行政院國家科學委員會補助專題研究計畫成果報告

免疫抑制劑對大鼠肝臟部分切除後肝細胞再生的影響
The effects of immunosuppressants on liver regeneration after partial hepatectomy in rats.

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計畫主持人：賴鴻緒
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一、中文摘要

近年來肝臟移植救治肝病末期病患之成功率已高達 90%以上。但由於捐肝者不足，由活人以部份肝臟移植之方法逐漸盛行。小塊肝臟是否能再生，以備長期之需當然十分重要。而術後各種抗排斥藥物如 Cyclosporin A，Tacrolimus （FK506），Myophenolate Mofetil （CellCept）及類固醇等，對肝細胞再生是否有重大影響，目前仍未知。許多報告指出，肝臟部份切除後，局部或全身性免疫系統會有變化，Sato 及 Hirano 等人的實驗均證明，諸如肝臟內之 extrathymic T cell，lymphokine activated killer cells 均與免疫變化有關。多年來我們深入研究肝細胞再生，以老鼠做動物實驗，已知肝臟部份切除術後，肝細胞分裂及細胞內代謝活動力，於術後 6 至 24 小時達到頂點，至 72 小時漸回復至基礎線。我們也發現切肝後，血液 T 型淋巴球次群，包括 T3、T4、T8、T-IL2R、NK 等，均明顯增加，血清 γ-IFN 及剩餘肝內 NK 細胞均明顯上升，而血清及尿中 neopterin 之濃度亦大大提高。然而免疫抑制劑的使用，是否能藉著對抗免疫細胞而反倒促進肝細胞再生，則仍需進一步證實。

本計畫以重約 200 公克之 Wistar 雄性大鼠作研究，分為實驗組及對照組，施行百分之七十部份肝臟切除術，分別在術前 2 天開始，給予 Cyclosporin A (CyA)，Tacrolimus (Ta)，Mycophenolate Mofetil (MM)，及 Solu-Medrol (S)單獨使用，或三合一使用 CyA+MM+S(CMS)或 Ta+MM+S (TMS)等免疫抑制劑，比較均不給予藥物之大鼠，在術後 6、24、48 及 72 小時犧牲取樣，測定(1) 剩餘肝臟內含 Thymidine Kinase(TK) 及 Ornithine Decarboxylase(ODC)等肝細胞再生指標酵素之活力；(2) 週邊血液之白血球及淋巴球數；(3) 血清肝功能指標變化；及 (4) 檢視剩餘肝臟組織學變化、細胞分裂情形等。

結果發現：(1) TK 與 ODC 活性在術後早期明顯上升，各種免疫抑制劑均使 TK 及 ODC 活性有意義增加($P<0.05$)，而三合一(CMS 及 TMS)更使增加明顯($P<0.01$)；(2) 單用 Solu-Medrol 者不影響 TK 及 ODC 活性；(3) 免疫抑制劑使用會使週邊血液淋巴球數明顯減少($P<0.05$)；(4) 組織學檢查可見到細胞分裂數目增加，因免疫抑制劑使用則增加較明顯，尤其 TK 之染色在 CMS 及 TMS 組更加明顯。本實驗證實，免疫抑制劑的使用，對促進肝臟部分切除後之肝細胞再生有所幫助。

關鍵詞：免疫抑制劑，肝臟部份切除，肝細胞再生，免疫反應，部分肝臟移植

ABSTRACT

Liver transplantation, with more than 90% successful rate, is one of the standard methods to treat the patients with end stage liver disease. Transplantation with partial

