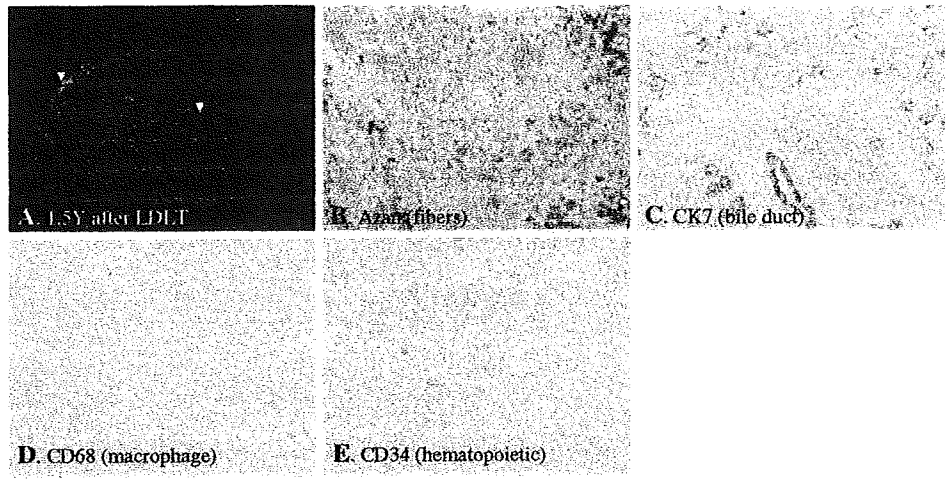


Fig. 5 Immunohistochemical stainings in case 15, secondary biliary cirrhosis after LDLT. **a** FISH showing Y-chromosome-positive cells (white square), **b** Azan staining was positive, showing the presence of liver fibrosis, **c** CK7 (cytokeratin 7, bile duct) was not positive in the black square, **d** CD68 (macrophage) was partially positive in the black square, **e** CD34 (hematopoietic cell) was not positive in the black square. *LDLT* living-donor liver transplantation

Table 1 Demographics of male recipients with female donors

Case no.	Age	Gender	Etiology	Donor	Blood type match	Graph type	Biopsy period after LDLT	Comments	Outcome
1	16	M	FHF	Mother	Identical	L	3d, 4Y	None	Survived
2	5	M	BA	Mother	Identical	LL	2M, 8Y	Cholestasis	Survived
3	56	M	LC-B/HCC	Sister	Identical	R	1.5M, 1.8Y, 2Y mild ACR	None	Survived
4	20	M	FHF	Aunt	Identical	R	1M, 5M, 2Y	Cholestasis	Survived
5	58	M	LC-C	Sister	Identical	R	2M	Hepatitis	Survived
6	56	M	LC-B/HCC	Daughter	Identical	R	8M	Vanishing BD	Survived
7	56	M	LC-B/HCC	Daughter	Identical	R	9M (Re-LDLT)	Poor quality	Survived
8	56	M	LC-B/HCC	Wife	Identical	R	6M	Mild ACR	Survived
9	58	M	LC-C/HCC	Daughter	Incompatible	R	3W	Hepatitis	Survived
10	62	M	LC-C	Sister	Compatible	L	1.5M	Moderate ACR	Survived
11	41	M	PBC	Wife	Identical	R	1W, 1M (autopsy)	Severe ACR	Died (2M)
12	50	M	LC-B	Wife	Identical	R	10d (graft failure)	Malcirculation	Died (1M)
13	57	M	LC-C/HCC	Wife	Identical	R	10d, 2M (graft failure)	Moderate ACR	Died (2M)
14	47	M	LC-Al	Sister	Identical	R	3.8Y (liver cirrhosis)	Poor quality	Died (3.8Y)
15	51	M	LC-C	Sister	Identical	R	2.5Y (chronic liver failure)	Biliary cirrhosis	Died (2.5Y)

FHF fulminant hepatic failure, *BA* biliary atrasia, *ACR* acute cellular rejection, *LC-B* liver cirrhosis due to hepatitis B, *LC-C* liver cirrhosis due to hepatitis C, *LC-Al* liver cirrhosis due to alcohol hepatitis, *HCC* hepatocellular carcinoma, *PBC* primary biliary cirrhosis, *d* days, *M* months, *Y* years, *LDLT* living-donor liver transplantation

Table 2 Summary of results

	Normal regeneration	Acute graft failure	Chronic graft failure
Y chromosome	–	++	+
Hepatocyte antigen	–	–	–
CK7 (bile duct)	–	–	–
CD68 (macrophage)	–	Partial +	–
CD34 (hematopoietic)	–	+	+

because some cells were positive for CD68, which we used to identify macrophages. However, CD34, used for hematopoietic cells, was negative, which indicated that those Y-positive cells did not have hematopoietic origins. In addition, there may be significant sampling variability in liver biopsy specimens from a single liver biopsy, which may not necessarily be representative of the entire liver. In liver chronically damaged by biliary complication, Y-chromosome-positive cells were not as numerous as seen in the case of acute graft failure. In addition, despite the information about expression of progenitor cell markers such as c-kit and Thy-1, we did not investigate this in this study; this awaits further investigation. With regard to CD68(+) Y chromosome(+) cells, we presume that they are regular macrophages from recipient side to dispose of damaged cells in failing liver, not special multipotent stem cells expressing CD68.

In conclusion, in adult-to-adult LDLT, vigorous liver regeneration occurs in graft livers. Involvement of extrahepatic cells in normal adult-to-adult liver regeneration seems limited.

References

1. Eguchi S, Yanaga K, Sugiyama N, Okudaira S, Furui J, Kanematsu T. Relationship between portal venous flow and liver regeneration in patients after living donor right-lobe liver transplantation. *Liver Transpl.* 2003;9:547–551. doi:10.1053/jlts.2003.50128.
2. Akamatsu N, Sugawara Y, Kaneko J, et al. Effects of middle hepatic vein reconstruction on right liver graft regeneration. *Transplantation.* 2003;76:832–837. doi:10.1097/01.TP.0000085080.37235.81.
3. Marcos A, Fisher RA, Ham JM, et al. Liver regeneration and function in donor and recipient after right lobe adult to adult living donor liver transplantation. *Transplantation.* 2000;69:1375–1379. doi:10.1097/00007890-200004150-00028.
4. Fujii H, Hirose T, Oe S, et al. Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. *J Hepatol.* 2002;36:653–659. doi:10.1016/S0168-8278(02)00043-0.
5. Conzelmann LO, Hines IN, Kremer M, Perry AW, Lemasters JJ, Wheeler MD. Extrahepatic cells contribute to the progenitor/stem cell response following reduced-size liver transplantation in mice. *Exp Biol Med (Maywood).* 2007;232:571–580.
6. Tomiyama K, Miyazaki M, Nukui M, et al. Contribution of cells of intact extrahepatic tissue origin to hepatocyte regeneration in transplanted rat liver. *Transplantation.* 2007;83:624–630. doi:10.1097/01.tp.0000253942.16061.d9.
7. Di Campli C, Piscaglia AC, Giuliante F, et al. No evidence of hematopoietic stem cell mobilization in patients submitted to hepatectomy or in patients with acute on chronic liver failure. *Transplant Proc.* 2005;37:2563–2566. doi:10.1016/j.transproceed.2005.06.072.
8. Moritoki Y, Ueno Y, Kanno N, et al. Lack of evidence that before marrow cells contribute to cholangiocyte repopulation during experimental cholestatic ductal hyperplasia. *Liver Int.* 2006;26:457–466. doi:10.1111/j.1478-3231.2006.01250.x.
9. am Esch JS 2nd, Knoefel WT, Klein M, et al. Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. *Stem Cells.* 2005;23:463–470.
10. Ferry N, Hadchouel M. Liver regeneration: with a little help from marrow. *J Hepatol.* 2002;36:695–697. doi:10.1016/S0168-8278(02)00054-5.
11. Eguchi S, Okudaira S, Azuma T, et al. Changes in liver regenerative factors in a case of living-related liver transplantation. *Clin Transplant.* 1999;13:536–544. doi:10.1034/j.1399-0012.1999.130616.x.
12. Theise ND, Badve S, Saxena R, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology.* 2000;31:235–240. doi:10.1002/hep.510310135.
13. Baccarani U, Donini A, Risaliti A, Bresadola F. Replacement of liver venous endothelium. *Lancet.* 2001;357:2137. doi:10.1016/S0140-6736(00)05209-0.

Surg Today (2009) 39:1–4
DOI 10.1007/s00595-008-3836-1

SURGERY TODAY

Review Article

What Is the Real Contribution of Extrahepatic Cells to Liver Regeneration?

