocytes. All findings indicating hepatocyte regeneration mentioned above were most significant in Group A (IHAT + PHGF) (Fig 1, 2).

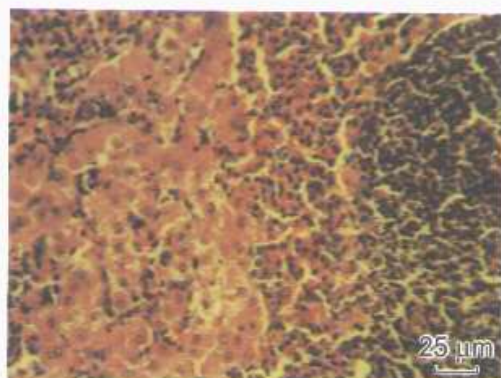


Fig 1. Intrasplenic hepatocellular islets and cords of group A (IHAT + PHGF) at the 12th week. Some hepatocytes had almost the same morphology as normal hepatocytes. (HE × 400).



Fig 2. Intrasplenic hepatocellular islets of group A at the 12th week, with regular nuclei and clearly discernible nucleoli. (EM × 2950).

The content of alanine transaminase (ALT) in hepated spleen homogenate It is shown in Tab 2. ALT was present in constantly low amounts in the spleen of group D (70 % hepatectomy without IHAT or application of any drugs) which was not treated by hepatocytes transplantation. There was a significant difference between group D and the other groups regardless of the time of postoperation. In second week post-operation, the ALT content of group B (IHAT + PNGS) ($928 \text{ U/g} \pm 268 \text{ U/g}$) was higher than that in Group C ($P < 0.01$) but in the 12th week post-operation, in group A (IHAT + PHGF) ($2325 \text{ U/g} \pm 401 \text{ U/g}$) ALT content was higher than that of group C ($P < 0.05$). Furthermore, there was a significant difference between 12-week and 2-week treatment in every group with IHAT. ALT content of every group with IHAT was about 2-to 3-fold higher at the 12th week than that at the 2nd week after transplantation.

Tab 2. The content of ALT in hepated spleen homogenate (U/g). $n=8$. $\bar{x} \pm s$. * $P > 0.05$, $^{\#}P < 0.01$ vs group C.

	ALT/U·g ⁻¹	
	2 weeks	12 weeks
Group A (IHAT + PHGF)	611 ± 172 [#]	2325 ± 401 [#]
Group B (IHAT + PNGS)	928 ± 268 [#]	1900 ± 424 [#]
Group C (IHAT)	639 ± 138	1839 ± 368
Group D	203 ± 47	220 ± 30

Uptake of ^{99m}Tc-HIDA by hepated spleen

^{99m}Tc-HIDA is a radiographic agent which can be absorbed particularly by hepatocytes. Normally, 98 % of ^{99m}Tc-HIDA will be absorbed by hepatocytes and then excreted through biliary system. Therefore, ^{99m}Tc-HIDA splenic scintiphotography can be used to monitor the quantitation and function of transplanted hepatocytes. This test was carried out in the 12th week post-transplantation in our study. The liver appeared clear but the spleen was invisible *in vivo* under SPECT scanning. Then the spleen was scanned directly by SPECT *in vitro* and its radioactivity accumulation of ^{99m}Tc-HIDA was analyzed statistically among groups. The result showed that the appearance of hepated spleen *in vitro* was apparently clear in group A (IHAT + PHGF) compared with group B (IHAT + PNGS) and group C (IHAT without drugs) (Fig 3), and the radioactivity accumulation of ^{99m}Tc-HIDA was also significantly higher in group A than that of group B or group C ($P < 0.05$, Tab 3).

Detection of proliferation index of transplanted hepatocytes The proliferation index of intrasplenic hepatocytes was detected by flow cytometry at the 12th week after operation. The result showed that the PI of group A ($3.8\% \pm 0.4\%$) was higher than that of group B ($3.0\% \pm 0.4\%$) or group C ($2.9\% \pm 0.3\%$). There was no significant difference between group B and group C (Tab 4).