liver resected from living people is needed due to non-enough donate livers. The regeneration of the small part of liver is very important for the long term survival. However, the effects of immunosuppressants such as cyclosporin A (CyA), tacrolimus (Ta), myophenolate mofetil (MM) and Solu-Medrol (S) on liver regeneration is still be unclear. Partial hepatectomy may raise the possibility of changes in local or systemic immune response. Several phenomena such as alterations of extrathymic T cells in the liver, lymphokine-activated killer cells were observed by Sato and Hirano et al in animal experiments after partial hepatectomy. Our previous studies have focused on the topic of liver. We have found that remnant liver mitosis and DNA synthetic activity increased markedly from 6 to 24 hours after partial hepatectomy in rats, then it recovered to normal level 72 hours postoperatively. We also found that all T-lymphocyte subpopulations in peripheral blood, serum γ-IFN, peripheral blood NK cells and the absolute number of NK cells in the remnant liver of hepatectomized rats increased markedly on the fifth and seventh posthepatectomized day. Serum and urinary neopterin concentrations can be used as markers of activated cell-mediated immunity in rats after partial hepatectomy. However, whether the administration of immunosuppressants can affect the regeneration of the remnant liver is still necessary to investigate for.

The main purpose of this project is to find out the effects of immunosuppressants on the liver regeneration after partial hepatectomy. Male Wistar rats around 200g was used as subject. Partial hepatectomies (about seventy percent hepatectomy) were performed on experimental groups rats. Cyclosporin A, tacrolimus, myophenolate mofetil, or Solu-Medrol were given separatedly or in three combine formula (CMS or TMS). Then they were sacrificed at 6, 24, 48 and 72 hours after hepatectomy. We measured (1) The activity of Thymidine Kinase (TK) and Ornithine decarboxylase (ODC) in the remnant liver which will be the indicators of the degree of liver regeneration; (2) leucocyte and absolute lymphocyte counts in peripheral blood; (3) serum liver function tests; (4) histological changes of the remnant liver (including mitosis and regeneration condition). The results were: (1) The increase of TK and ODC activity in remnant liver were significantly more in CyA, Ta and MM group rats ($p<0.05$), and even more in CMS and TMS groups ($p<0.01$); (2) There were no significant changes in S group rats; (3) The peripheral lympocytes counts decreased significantly in all immunosuppressive group rats ($p<0.05$); (4) Histological pictures showed that the mitosis, especially TK stained cell, increased significantly in CMS and TMS groups rats. We suggested that immunosuppressants can increase liver regeneration after partial hepatectomy in rats.

Keywords : immunosuppressant, partial hepatectomy, liver regeneration, immune response, partial liver transplantation

INTRODUCTION

Liver is a core organ in our body. Although it contains good regeneration ability, hepatic failure still can occur after massive hepatic injury or hepatectomy. The capacity of the mammalian liver to regenerate in response to numerous stimuli has been well documented[1-3]. Many studies have focused on factors which can promote or regulate liver regeneration. Several factors, such as hormones, growth factors, nutritional components, and pharmacological agents, have been demonstrated to directly or indirectly affect liver regeneration[4-6]. Much controversy continues on the initiation, regulation, metabolic changes, and termination of liver regeneration after partial hepatectomy that may well initiate proliferation of the remaining hepatocytes. Partial hepatectomy may raise the possibility of changes in local or systemic immunological response. During liver regeneration after partial hepatectomy, cell-mediated immunity is activated, and as a

result, autoreactive suppressor T cells[7-10], and natural killer (NK) cells [11,12] are activated against the allogeneic respinse.

Liver transplantation is already a standard therapy of choice for the patients with end stage liver disease. Immunosuppressants such as Cyclosporin A (Sandimmun Neoral), Tacrolimus (Prograf), Mycophenolate Mofetil (CellCept), and even steroid (Solu-Medrol) were usually used after liver (and other organs) transplantation. Liver regeneration is important for the graft liver, especially in the patients with partial liver transplantation. The small resected liver graft, after preservation and ischemic injury, can create good liver function for good only by enough regeneration. The relationship between immune response and liver regeneration has already been proved to be existed. The immune response was believed to be one of the factors which terminate the liver regeneration. Cyclosporin A, Tacrolimus and Mycophenolate Mofetil were proved to be powerful immunosuppressive agent and routinely used after partial liver transplantation. Steroid combine use could have synergistic effects. Kahn et al reported that oral intake of Cyclosporin A can augment the regeneration response[13]. However, there were no further study to compare the effects of several anti-rejection agents and the effects of combine use of these agents.