SUSUMU EGUCHI and TAKASHI KANEMATSU

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Abstract
Extrahepatic cells, especially bone marrow (BM) cells, might contribute to liver repair, but recent published evidence suggests that they do not play a role in the normally regenerating liver. The mechanism by which extrahepatic cells express a liver-specific function in the liver, whether by transdifferentiation or by cell fusion, remains unclear. In this review, we investigate the status of findings on this controversial subject and summarize the recent research.

Key words Extrahepatic stem cells · Liver regeneration · Transdifferentiation · Cell fusion

Introduction

It has been reported that extrahepatic cells, especially bone marrow (BM)-derived cells, are mobilized and involved in the repair of liver tissue, including after injury.[1–17] However, there are few details about the involvement of extrahepatic cells and how much they contribute to normal liver regeneration. Even if such involvement exists, it remains unclear whether liver-specific function is achieved through transdifferentiation or cell fusion. Thus, we reviewed the findings of recent published research on this subject.

Contribution of Extrahepatic Cells to Liver Regeneration from Injury

After partial hepatectomy, the liver mass is usually restored within 1–2 weeks in rats and 1–3 months in humans, in order to catch up with liver-specific function. DNA synthesis and cell division occurs first in hepatocytes, and then in nonparenchymal cells.[17,18] It was originally believed that only cells in the liver participate in this restoration; however, recent findings in liver transplant recipients who received BM transplantation have prompted investigations into the contribution of BM cells to liver repopulation, especially liver regeneration after liver damage or partial hepatectomy, but with controversial results (Tables 1 and 2).

An in vivo experiment in 2000 revealed that hepatocytes could be derived from BM cells after irradiation in the absence of severe acute injury.[1] Then, in 2001, Baccarani et al.[2] reported that replacement of female liver venous endothelium with male BM in humans showed the possible involvement of BM cells in liver rearrangement. This was followed by the finding by Körbling et al. of the differentiation of circulating stem cells into mature hepatocytes.[3] Since 2002, research has advanced in this area with the advent of green fluorescent protein (GFP) transgenic mice, which express green fluorescent protein throughout their bodies. The GFP-positive cell-transplant model allows researchers to detect transplanted or mobilized cells without complicated molecular biological methods. Using this model, after GFP-positive BM transplantation, Fujii et al. found that although BM cells participated in liver regeneration after hepatectomy, the majority was committed to sinusoidal endothelial cells (Fig. 1), probably through endothelial progenitor cell mobilization.[4,5] Using their GFP/carbon-tetrachloride (CCl_4) mouse model, in 2003 Terai et al. reported that autologous BM cells were an effective treatment for liver failure caused by persistent liver damage. They found the same results for liver cirrhosis.[6,7,16] In 2005, am Esch also reported that CD133, used as a hematopoietic stem cell marker, plus BM stem cells infused into the portal vein, accelerated hepatic regeneration.[8] Even more recently, Conzelmann et al., using their reduced-size liver transplantation model, reported that recipient-derived

Reprint requests to: S. Eguchi
Received: April 7, 2008 / Accepted: June 11, 2008

Table 1. Relationship between extrahepatic cells and liver regeneration/impairment (1)

First author[Ref.]	Year	Patient or model	Findings
Baccarani[2]	2001	Human	Replacement liver venous endothelium in livers of BM transplant recipients
Fujii[4]	2002	GFP transgenic mouse	BM cells participated in LR. The majority was committed to sinusoidal endothelial cells
Wu[25]	2003	Human	Only rare, isolated, and tentatively identified recipient hepatocytes
Cantz[20]	2004	GFP transgenic mouse	No evidence of BM cells in LR
Terai[6]	2005	GFP transgenic mouse	Autologous BM cells were effective for treatment of liver failure
Di Campli[12]	2005	Human	No evidence of hematopoietic stem cells in LR

BM, bone marrow; GFP, green fluorescent protein; LR, liver regeneration

Table 2. Relationship between extrahepatic cells and liver regeneration (2)

First author[Ref.]	Year	Patient or model	Findings
am Esch[8]	2005	Human	CD133(+) BM stem cells infused into portal vein accelerating LR
Moritoki[13]	2006	GFP transgenic mouse	BM cells transfer did not contribute to the differentiation of cholangiocytes in chronic cholestasis model. Scattered GFP(+) cells in hepatic parenchyma
Tomiyama[14]	2007	Rat	Limited contribution of cells of intact extrahepatic tissue origin to LR in transplanted liver. Liver injury did not increase the percentage of GFP(+) using LT model
Conzelmann[9]	2007	GFP transgenic mouse	Using reduced-size LT, recipient-derived progenitor cells were present and might contribute to LR
Beaudry[21]	2007	GFP transgenic mouse	Contribution of circulating endothelial progenitor cells with exogenous vascular endothelial growth factor

BM, bone marrow; GFP, green fluorescent protein; LR, liver regeneration; LT, liver transplantation

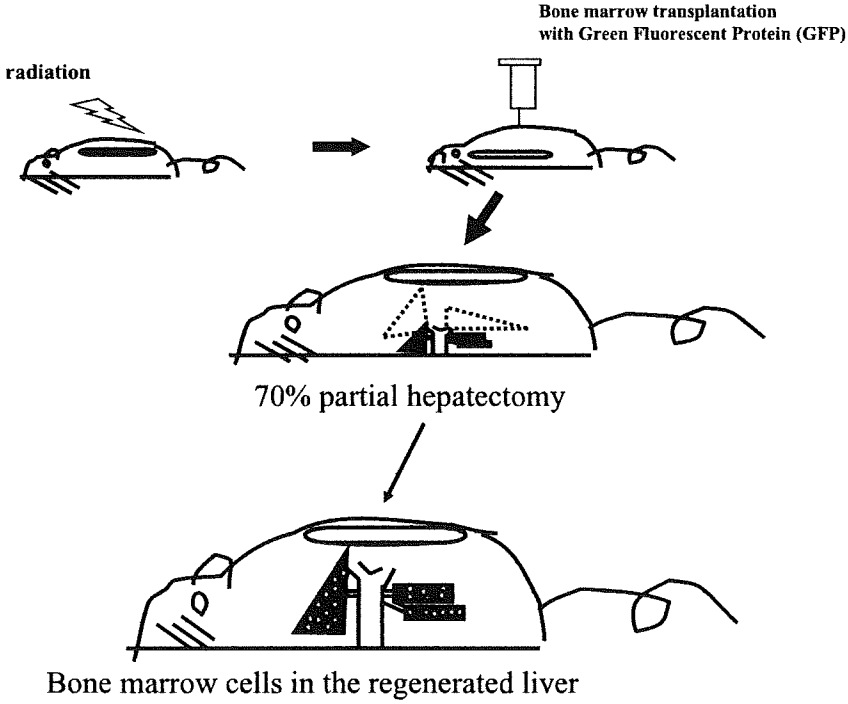


Fig. 1. Involvement of extrahepatic cells in liver regeneration. Transplanted bone marrow cells were involved in liver regeneration after partial hepatectomy in rats. The majority was committed to sinusoidal endothelial cells. Adapted from Fujii et al.[4]

progenitor cells were present and might contribute to liver regeneration in mice.[9] All these reports constitute encouraging data to support the notion that extrahepatic cells, and especially BM cells, are potent therapeutic resources for impaired liver regeneration. Furthermore, studies on partial hepatectomy using rats with liver regeneration impaired by retrorsine have revealed some positive results.[10,11] However, there is

Table 3. Controversies on involvement of extrahepatic cells in liver regeneration and repair

First author[Ref.]	Year	Species	Cells differentiated from extrahepatic cells
Yes			
Baccarani[2]	2001	Human	Hepatic endothelial cells
Fujii[4]	2002	Rat	Hepatic endothelial cells
Conzelmann[9]	2007	Mouse	9% of liver comprised within 28 days
Beaudry[21]	2007	Mouse	Hepatic endothelial cells
No			
Wu[25]	2003	Human	No endothelial cells from BM cells
Cantz[20]	2004	Mouse	Limited or no contribution
Di Campli[12]	2005	Human	No evidence of BM mobilization
Moritoki[13]	2006	Mouse	No cholangiocytes from BM cells
Tomiyama[14]	2007	Rat	Limited contribution