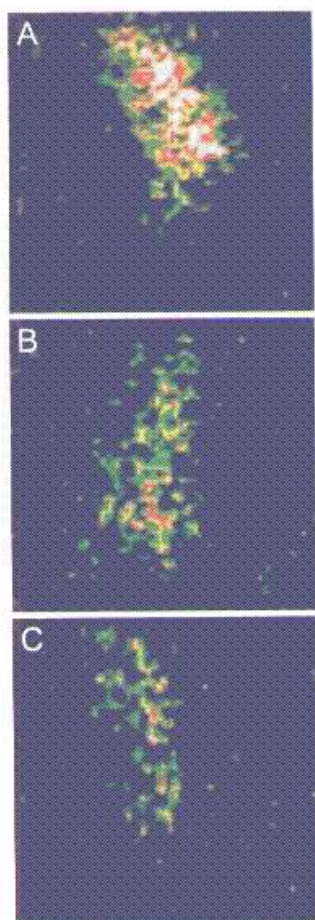


Fig 3. ^{99m}Tc -HIDA imaging of hepatized spleen *in vitro* at the 12th week. The clarity of group A is better than the other groups. A) IHAT + PHGF 5 mg/kg; B) IHAT + PNGS 25 mg/kg; C) IHAT.

DISCUSSION

Many experiments have indicated that intrasplenic hepatocellular transplantation has potential clinical application to compensate for impaired hepatic function or to correct congenital errors, even to carry out gene therapy.

Tab 3. The radioactivity accumulation of ^{99m}Tc -HIDA in hepatized spleen *in vitro*. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ vs group C.

	Radioactivity Bq
Group A (IHAT + PHGF)	50 ± 13^a
Group B (IHAT + PNGS)	23 ± 8^b
Group C (IHAT)	18 ± 4

Tab 4. Detection of proliferation index of transplanted hepatocytes. $n = 6$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ vs group C.

	Proliferation index (%)
Group A (IHAT + PHGF)	3.8 ± 0.4^a
Group B (IHAT + PNGS)	3.0 ± 0.4^b
Group C (IHAT)	2.9 ± 0.3

In acute hepatic function failure, hepatocytes transplantation could be regarded as a "bridge" for treatment before liver organ transplantation is performed. However, most of the studies have been limited in animal experiments, the main reason preventing it from being widely used in clinical practice is that the weight of liver tissue contained in spleen increases difficultly and the regeneration period of transplanted hepatocytes is too long^[9-12]. In view of above, we used PHGF and PNGS in the experiment of intrasplenic hepatocellular autotransplantation with 70% partial hepatectomy. PHGF is a strong stimulator of hepatocellular DNA synthesis and PNGS is a new calcium channel blocker. In this study, we expected an increase in the weight of liver tissue mass present in spleen and shortening in the regeneration period of transplanted hepatocytes by stimulating DNA synthesis and protecting against ischemic-reperfusion injury, hence to provide some experimental results for the clinical application of hepatocellular transplantation.

The mechanism for regeneration of intrasplenic transplanted hepatocytes is very complicated and is affected by many factors^[9,10], but mainly consisting of both hepatic hypertrophy and hyperplasia. Hepatic hypertrophy is manifested by an increase in cell size and function and with RNA synthesis and protein synthesis as its major features. Hepatic hyperplasia is characterized by an increased number of cells with DNA synthesis as its most striking feature. The so-called hepatic trophic factors

(including insulin, glucogen, EGF, PDGF, IL-6, TGF and corticoid, etc) have a role in the control of hepatic hypertrophy but can not initiate hepatic hyperplasia. Only hepatocellular growth factors (HGF) have the ability to promote hepatocyte proliferation, because they can stimulate hepatocellular DNA synthesis specifically^[11]. PHGF is a kind of HGF, which is isolated from fetal or regenerating liver tissue, and has been proved to be a strong stimulator of hepatocellular DNA synthesis^[1]. Our experimental results showed that hepatocellular regeneration in group A was better than that of other group, ALT content was higher than that in group C ($P < 0.05$), and the radioactivity accumulation of ^{99m}Tc-HIDA in group A was also higher than that in group C ($P < 0.05$), and proliferating index (which directly and specifically reflects the regenerating ability of transplanted hepatocytes), got to a high point of $3.8\% \pm 0.4\%$ (group A vs group C; $P < 0.05$) at the 12th week after transplantation, thus demonstrating that PHGF had great effects on increasing the weight of liver tissue contained in spleen and shortening the regeneration period of the transplanted hepatocytes.