MATERIALS AND METHODS

(1) Animals:

Male Wistar rats (purchased from Charles River, Osaka, Japan) weighing approximately 200 g will be used as subjects. All the rats will be randomly assigned to seven different groups.

(2) Grouping:

Group 1 (N=40), Partial hepatectomy with no medication (C)

Group 2 (N=40), Partial hepatectomy with Cyclosporin A (CyA)

Group 3 (N=40), Partial hepatectomy with Tacrolimus (Ta)

Group 4 (N=40), Partial hepatectomy with Mycophenolate Mofetil (MM)

Group 5 (N=40), Partial hepatectomy with Solu-Medrol (S)

Group 6 (N=40), Partial hepatectomy with combine administration of Cyclosporin A, Mycophenolate Mofetil and Solu-Medrol (CMS)

Group 7 (N=40), Partial hepatectomy with combine administration of Tacrolimus, Mycophenolate Mofetil and Solu-Medrol (TMS)

(3) Surgical Procedures

All rats are anesthetized by intraperitoneal pentobarbital (40mg/Kg) injection. A midline laparotomy was performed. Partial hepatectomy was then carried out by means of aseptic extirpation of the median and left lateral lobes, according to the procedure of Higgins and Anderson [14].

(4) Measurements

A. The degree of liver regeneation

The remnant livers were removed and cut in pieces. Hepatic thymidine kinase (TK) and Ornithine decarboxylase (ODC) activities will be used as indicators of liver regeneration.

a. Thymidine Kinase(TK) activity

The enzymatic activity of TK was assayed in the supernatant fractions of liver portions after homogenisation centrifugation at 105,000 g for 1 h at 4 ℃ with a Beckman model L5-75 ultracentrifuge, according to the method described by Kahn et al[15].

b. Ornithine decarboxylase(ODC) activity

The supernatant fractions of liver portions after homogenisation and centrifugation for analysis of ODC were obtained as described same in TK activity innitial procedure. The ODC activity will be measured by ELISA method (ODC Ab-1, Clone MP 16-2, #MS-464-PO, Purified, Neomarkers, CA, U.S.A.)[16].

B. The leucocyte and lymphocyte counts of peripheral blood

C. Serum biochemical indicators of liver function test

D. Histological examination of remnant

liver

The small pieces of liver tissue for hisopathological examination was fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin for microscpic observation. TK stained cell was also done by methods[17]

RESULTS

All rats were survived after hepatectomy and lived well during the experimental periods.

The thymidine kinase activity which was detected at mitotic stage were shown as Figure 1. The TK incorporated cell could not be seen in normal liver and at 6h posthepatectomized stage in control and all experimental groups rats. The TK incorporated all began to show at 24h stage, and increased marbedly at 48h stage, then decreased in number but still be much at 72h stage after partial hepatectomy in control group rats (Fig.1A). It was demonstrated that the number of TK stained hepatocytes in creased in experimental groups rats, especially at 48h stage in CMS and TMS groups rats (Fig.1B, 1C). When calculated the TK stained hepatocytes as mitotic index by 50 different fields under 400x light microscope, it showed that the number of TK enhanced mitotic index were 4±2, 108±12 and 26±5 at 24h, 48h and 72h posthepatectomized stages in control group rats (Table 1). The in creased mitotic index number were significantly more in group CyA (141±18, $p<0.05$), group Ta (144±19, $p<0.05$), group MM (152±22, $p<0.05$), group CMS (168±24, $p<0.01$) and group TMS (160±25, $p<0.01$) at 48h postoperative stage. Then the mitotic index number began to decrease at 72h postoperative stage, but still be significantly more in groups Ta, CMS and TMS (36±7, 40±8 and 42±10) rats when compare to control group rats. The TK mitotic index number in group S rats were similar to group C rats at all stages.