BM, bone marrow

still controversy regarding the degree and mechanism of involvement of the cells. Thus, we investigated the studies focusing on this issue (Table 3). In 2005, Di Campli et al. reported no evidence of hematopoietic stem cell mobilization in patients who underwent hepatectomy or in patients with acute liver failure. They observed no CD34-positive cells in the blood after hepatectomy for acute decompensation of a cirrhotic liver.[12] Similarly in 2006, a study by Moritoki et al. using GFP transgenic mice demonstrated that BM cell transfer seemed not to contribute to the differentiation of cholangiocytes in a chronic cholestasis model. These authors also found scattered GFP-positive cells in the hepatic parenchyma.[13] In 2007, Tomiyama et al. reported the limited contribution of cells originating from intact extrahepatic tissue in hepatocyte regeneration in transplanted rat livers. They reported that even in the non-injured liver, GFP-positive hepatocytes increased by 0.0048% per week: in other words, 5×10^3 were generated per day. However, liver injury did not trigger an increase in the percentage of GFP-positive hepatocytes in their liver transplantation model,[14] as Popp et al. reported similar findings in 2007.[15]

Based on these findings, it seems that limited involvement is possible in normal liver regeneration after partial hepatectomy. However, evidence from an in vivo liver injury model suggests that BM cells may be involved in the regeneration of an impaired liver. Conversely, an investigation on liver regeneration using a specific model in which liver cells cannot divide, using retrorsine, showed no contribution of multipotent mesenchymal stromal cells in liver regeneration. Whether extrahepatic cells migrate to the regenerating liver to function as liver cells and if so, how long they can survive, are still subjects of debate. Nevertheless, preliminary clinical studies with autologous BM cells or $CD34^+$ cells have been conducted to treat liver insufficiency, with moderate effects observed.[19]

Transdifferentiation or Cell Fusion

It has been intensely debated whether transdifferentiation or fusion accounts for the mechanism by which BM cells become hepatocytes. In 2004, Lee et al. described the differentiation of human mesenchymal stem cells into hepatocytes in vitro.[23] According to the transdifferentiation theory, the phenotype of BM cells changes to that of hepatocytes through coordinated changes in the transcriptional activities of many genes. The mesenchymal stem cell component in BM cells or other specific stem cells are candidates for this ability to transdifferentiate. Although transdifferentiation of the peripheral blood monocyte-derived subset into hepatic transdifferentiated cells has been described, it has not been established which cells are involved. Using their mouse model, Brulport et al. presented evidence not for transdifferentiation, but for a complex situation including partial differentiation and possible horizontal gene transfer.[24] In 2005, Wu et al. also reported minimal evidence of transdifferentiation from recipient BM to parenchymal cells, regenerating with long-term survival in human allografts.[25] On the other hand, "cell fusion" between BM stem cells and hepatocytes was reported and is thought to be a major mechanism based on an experiment repeated by many researchers, in which new hepatocytes appear after the infusion of BM cells.[26–30] We are still unsure if hepatocytes cannot be produced more effectively through "transdifferentiation" or "fusion." Further research on liver regeneration is needed to resolve these issues.[31]

In conclusion, based on our review of the recent literature, extrahepatic cells, especially BM cells, might contribute to repair of the injured liver but not to repair of the normally regenerating liver. The mechanism by which extrahepatic cells express a liver-specific function, whether transdifferentiation or cell fusion, has not been established.

Search Strategy

Recent data for this review were collected by PubMed searches.

References

1. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 2000;31: 235–40.
2. Baccarani U, Donini A, Risaliti A, Bresadola F. Replacement of liver venous endothelium. Lancet 2001;357:2137.
3. Körbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albitar M, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Eng J Med 2002;346:738–46.
4. Fujii H, Hirose T, Oe S, Yasuchika K, Azuma H, Fujikawa T, et al. Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. J Hepatol 2002;36:653–9.
5. Ferry N, Hadchouel M. Liver regeneration: with a little help from marrow. J Hepatol 2002;36:695–7.
6. Terai S, Sakaida I, Nishina H, Okita K. Lesson from the GFP/ CCl4 model–translational research project: the development of cell therapy using autologous bone marrow cells in patients with liver cirrhosis. J Hepatobiliary Pancreat Surg 2005;12:203–7.
7. Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, et al. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 2004;78;83–88.
8. am Esch JS 2nd, Knoefel WT, Klein M, Ghodsizad A, Fuerst G, Poll LW, et al. Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 2005;23:463–70.
9. Conzelmann LO, Hines IN, Kremer M, Perry AW, Lemasters JJ, Wheeler MD. Extrahepatic cells contribute to the progenitor/ stem cell response following reduced-size liver transplantation in mice. Exp Biol Med (Maywood). 2007;232:571–80.
10. Dahlke MH, Popp FC, Bahlmann FH, Aselmann H, Jäger MD, Neipp M, et al. Liver regeneration in a retrorsine/CCl_4-induced acute liver failure model: do bone marrow-derived cells contribute? J Hepatol 2003;39:365–73.
11. Hunter Best D, Coleman WB. Cells of origin of small hepatocyte-like progenitor cells in the retrorsine model of rat liver injury and regeneration. J Hepatol 2008;48:369–71.
12. Di Campli C, Piscaglia AC, Giuliante F, Rutella S, Bonanno G, Zocco MA, et al. No evidence of hematopoietic stem cell mobilization in patients submitted to hepatectomy or in patients with acute on chronic liver failure. Transplant Proc 2005;37:2563–6.
13. Moritoki Y, Ueno Y, Kanno N, Yamagiwa Y, Fukushima K, Gershwin ME, et al. Lack of evidence that bone marrow cells contribute to cholangiocyte repopulation during experimental cholestatic ductal hyperplasia. Liver Int 2006;26:457–66.
14. Tomiyama K, Miyazaki M, Nukui M, Takaishi M, Nakao A, Shimizu N, et al. Contribution of cells of intact extrahepatic tissue origin to hepatocyte regeneration in transplanted rat liver. Transplantation 2007;83:624–30.
15. Popp FC, Slowik P, Eggenhofer E, Renner P, Lang SA, Stoeltzing O, et al. No contribution of multipotent mesenchymal stromal cells to liver regeneration in a rat model of prolonged hepatic injury. Stem Cells. 2007;25:639–45.
16. Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, et al. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 2006;24:2292–8.
17. Eguchi S, Kamlot A, Ljubimova J, Lebow LT, Demetriou AA, Rozga J. A novel experimental animal model of fulminant hepatic failure. Survival and effect on biochemistry and liver regeneration. Hepatology 1996:24;1452–9.
18. Eguchi S, Yanaga K, Sugiyama N, Okudaira S, Furui J, Kanematsu T. Changes in serum levels of hepatocyte growth factor in patients undergoing adult-to-adult living donor liver transplantation. Transplantation 2003:76;1769–70.
19. Gordon MY, Levicar N, Pai M, Bachellier P, Dimarakis I, Al-Allaf F, et al. Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells 2006;24:1822–30.
20. Cantz T, Sharma AD, Jochheim-Richter A, Arseniev L, Klein C, Manns MP, et al. Reevaluation of bone marrow-derived cells as a source for hepatocyte regeneration. Cell Transplant 2004;13: 659–66.
21. Beaudry P, Hida Y, Udagawa T, Alwayn IP, Greene AK, Arsenault D, et al. Endothelial progenitor cells contribute to accelerated liver regeneration. J Pediatr Surg 2007;42:1190–8.
22. Lai HS, Chen Y, Lin WH, Chen CN, Wu HC, Chang CJ, et al. Quantitative gene expression analysis by cDNA microarray during liver regeneration after partial hepatectomy in rats. Surg Today 2005;35:396–403.
23. Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, et al. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40;1275–84.
24. Brulport M, Schormann W, Bauer A, Hermes M, Elsner C, Hammersen FJ, et al. Fate of extrahepatic human stem and precursor cells after transplantation into mouse livers. Hepatology 2007;46:861–70.
25. Wu T, Cieply K, Nalesnik MA, Randhawa PS, Randhawa PS, Sonzogni A, et al. Minimal evidence of transdifferentiation from recipient bone marrow to parenchymal cells in regenerating and long-surviving human allografts. Am J Transplant 2003;3:1173–81.
26. Camargo FD, Finegold M, Goodell MA. Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. J Clin Invest 2004;113:1266–70.
27. Willenbring H, Bailey AS, Foster M, Foster M, Akkari Y, Dorrell C, et al. Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 2004;10:744–8.
28. Quintana-Bustamante O, Alvarez-Barrientos A, Kofman AV, Fabregat I, Bueren JA, Theise ND, et al. Hematopoietic mobilization in mice increases the presence of bone marrow-derived hepatocytes via in vivo cell fusion. Hepatology 2006;43:108–16.
29. Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature 2003;422:901–4.
30. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003;422:897–901.
31. Kawashita Y, Guha C, Yamanouchi K, Ito Y, Kamohara Y, Kanematsu T. Liver repopulation: A new concept of hepatocyte transplantation. Surg Today 2005;35:705–10.