Generally speaking, isolated hepatocytes after ectopic transplantation into the spleen will also undergo a process of ischemic-reperfusion injury, which will cause Ca^{2+} -overload, thus hindering the regeneration and proliferation of transplanted hepatocytes and even cause cells death. Blockers of calcium channels can protect against this injury^[12]. Usually voltage-operated calcium channel (VOC) were regarded to be the most important for hepatocytes, but recent studies showed that receptor operated calcium channel (ROC) was much more important than VOC. This can explain why verapamil, a classical blocker of calcium channel, could not decrease the ischemic-reperfusion injury significantly after liver ectopic transplantation because verapamil had almost no effects on ROC. PNGS are new blockers for ROC which were purified and obtained from a traditional Chinese medicine recently. Guan *et al* have proved that PNGS could block ROC particularly and strongly by inhibiting the transmitter release at mouse motor nerve terminal and the ⁴⁵Ca-influx and efflux induced by activation of α_1 adrenoceptor of vascular smooth muscle^[2,13,14]. So, we used PNGS in our study. The results showed that the extent of hepatocellular degeneration appeared to be slight in group B and the degree of PAS staining was also relatively positive in group B compared with the other groups, and the ALT content was markedly higher than that of group C ($P <$

0.05) in the 2nd week post-operation. This indicated that PNGS had certain effects on protecting the transplanted hepatocytes from ischemic-reperfusion injury in the early stage of transplantation. But at the 12th week post-operation, there were no significant differences between group B and group C.

With the rapid development of molecular biology, if transplanted hepatocytes could self-express and self-secrete some cell factors by techniques of genetic engineering to enhance their regeneration and proliferation, there will be a hope to make a breakthrough in promoting hepaticized spleen. In addition, the reliability of hepatocellular transplantation is essential for clinical application of *ex situ* hepatic gene therapy. Intrasplenic hepatocellular transplantation provides a new way for hepatocellular gene therapy^[15,16].

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肝细胞生长因子与三七总皂甙对自体肝细胞脾内移植的影响¹

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关键词 肝再生; 异位移植; 肝细胞生长因子; 人参

目的: 探索通过促进肝细胞 DNA 合成及对抗缺血再灌注损伤以加速肝化脾增量及缩短其再生周期的新途径. 方法: 应用肝细胞生长因子(PHGF)与三七总皂甙(PNGS)于自体肝细胞脾内移植(IHAT)的动物模型上, 分别于移植术后 2 周、12 周对病理组织学、电镜形态、肝化脾匀浆谷丙转氨酶(ALT)含量、^{99m}Tc-HIDA 摄取试验及脾内肝细胞增殖指数等进行观察分析. 结果: 移植术后 2 周, 三七总皂甙组脾内肝细胞水肿、变性程度较轻, 肝化脾匀浆 ALT 含量达(928 ± 268)U/g, 明显高于对照组(P < 0.05); 移植术后 12 周, 肝细胞生长因子组脾内肝细胞生长好, 数量、面积大, 肝化脾匀浆 ALT 含量达(2325 ± 401)U/g, 增殖指数达 3.8 % ± 0.3 % . 对照组分别为(1839 ± 368) U/g, 2.9 % ± 0.4 % , P < 0.05, 且在^{99m}Tc-HIDA 摄取试验中 PHGF 组离体肝化脾显影较清晰, 其放射性计数明显高于对照组(P < 0.05) 结论: PNGS 在移植早期对脾内肝细胞有一定的抗损伤保护作用, 而 PHGF 对加速肝化脾增量及缩短其再生周期有明显作用.

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