As for the changes of ornithine decarboxidase, the contents in the remnant liver increased significantly from 225±30 mg/dL preoperative to 656±44, 604±45, 460±51, and 392±42 mg/dL at 6h, 24h, 48h and 72h after partial hepatectomy in group C rats. The changing curves in experimental groups rats were about the same. However, the increase were significantly more in CyA, Ta and MM groups rats ($p<0.05$) and ever more markedly in CMS and TMS groups rats ($p<0.01$) at 6h stage (Table 2). The increase were still significantly more in groups Ta, MM ($p<0.05$) and groups CMS, TMS ($p<0.01$) at 24h stage, and in group MM ($p<0.05$), groups CMS and TMS ($p<0.01$) at 48h stage. Then the ODC contents in remnant liver returned to about same level of control group 72h after partial hepatectomy. The ODC contents in group S rats were similar to group C rats at all stages.

The patterns of peripheral white blood cells at each stage were similar in all groups rats. There were no special changes could be detected. As for lymphocyte count in peripheral blood, it increased significantly at 48h and 72h ($p<0.05$). The changes could also in control group rats be seen in experimental group rats. However, the lymphocyte counts were significantly less even at preoperative stage in CMS and TMS groups rats ($p<0.05$) (Table 3). The increase of posthepatectomized lymphocyte counts were significantly less at 48h and 72h stage in Ta group rats, and all stages in CMS and TMS groups rats. The lymphocytes counts level of group S rats were similar to control group rats.

The serum parameters of the liver function test showed similar patterns in control and all experimental groups rats. It revealed a somewhat elevated level of AST, ACT, and alkaline phosphatase within the first 48 hours after partial hepatectomy. No significant changes could be found in the bilirubin, albumin and globulin levels. These were no any significant difference could be detected between control and each experimental group rats.

Observation of the paraffin sections of the liver tissue under microscope were performed. It demonstrated that many lipid droplets appeared since 6h, then persisted to 72h posthepatectomized stage. There were no mitoses could be seen in control group rats at the preoperative and 6h postoperative stages. Few mitoses could be observed at 24h stage. Then, the mitotic index of the regenerating liver increased markedly at 48h (up to around 100 in 50 different fields under 400X high power field). The number decreased to around one-fourth at the 72h stage. There were no any significant difference of the changes of the mitotic hepatocytes between control group and each experimental group rats.

DISCUSSION

Liver regeneration is one of the most raped forms of tissue growth in mammals. The intracellular activities which include the completion of DNA replication and the onset of mitoses in the regenerating hepatocytes, are affected by many factors [1,2,11,13]. The mechanism of the termination of the regenerating process has been widely studied but is still not clear. Liver has the function of a haematolymphoid organ being closely associated with the systemic haematolymphoid system [9]. An immunological response might regulate or even inhibit liver regeneration after hepatectomy [12,13], and cell-mediated immunity involving γ-IFN, NK cells, and macrophages has an important role in this immunological response. Several lymphokines and extrathymic T-cells are also involved [9,11]. However, whether the immunological response and liver regeneration are two independent events or one connected to the other is still controversial. The effect of immunosuppressants on the regenerative response may also be relevant in certain cases of pediatric liver transplantation where, because of the critical shortage of suitable pediatric donors, many centers have resorted to using reduced-size adult liver grafts. A normal regenerative response may be necessary for the successful outcome of these grafts.

ODC is the rate-limiting enzyme in polyamine synthesis, and increased polyamine synthesis is thought to play a critical role in the stimulation of hepatic regeneration after partial hepatectomy. In addition, regeneration of the liver involves an increase in DNA synthesis in the liver, which requires an induction of hepatic TK activity. Thus, these two enzymes were used as indices of liver regeneration. Both TK activity and ODC activity were significantly higher after partial hepatectomy in rats pretreated with CsA.

Thus, there appears to be increased hepatic regeneration after partial hepatectomy in rats treated with CsA. CsA is known to be hepatotoxic and this may account for the greater regenerative response. However, preoperative liver function tests in the rats pretreated with CsA were the same as control values. On the other hand, the injury may be subclinical and not detected by routine tests of liver function.