2. 肝細胞癌に対する肝移植
——最近分かったこと——

長崎大学大学院　移植・消化器外科　江口　晋，日高　匡章，兼松　隆之

はじめに

肝細胞癌(以下，HCC)に対する肝移植の適応としてはミラノ基準(Milan criteria)が汎用されており，本邦のHCCに対する肝移植の保険適応にも使用されている。ミラノ基準では術前画像で遠隔転移(−)，脈管侵襲(−)，単発HCC 5 cm以下もしくは多発HCC個数3個までで最大径3 cm以下を肝移植の良い適応としている[1]。この基準を遵守すると，移植後の5年患者生存率が70%以上，5年無再発率が15%以下，待機リストからの脱落の危険率が月間4%と良好な成績が得られる[1]。また，生体肝移植でもHCCに対するミラノ基準の妥当性が示されている。2004年，カリフォルニア大学サンフランシスコ校(以下，UCSF)からUCSF criteriaが提案され[2]，この基準を遵守するとミラノ基準内HCCに対する肝移植と同等の成績が維持できるとし，最近，ミラノ基準内HCCと予後が同等であることがprospectiveに確認された[3]。最近，さらなる適応拡大に向けて種々の検討が行われており[4,5]，HCCの悪性度マーカー(腫瘍マーカーなど)を組み込んだ適応が諸施設より提案されているが[6,7]，現在のところ，特に生体肝移植を考える上では，ミラノ基準遵守が最も妥当な安全域であると考えられている。また，本邦では非代償性肝硬変に合併したHCCのみが肝移植の保険適応となっていることが，欧米の考え方と異なる。肝機能良好例でのミラノ基準内HCCに対する初回治療としての肝移植の意義については議論のあるところであるが，本邦では肝切除が第一選択となることが多い。本稿では，長崎大学での肝移植成績を示し，摘出肝の全肝病理学的検索により，最近明らかとなった事項を紹介する。

1. 肝細胞癌に対する長崎大学チームの肝移植成績

長崎大学では1997年より肝移植を開始し，2008年12月までに96例に生体肝移植を施行した。その中の39例(40.6%，3例のincidental HCCを含む)がHCCの症例であり，基本的にはミラノ基準を遵守して肝移植を施行している。その3年患者生存率はミラノ基準内HCC(n=36)で73.2%，ミラノ基準外HCC(n=3)は66.7%，HCC無しの症例は84.4%(n=57)であった。各群間に今のところ有意差はない。HCCの再発はミラノ基準内HCCでは1例のみで，ミラノ基準外HCCでは1例に肺転移再発している。背景のウイルス制御も重要で，移植後のB型肝炎の再発は抗ウイルス剤，抗B型肝炎ウイルス免疫グロブリン(HBIG)にて完全に制御可能である。一方，C型肝炎ウイルスは多くの場合移植後にインターフェロン，リバビリン療法が必要となる。移植後の合併症としては，拒絶反応，感染症，動脈塞栓などが考えられ，細やかな管理が必要となる。

2. 摘出肝の全肝病理学的検索

肝硬変症例での肝細胞癌(HCC)に対する治療法は限られており，肝切除よりも局所療法(TAE，RFA等)が選択されることが多い。治療後肝硬変が進行すると肝移植が選択されるが，その際ミラノ基準を当て嵌めてよいか議論の余地がある。またHCC合併肝硬変における肝移植適応は，現在ミラノ基準(術前診断)によって行われているが，前治療部位をどのように扱うかが問題となっている。そこで局所療法を行った部位を中心に摘出肝を用いて解析し，前治療による適応の影響を検討した。

【方法】当科で1997年から2008年12月までに施

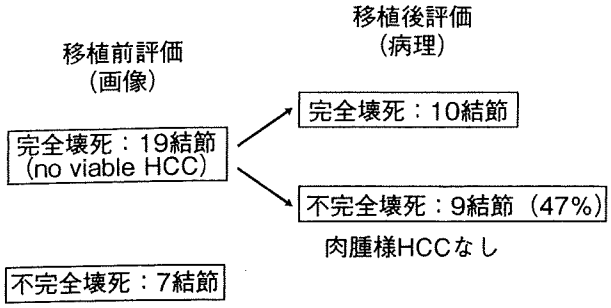


図 1　HCC に対する移植前治療の効果の検証；
26HCC 結節（患者 20 人）

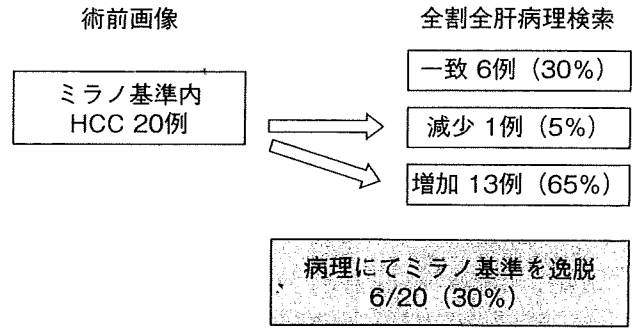
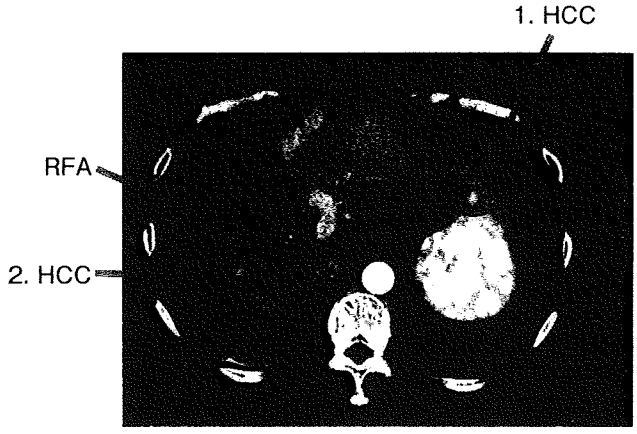


図 2　全割全肝病理検索の結果



移植前評価：viable HCC 2個

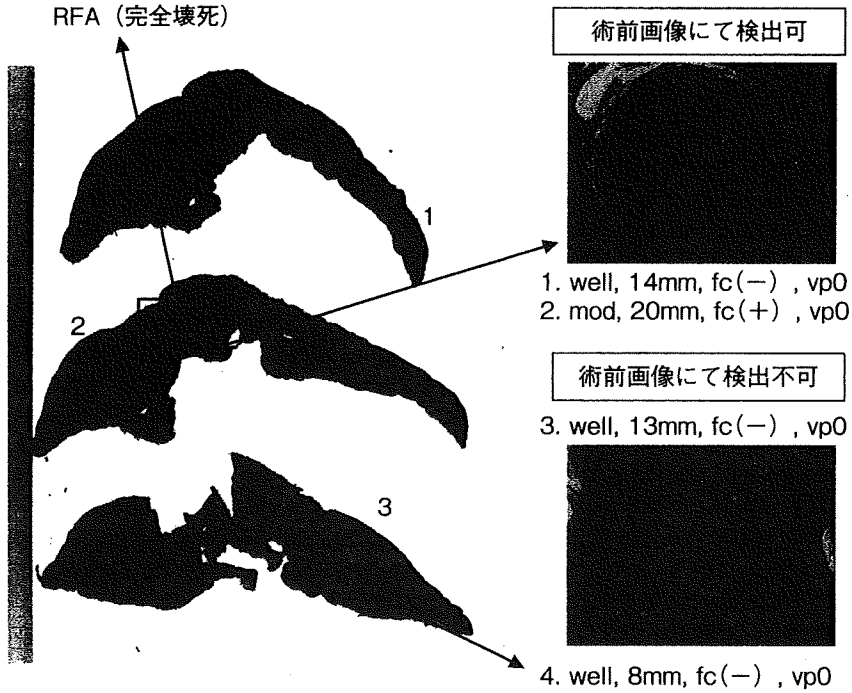


図 3　症例：59 歳，男性，C 型肝硬変，HCC に対して RFA 歴あり

行した生体肝移植96例のうち，術前および術後HCCと診断された39例中移植摘出肝の全割を行った16例について検討した。1症例あたり約200枚のプレパラートを作成し，病理学的検討を行い解析した。

【結果】36例(92%)がミラノ基準内のHCCで，そのうち20例(55%)に移植前治療が施行されていた。20例26結節中，術前画像にて局所再発なしと判断されたのは19結節例(73%)であった。そのうち完全壊死をきたしていたのは10結節のみで，9結節(47%)はviableなHCCが残存していた(**図1**)。しかし，肉腫様HCCや腫瘍の門脈内への押し出しは認めなかった。また，前治療の有無でHCCの再発率に差はなかった。