It may be that CsA induces both ODC and TK. This would account for the significantly higher preoperative levels of activity of both enzyme in rats pretreated with CsA and would explain the significant elevation in ODC at six hours after sham operation.

The data presented in this study demonstrate that CsA at the dosage used did not adversely affect hepatic regeneration after partial hepatectomy in rats and actually resulted in a greater regenerative response.

The effect of each immunosuppressant agents and combined formula on lymphocyte counts in this study could be observed clearly. The changes of TK enhanced mitotic index and ODC content in remnant liver could also be detected to show the effect of immunosuppressants on the liver regeneration after partial hepatectomy. The administration of corticosteroid was unable to effect the lymphocyte counts and the

regenerating status after > 0 % partial hepatectomy. The regenerating status, which was indicated by TK enhanced mitotic index and ODC content in remnant liver, became more active in cyclosporin A, tacrolimus and mycophenolate mofetil administrating rats in whom the decrease of lymphocyte counts could also be detected. The regenerating status appeared even more marked significantly in CMS and TMS group rats. From these results, we suggested that there is a positive effect of immunosuppressants (including cyclosporin A, tacrolimus and mycophenolate mofetil) on the liver regeneration after partial hepatectomy. The synergestic effect can be found markedly when combined use of multiple immuno-suppressants and steroid were tried. Further study of the mechanism and the best formula of the immunosuppressants for the effect on liver regeneration is needed.

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Table1. Thymidine kinase enhanced mitotic index number

Time\Gr	C	CyA	Ta	MM	S	CMS	TMS
Preop.	0	0	0	0	0	0	0
6h	0	0	0	0	0	0	0
24h	4±2	6±2	5±2	5±2	4±2	16±5#	18±5#
48h	108±12	141±18*	144±19*	152±22*	100±11	168±24#	160±25#
72h	26±5	34±8	36±7*	34±9	24±6	40±8*	42±10*

Mean ± SD; 400X, Sum of 50 different fields; h, hour; $p<0.05$, vs Gr. C; # $P<0.01$, vs Gr. C.

Table2. The alternations of ornithine decaboxidase activity in each group rats

Time\Gr	C	CyA	Ta	MM	S	CMS	TMS
Preop.	255±30	215±28	222±28	230±30	218±22	209±29	227±32
6h	656±44	741±48*	742±45*	741±50*	647±68	1028±71#£	1122±70#◎
24h	604±45	660±58	682±50*	698±52*	586±49	970±66#£	908±69#◎
48h	460±51	511±62	513±48	533±57*	407±41	623±55#£	632±64#◎
72h	392±42	385±42	375±42	360±38	388±40	366±39	390±40

*p<0.05, vs Gr. C; # p<0.01, vs Gr. C; £ p<0.01, vs Gr. CyA; ◎ p<0.01, vs Gr.Ta.

Table 3. Changes of peripheral white blood cells (WBC) and lymphocytes counts(/cmm)

Time\Gr	C	CyA	Ta	MM	S	CMS	TMS
WBC							
Preop.	6170±681	6088±708	5862±697	5689±683	6185±701	5682±607	5219±621
24h	6600±817	5617±694	5912±821	5811±608	6088±699	5444±594	5402±666
48h	5638±752	5602±702	6032±808	5733±574	6080±714	5349±612	5220±588
72h	5933±823	5022±519	5433±721	5292±621	6144±612	5011±529	5214±684
Lymphocytes							
Preop.	1728±186	1483±177	1388±180	1409±111	1828±199	1258±144#	1189±190#
24h	2111±284	1662±202	1708±199	1682±281	2158±288	1413±205#	1482±216#
48h	2466±296*	2022±249	1897±242*#	1844±229	2602±284*	1445±229#	1409±284#
72h	2618±305*	2228±285	2049±282*#	2195±300	2700±317*	1617±294#	1587±266#

* <0.05 vs Preop. ; # <0.05 vs Gr. C

Fig 1: Histological pictures of TK incorporated hepatocytes at each postoperative period in control (1A), CMS (1B) and TMS (1C) groups rats.