次の残肝を病理学的に検索してみると，術前と比べて腫瘍数の増加13例(65%)，不変6例(30%)，減少1例(5%)であった(**図2**)。術前画像にて指摘され得ない結節は，境界不明瞭，高分化が多く，腫瘍径は2-15 mm(中央値6 mm)であった(典型例を**図3**に示す)。全例再発は認めていない(観察期間中央値21.4カ月)。結果的に20例中6例(30%)がミラノ基準を超えていた。

【結語】局所療法にて画像上制御されている肝細胞癌の中で，治療周囲に癌が残存，再発している可能性があり，それによってミラノ基準を逸脱することもある。しかし再発には寄与しない可能性が示唆された。非代償性硬変肝内のオカルトHCCは，高分化，脈管侵襲を認めない超早期癌と考えられた。非代償性硬変肝でミラノ基準内のHCCとは別にオカルトHCCが存在していても，現在の画像診断でミラノ基準内に収まっていれば，移植後予後には関係せず，十分長期予後を期待できるものと考えられる。よって，最近のMD-CT等の高感度画像診断を用いミラノ基準を評価することは，移植基準として非常にstrictではあるが，十分な安全域を確保できるものと考えられる。また，今後の検出モダリティーの精度上昇による，微小HCCの検出は再発に関連しないため，移植適応を吟味する際には個数に数えないなどの工夫が必要になってくると考えられる。

以上，HCCに対する肝移植の現状につき，当科での経験も含め概説した。

文　献

1) Mazzaferro V, Regalia E, Doci R, *et al*: Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 1996; **334**: 693-699.
2) Todo S, Furukawa H: Japanese Study Group on Organ Transplantation. Living donor liver transplantation for adult patients with hepatocellular carcinoma: experience in Japan. Ann Surg. 2004; **240**: 451-9.
3) Yao FY, Ferrell L, Bass NM, *et al*: Liver transplantation for hepatocellular carcinoma: comparison of the proposed UCSF criteria with the Milan criteria and the Pittsburgh modified TNM criteria. Liver Transpl 2002; **8**: 765-774.
4) Yao FY, Xiao L, Bass NM, Kerlan R, Ascher NL, Roberts JP.: Liver transplantation for hepatocellular carcinoma: validation of the UCSF-expanded criteria based on preoperative imaging. Am J Transplant. 2007; **7**: 2587-96.
5) Duffy JP, Vardanian A, Benjamin E, *et al*: Liver transplantation criteria for hepatocellular carcinoma should be expanded: a 22-year experience with 467 patients at UCLA. Ann Surg. 2007; **246**: 502-9.
6) Sugawara Y, Tamura S, Makuuchi M: Living donor liver transplantation for hepatocellular carcinoma: Tokyo University series. Dig Dis. 2007; **25**: 310-2.
7) Soejima Y, Taketomi A, Yoshizumi T, *et al*: Extended indication for living donor liver transplantation in patients with hepatocellular carcinoma.Transplantation. 2007; **83**: 893-9.
8) Takada Y, Ito T, Ueda M, *et al*: Living donor liver transplantation for patients with HCC exceeding the Milan criteria: a proposal of expanded criteria. Dig Dis. 2007; **25**: 299-302.

Original Paper

Low-dose Recombinant Human Hepatocyte Growth Factor Enhances Effect of Hepatocyte Transplantation in Rats Treated with Retrorsine

Takayuki Hamada[1], Susumu Eguchi[1], Mitsuhisa Takatsuki[1], Kosho Yamanouchi[1], Nozomu Sugiyama[1], Yujo Kawashita[1], Sadayuki Okudaira[1], Yoshitsugu Tajima[1], Takehisa Ishii[2], Takashi Kanematsu[1]

[1]Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. [2]Mitsubishi Pharma Corporation Discovery Technology Laboratory, 1000 Kamoshida-cho, Aoba-ku, Yokohama, Japan

Corresponding Author: Susumu Eguchi, Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Tel: +81958197316, Fax: +81958197319, E-mail: sueguchi@nagasaki-u.ac.jp

KEY WORDS: Hepatocyte transplantation; Hepatocyte growth factor; Regeneration; Retrorsine

ABBREVIATIONS: Recombinant Human Hepatocyte Growth Factor (rhHGF); Nagase Analbuminemic Rats (NARs); Hepatocyte Transplantation (HcTx); Orthotopic Liver Transplantation (OLT); Hepatocyte Growth Factor (HGF); Sprague-Dawley (SD); Continuous Systemic Administration (c.s.a.)

ABSTRACT

Background/Aims: The aim of this study was to regenerate transplanted hepatocytes selectively in a recipient using retrorsine and recombinant human hepatocyte growth factor (rhHGF).

Methodology: Nagase analbuminemic rats (NARs) received pretreatment with retrosine and were divided into three experimental groups. Group1: Hepatocyte transplantation (HcTx) + 50μg/kg/day rhHGF. Group2: HcTx + 250μg/kg/day rhHGF. Group3: HcTx + normal saline. The serum levels of albumin and the albumin-positive hepatocytes in the liver were investigated. The rat endogenous HGF of the rats given only retrorsine was measured.

Results: The serum albumin levels of Group1 were higher than those of Group2, while there was no significant difference between Group2 and Group3. Histological examination of Group1 and 3 showed the presence of a large number of albumin-positive hepatocytes, which frequently consisted of large clusters and occupied 53.90±2.31% and 31.25±5.36% of host liver, respectively. The liver sections of Group2 showed numerous albumin-positive hepatocyte, which were not seen as clusters. The rat endogenous HGF concentration was extremely high.

Conclusion: Low-dose rhHGF enhances the effect of HcTx under the suppressive state of proliferation of host hepatocytes. Because of the high endogenous HGF, the administration of a high concentration of rhHGF suppressed the regenerative activity of the transplanted hepatocytes.

INTRODUCTION

Although the therapy for hepatic metabolic deficient disease still remains orthotopic liver transplantation (OLT), the donor organ shortage has been problematic. HcTx has been proposed as an alternative therapy to OLT, but it is difficult to regenerate transplanted hepatocytes and to achieve the long term correction of a liver-related metabolic defect (1-3). Therefore, partial hepatectomy and portal branch ligation have been added as a growth stimulus for transplanted hepatocytes (4-8). However, these invasive methods are not suitable in a clinical setting for patients with congenital hepatic metabolic deficiency disease.

Human hepatocyte growth factor (HGF) was isolated and purified from the plasma of patients with fuluminant hepatic failure and was found to stimulate DNA synthesis even in adult rat hepatocytes (9, 10). A recombinant form of human rhHGF has also been developed and is as effective as the native HGF in terms of proliferative activities in rat and human hepatocytes (11). Furthermore, the cytoprotective effect of HGF through the immunoregulation action and antiapoptosis action has also been reported (12-15).

Recently, nearly total liver replacement by transplanted normal hepatocytes was reported in rats treated with retrorsine (16-18). Retrorsine is a naturally occurring pyrrolizidine alkaloid that is taken up selectively by the liver and metabolized to bioactive compounds that alkylate DNA, which causes a long-lasting block of hepatocyte cell division (19-23). To selectively enhance the proliferation of transplanted hepatocytes, combined therapy with HGF and retrorsine seems to be an attractive strategy, although it has never been tested in vivo.

In the present study, was investigated the effect of rhHGF for HcTx in rats treated with retrorsine.

METHODOLOGY

Animals

All rats used were purchased from Japan SLC Inc.(Shizuoka, Japan) and were maintained at the

Hepato-Gastroenterology 2009; 56:1466-1470

Animal Center at Nagasaki University School of Medicine. NARs were used as the recipients, while male Sprague-Dawley (SD) rats were used as the hepatocyte donors. All animals were maintained in a climate-controlled (24°C) room with a 12-hour light-dark cycle and were provided tap water and standard laboratory chow *ad libitum*. All procedures were done in accordance with the guidelines of the University of Nagasaki Research Animal Resources.

Chemicals

rhHGF was manufactured and provided by Mitsubishi Pharma Corporation (Yokohama, Japan). Retrorsine, Collagenase type IV, bovine serum albumin, and purified rat albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Purified rabbit anti-rat albumin IgG antibodies and peroxidase conjugated rabbit anti-rat albumin were obtained from ICN Pharmaceuticals Inc (Aurora, OH).

Hepatocyte Isolation and transplantation

Hepatocytes were isolated from SD rats according to a standard two-step collagenase perfusion technique (24). After enrichment through a Percoll gradient, hepatocytes viability was determined by the trypan blue exclusion test. The final viability of the purified hepatocytes suspension was always 90-95%. A suspension of 2×107 viable hepatocytes in one milliliter of saline was directly infused via the portal vein. The NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally, 2 weeks apart. Four weeks after the second injection, each animal received 2×107 viable hepatocytes via the portal vein. After the HcTx, the continuous systemic administration (c.s.a.) of rhHGF or normal saline was done using an osmotic pump as described below. All animals were given Cyclosporin A, at a dose of 15mg/kg, intramuscularly one day prior to HcTx and every other day until sacrifice to limit rejection of the transplanted cells.

Continuous systemic administration of rhHGF

Alzet osmotic pumps were purchased from ALZA Co.(Palo Alto, CA). The pump was connected to the catheter canulated into the jugular vein and placed in the subcutaneous layer of the anterior wall. Continuous infusion of rhHGF or normal saline was done using an osmotic pump.

Experimental designs

The rats were divided into three experimental groups (**Figure 1**). The Group 1 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 50μg/kg/day of rhHGF for 7 days. The Group 2 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 250μg/kg/day of rhHGF for 7 days. The Group 3 (n=5) animals received retrorsine+HcTx followed by c.s.a. of normal saline for 7 days. HGF concentration was set according to our previous study (25). Blood samples were obtained from the tail vein on days 0 (before HcTx), 3, 7, 14, 21, 28, 35, 42 and 56, respectively, for the determination of serum albumin. All animals were euthanized at the 56th day. At sacrifice, the liver specimens were fixed in 10% buffered formalin.

Serum Albumin determination

Quantitative analysis of the serum levels of albumin was carried out by sandwich ELISA using rabbit anti-rat albumin IgG described previously (26).

Investigation of albumin positive hepatocytes

Serial sections of the liver were immunostained utilizing rabbit anti-rat albumin IgG antibody and an immunoperoxidase avidin-biotin peroxidase complex method. Albumin-positive area in the liver was quantitated with the help of a computer-assisted image analyzer.

Measurement of serum human HGF concentration

The human HGF concentration was measured by the Quantikine Human HGF EIA kit (R&D Systems, Inc., Minneapolis, MN) on day 3 to confirm the rise of the human HGF level for each administration of rhHGF.

Measurement of serum rat endogenous HGF concentration

The rat endogenous HGF concentration of rats given only retrorsine was measured by a rat HGF EIA kit (Institute of Immunology Co., Tokyo, Japan) on days 0 and 56 to check the host response

FIGURE 1 NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally (i.p.), 2 weeks apart. Four weeks after the second injection, each animal received HcTx. After theHcTx, c.s.a. ofrhHGF or normal saline was done.

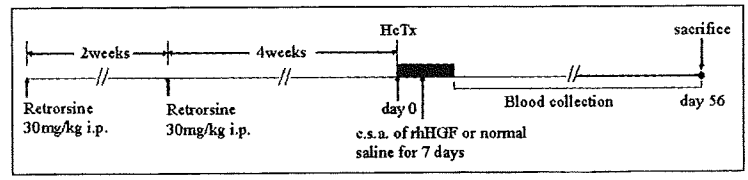
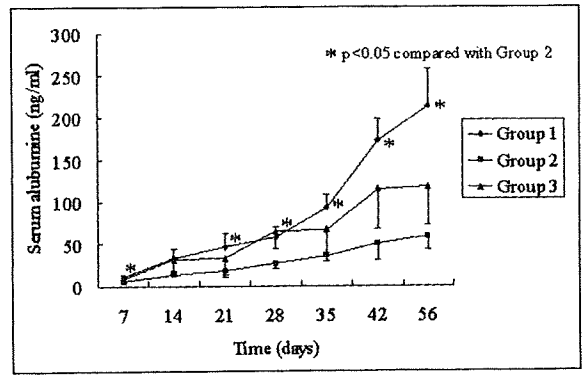
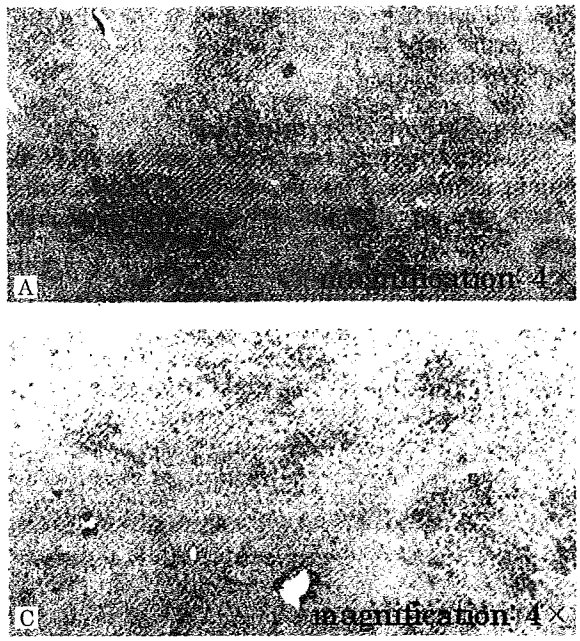


FIGURE 2 A significant increases in the serum albumin level was observed in each group following hepatocyte transplantation. The serum albumin level of Group1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference between Groups 2 and 3.





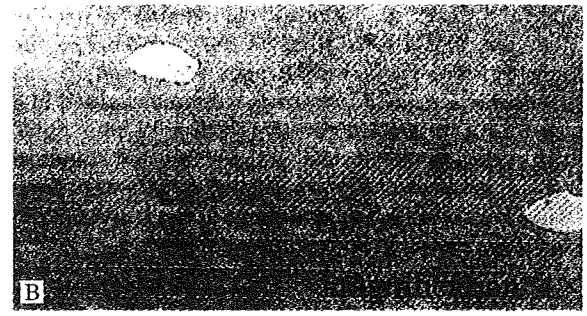


FIGURE 3
(**A**) A large number of albumin–positive hepatocytes can be seen in Group 1, frequently as large clusters, which occupied 53.90±2.31% of host liver.
(**B**) There are numerous albumin–positive hepatocytes in Group 2, which are distributed throughout the liver parenchyma, but not seen as clusters.
(**C**) A large number of albumin–positive hepatocytes can be seen in Group 3. They are frequently seen as large clusters, which occupied 31.25±5.36% of host liver.

against retrorsine (n=5).

Statistical Analysis

All data were expressed as a median and their range. Mann-Whitney's test was used for the data analysis. The differences were considered statistically significant when the p values were less than 0.05.

RESULTS

Serum albumin level

A significant increase in serum albumin levels were observed in each group following hepatocyte transplantation (v.s. base line levels; 1.26±0.04ng/ml) (**Figure 2**). The serum albumin level of Group 1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference in the serum albumin level between Groups 2 and 3.

Albumin-positive hepatocytes in the liver

The liver sections obtained from Group 1 showed a markedly large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 53.90±2.31% of host liver (**Figure 3A**). The liver sections obtained from Group 2 showed sparse albumin-positive hepatocytes. They were uniformly distributed throughout the liver parenchyma, and were not observed as clusters (**Figure 3B**). The liver sections obtained from Group 3 showed a large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 31.25±5.36% of host liver, although there were fewer clusters than in Group 1 (**Figure 3C**). There was statistically difference in the albumin-positive hepatocyte area between group 1 and 3 ($p<0.05$).

Serum human HGF concentration

The serum human HGF concentration through a c.s.a. of 50 and 250μg/kg/day of rhHGF were 5.59±1.20 and 8.44±0.51 ng/ml, respectively ($p<0.05$).

Serum endogenous rat HGF concentration

The endogenous rat HGF concentration on days 0 and 56 were 25.01±0.19 and 20.14±1.29 ng/ml, respectively, after retrorsine treatment. There was no statistically significant difference between the concentration on day 0 and that on day 56.

DISCUSSION

The present study demonstrated the enhanced effect of hepatocyte transplantation by the administration of low- dose rhHGF in rats treated with retrorsine as compared to that with high-dose rhHGF.

The hepatocytes were transplanted under the host's hepatocyte proliferation potency control by retrorsine processing, and attempted selective proliferation of the transplanted hepatocytes. Pyrrolizidine alkaloids including retrorsine were studied originally because of their toxicity in animals, particularly sheep and cattle, in which they cause both acute and chronic injury (19, 20). The administration of a high dose of pyrrolizidine alkaloid is lethal, but a low dose induces only chronic hepatic megalocytosis (27). In our study, there was no death in the rats that only received retrorsine. Pyrrolizidine alkaloids are established hepatocarcinogens (28-32). However, Laconi E *et al.* (16) and Laconi S *et al.* (18, 33) maintained that animals treated with retrorsine for a long time without neoplastic change in the host's hepatocytes and transplanted hepatocytes. To date, in order to inhibit the proliferation of endogenous hepatocytes, irradiation (34, 35) and anti-cancer drugs (36) have been used. Nevertheless, it is thought that those are not suitable in a clinical setting for co-effects to cells except hepatocytes. For the above reasons, we thought that retrorsine could likely be a drug which would the inhibit host's hepatocyte proliferation potency in clinical application.

In the present study, as a proliferation stimulus, instead of partial hepatectomy or portal branch ligation, rhHGF was administered, which was thought to be less invasive. In addition, carbon tetrachloride

has been used as a proliferation stimulus for transplanted hepatocytes (37, 38), but it is not thought to be suitable for use in a clinical setting. Strain *et al.* (11) reported that 0.63ng/ml of rhHGF was the minimum concentration needed to stimulate hepatocyte proliferation in a culture system. According to our previous study (25), when the rats were given a continuous systemic administration of rhHGF in a dose of 50μg/kg/day, the mean concentration of rhHGF in the portal blood was almost similar to the minimum concentration needed to stimulate hepatocyte proliferation in vitro. Based on this investigation, we considered that 50μg/kg/day should be given. We set the concentration of 250μg/kg/day as the high dose HGF, but could have set various concentrations of rhHGF between 50μg/kg/day and 250μg/kg/day, or lower concentration than 50μg/kg/day. Furthermore, we planned to administer rhHGF by continuous systemic infusion because the half-life of HGF in plasma was very short in vivo and vitro (39-42).

In the present study, the cell proliferation and function of the transplant hepatocytes of low-dose rhHGF were better than that with high-dose rhHGF. For that reason, since the rat endogenous HGF was already elevated by retrorsine treatment itself, high dose exogenous rhHGF administration was thought to counteract the proliferation of the transplanted hepatocytes. In fact, it was reported that there is a synergistic effect between rat HGF and rhHGF (40, 43). Furthermore, on the HGF concentration of 5-10ng/ml as growth stimuli, the DNA synthesis of the hepatocyte reaches a plateau, while the DNA synthesis is suppressed with an HGF concentration of more than 10ng/ml (44). This is presumably the reason why the elevation of serum albumin was observed in Group 2 in the present study. In addition, cytoprotective effect rhHGF may be affecting the data. Whether infused rhHGF affect only transplanted hepatocytes, or only native hepatocytes injured by retrorsine, or both awaits further investigation.

In conclusion, low-dose rather than high-dose rhHGF enhances the therapeutic effect of hepatocyte transplantation under retrorsine treatment. Since we only investigated 50μg/kg/day and 250μg/kg/day, further investigation is needed using a more optimal concentration of rhHGF.

REFERENCES

1. **Strom SC, Chowdhury JR, Fox IJ:** Hepatocyte transplantation for the treatment of human disease. Seminars in Liver Disease 1999; 19:39-48.
2. **Strom SC, Fisher RA, Rubinstein WS, et al:** Transplantation of human hepatocytes. Transplant Proc 1997; 29:2103-2106.
3. **Fox IJ, Roy Chowdhury JR, Kaufman SS, et al:** Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 1998; 338:1422-1426.
4. **Raper SE, Wilson JM:** Cell transplantation in the liver directed gene therapy. Cell Transplant 1993; 2:381-400.
5. **Demetriou A:** Experimental hepatocyte transplantation and liver gene therapy. Cell Transplant 1993; 2:401-403.
6. **Eguchi S, Rozga J, Lebow LT, et al:** Treatment of hypercholesterolemia in Watanabe rabbits using allogenic hepatocellular transplantation under a regeneration stimulus. Transplantation 1996; 62:588-593.
7. **Moscioni AD, Rozga J, Chen SC, et al:** Long-term correction of albumin levels in the Nagase analbuminemic rat: repopulation of the liver transported normal hepatocyte under a regeneration response. Cell Transplant 1996; 5:499-503.
8. **Rozga J, Jeppsson B, Bengmark S:** Portal branch ligation. Reevalution of a model. Am J Patho 1986; 125:300-308
9. **Gohda E, Tsubouchi H, Nakayama H, et al:** Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. Exp Cell Res 1986; 166:139-150.
10. **Gohda E, Tsubouchi H, Nakayama H, et al:** Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J Clin Invest 1988; 81:414-419.
11. **Strain AJ, Ismail T, Tsubouchi H, et al:** Native and recombinant human hepatocyte growth factor are highly potent promoters of DNA synthesis in both human and rat hepatocytes. J Clin Invest 1991; 87:1853-1857.
12. **Ichiguchi O, Yamaguchi Y, Miyanari N, et al:** Enhanced hepatocyte growth factor expression associated with prolonged rat hepatic allograft survival in recipients pretreated with donor-specific blood. Transplantation 1999; 67:115-123.
13. **Uchiyama H, Yanaga K, Nishizaki T, et al:** Effects of deletion variant of hepatocyte growth factor on reduced-size liver transplantation in rats. Transplantation 1999; 68:39-44.
14. **Ikegami T, Nishizaki T, Uchiyama H, et al:** Deletion variant of hepatocyte growth factor prolongs allograft survival after liver transplantation in rats. Surgery 1999; 125:602-607.
15. **Sakakura Y, Kaibori M, Oda M, et al:** Recombinant human hepatocyte growth factor protects the liver against hepatic ischemia and reperfusion injury in rats. J Surg Res 2000; 92:261-266.
16. **Laconi E, Oren R, Mukhopadhyay DK, et al:** Long-term, near-total liver replacement by transplantation of isolated hepatosytes in rats treated with retrorsine. Am J Patho 1998; 153:319-329.
17. **Oren R, Dabeva MD, Petkov PM, et al:** Restoration of serum albumin levels in Nagase analbuminemic rats by hepatocyte transplantation. Hepatology 1999; 29:75-81.
18. **Laconi S, Pillai S, Porcu PP, et al:** Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. Am J Patho 2001; 158:771-777.
19. **McLean EK:** The toxic actions of pyrrolizidine (senecio) alkaloids. Pharmacol Rev 1970; 22:429-483.
20. **Mattocks AR:** Chemistry and toxicology of pyrrolizidine alkaloids. Orlando, FL, Academic Press, 1986.
21. **Laconi S, Curreli F, Diana S, et al:** Liver regeneration in response to partial hepatectomy in rats treated with retrorsine: a kinetic study. J Hepatol 1999; 31:1069-1074.
22. **Picard C, Lambotte L, Starkel P, et al:** Retrorsine: a kinetic study of its influence on rat liver regeneration in the portal branch ligation model. J Hepatol 2003; 39:99-105.
23. **Pitzalis S, Doratiotto S, Greco M, et al:** Cyclin D1 is up-regulated in hepatocytes in vivo following cell-cycle block induced by retrorsine. J Hepatol 2005; 43:485-490.
24. **Seglen PO:** Preparation of isolated rat liver cells. Methods Cell Biol 1976; 13:29-83.
25. **Sugiyama N, Eguchi S, Kawazoe Y, et al:** Intraportal administration of low-dose recombinant human hepatocyte growth factor enhances effects of hepatocellular

transplantation. Hepato-Gastroenterology 2000; 47:1245-1249.
26. **Holzman MD, Rozga J, Neuzil DF, et al:** Selective intraportal hepatocyte transplantation in analbuminemic and Gunn rats. Transplantation 1993; 55:1213-1219.
27. **Jago MV:** The development of the hepatic megalocytosis of chronic pyrrolizidine alkaloid poisoning. Am J Pathol 1969; 56:405-422.
28. **Svoboda DJ, Reddy JK:** Malignant tumors in rats given lasiocarpine. Cancer Res 1972; 32:908-912.
29. **Rao MS, Reddy JK:** Malignant neoplasms in rats fed lasiocarpine. Br J Cancer 1987; 37:289-293.
30. **Schoental R, Magee PN:** Further observation on the subacute and chronic liver change in rats after a single dose of various pyrrolizidine (senecio) alkaloids. J Pathol Bacteriol 1959; 78:471-482.
31. **Chou MW, Yan J, Nichols J, et al:** Correlation of DNA adduct formation and riddelline-induced liver tumorigenesis in F344 rats and B6C3F1 mice. Cancer Lett 2003; 193:119-125.
32. **Chan PC, Haseman JK, Prejean JD, et al:** Toxicity and carcinogenicity of riddelline in rats and mice. Toxicol Lett 2003; 144:295-311.
33. **Laconi S, Montisci S, Doratiotto S, et al:** Liver repopulation by transplanted hepatocytes and risk of hepatocellular carcinoma. Transplantation 2006; 82:1319-1323.
34. **Guha C, Sharma A, Gupta S, et al:** Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res 1999; 59:5871-5874.
35. **Malhi H, Gorla GR, Irani AN, et al:** Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci USA 2002; 99:13114-13119.
36. **Kim KS, Joseph B, Inada M, et al:** Regulation of hepatocyte engraftment and proliferation after cytotoxic drug-induced perturbation. Transplantation 2005; 80: 653-659.
37. **Dahlke MH, Popp FC, Bahlmann FH, et al:** Liver regeneration in a retrorsine/CCl4-induced acute liver failure model: do bone marrow-derived cell contribute? J Hepatol 2003; 39:365-373.
38. **Guo D, Fu T, Nelson JA, et al:** Liver repopulation after cell transplantation in mice treated with retrorsine and carbon tetrachloride. Transplantation 2002; 73:1818-1824.
39. **Naka D, Shimomura T, Yoshiyama Y, et al:** Internalization and degradation of hepatocyte growth factor in hepatocytes with down-regulation of the receptor / c-Met. FEBS Lett 1993; 329:147-152.
40. **Fujiwara K, Nagoshi S, Ohno A, et al:** Stimulation of liver growth by exogenous human hepatocyte growth factor in normal and partially hepatectomized rats. Hepatology 1993; 18:1443-1449.
41. **Ishii T, Sato M, Sudo K, et al:** Hepatocyte growth factor stimulates liver regeneration and elevates blood protein level in normal and partially hepatectomized rats. J Biochem (Tokyo) 1995; 117:1105-1112.
42. **Liu KX, Kato Y, Narukawa M, et al:** Importance of the liver in plasma clearance of hepatocyte growth factor in rats. Am J Physiol 1992; 263:G642-649.
43. **Ishiki Y, Ohnishi H, Muto Y, et al:** Direct evidence that hepatocyte growth factor is a hepatototropic factor for liver regeneration and has a potent antihepatitis effect in vivo. Hepatology 1992; 16:1227-1235.
44. **Gomez-Lechon MJ, Castelli J, Guillen I, et al:** Effects of hepatocyte growth factor on the growth and metabolism of human hepatocytes in primary culture. Hepatology 1995; 21:1248-1254.

Journal of Surgical Research ■, 1–9 (2009)
doi:10.1016/j.jss.2009.06.054

Diverse Effects of FK506 on the Apoptosis of Hepatocytes and Infiltrating Lymphocytes in an Allografted Rat Liver

Hiroki Moriuchi, M.D.,*,[1] Yukio Kamohara, M.D., Ph.D.,* Susumu Eguchi, M.D., Ph.D.,* Weili Gu, M.D., Ph.D.,* Hikaru Fujioka, M.D., Ph.D.,* Takao Yamamoto, M.D., Ph.D.,* Yoshitsugu Tajima, M.D., Ph.D.,* Takashi Kanematsu, M.D., Ph.D.,* and Takehiko Koji, Ph.D.†

*Department of Surgery; and †Department of Surgery Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Saga, Japan

Submitted for publication March 22, 2009

Background. **The current study investigated whether FK506 (FK) regulates the apoptotic systems in allografted rat liver and the contribution of Fas/Fas-ligand system and Bcl-2 family during acute rejection.**

Materials and Methods. **The recipients were divided into three groups, the allo, the allo-FK, and the syn group. Rats were euthanized 1, 3, 5, and 7 d after OLT. Apoptotic activity was explored using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The expression of Fas/Fas-ligand and Bcl-2/Bax in the grafted livers was investigated by Western blotting and immunohistochemistry.**

Results. **The apoptotic index (AI) of hepatocytes in the allo-FK group was less than that in the allo group. Fas in the allo group was more intense than that in the allo-FK group in the periportal areas on day 1 and 3, while Bcl-2 in the allo group was less intense than that in the allo-FK group in the pericaval areas at all time-points after OLT.**

Conclusion. **FK provides beneficial antiapoptotic effects on hepatocytes in the grafted rat livers through both the down-regulation of Fas expression in the periportal areas and the up-regulation of Bcl-2 expression in the pericaval areas.**

Key Words: **FK506; apoptosis; liver transplantation; Fas; Fas ligand; Bax; Bcl-2.**

INTRODUCTION

Apoptosis occurs in various organs to maintain normal development and homeostasis. Moreover, apoptosis is involved in several human diseases, including autoimmune diseases, viral hepatitis, hepatocellular carcinoma, ischemia-reperfusion injury (I/R injury), and gastrointestinal malignancies [1–5]. In the case of liver transplantation, apoptosis in liver allografts was first reported in a porcine model in 1974 [6]. Recent studies suggest that apoptosis of infiltrating lymphocytes plays an important role in acquiring spontaneous tolerance to liver allografts [7, 8].

FK506 (FK), like cyclosporine A, is a widely accepted immunosuppressant. The use of FK is well standardized in liver transplantation. It belongs to the family of calcineurin inhibitors, which reduces IL-2 production through NF-AT in T lymphocytes [9, 10]. In addition, FK enhances apoptosis of activated T cells *in vivo* [11].

Apoptosis is regulated by several factors, including the Fas/Fas ligand (FasL) system, and Bcl-2 related proteins. However, in liver transplantation, the relationship between FK and apoptosis associated factors remains unclear, especially in hepatocytes.

The present study investigated the effects of FK on the induction of apoptosis in hepatocytes and infiltrating lymphocytes, and their correlation with the expression of those apoptosis-related molecules using rat models of allogeneic OLT.

MATERIALS AND METHODS

Animals

Male Dark Agouti ($RT1^a$), and Lewis ($RT1^l$) rats were purchased from SLC (Shizuoka, Japan) and Charles River Japan (Atsugi, Japan), respectively. All animals were housed in a climate controlled room with a 12 h light, 12 h dark schedule, and maintained in the pathogen-free facility at the Nagasaki University School of Medicine.

[1] To whom correspondence and reprint requests should be addressed at, Department of Surgery, National Hospital Organization Saga National Hospital, 20-1, Hinodemachi, Saga 849-8577, Japan. E-mail: mkaki1@mac.com.

0022-4804/09 $36.00

They were provided rat chow (Oriental Koubo Kougyo, Tokyo, Japan) and tap water *ad libitum*, and were used as donors or recipients for OLT at a weight 250–300 g. All experiments were performed in accordance with the University of Nagasaki Research Animal Resources guidelines.

Orthotopic Liver Transplantation

Donor livers were harvested with flushing by 5 mL of lactated Ringer's solution and preserved in a bath of lactated Ringer's solution at 0–4°C for 30 min. The liver was transplanted orthotopically, using the cuff technique for the anastomosis of the portal vein, infrahepatic vena cava, and bile duct. The hepatic artery was not reconstructed, as previously described [12]. The transplant procedures were finished within 15 min in all instances.

Experimental Design

The recipients were divided into three groups, (1) allo group; allogeneic combination without any immunosuppressant (DA to LEW, $n = 20$), (2) the allo-FK group; allogeneic combination with FK (0.2 mg/kg/d) in a subcutaneous injection (s.c.; DA to LEW, $n = 20$), (3) syn group; syngeneic combination (LEW to LEW, $n = 12$). In the allo-FK group, FK was dissolved in normal saline at a concentration of 0.1 mg/mL and kept at 4°C. Other groups received normal saline only at the same amount used for the allo-FK group. Five animals from each group were euthanized and the grafted liver was harvested 1, 3, 5, and 7 d after liver transplantation, respectively. Harvested livers were frozen in liquid nitrogen for protein extraction, or fixed in 10% formalin. The tissues were embedded with paraffin, sectioned at 4 μm and placed on silane-coated glass slides for hematoxylin and eosin (H and E) staining, immunohistochemistry, and the DNA double-strand break assay. Peripheral blood was obtained for measurements of the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.Bil) levels.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay (TUNEL)

TUNEL was performed to detect DNA fragmentation according to the method of Hakuno N *et al.* [13], using proteinase K (Wako Pure Chemicals, Osaka, Japan), terminal deoxynucleotidyl transferase (TdT) buffer (Roche Diagnostics, Mannheim, Germany), and biotin-16-dUTP (Roche Diagnostics). Lastly, the sites of horseradish peroxidase (HRP) were visualized by 3, 3'-diaminobenzidine tetrachloride (DAB; Wako Pure Chemicals, Osaka, Japan) and H_2O_2 with nickel and cobalt enhancement [14] for 5 min. Thymidine triphosphate (TTP) was used instead of biotin-16-dUTP as a negative control.

Apoptotic Index

The apoptotic index (AI) was calculated in each grafted liver. More than 5000 cells were counted in one section of the grafted liver and the number of TUNEL positive hepatocytes or lymphocytes per 1000 each cells was expressed as an AI.

Western Blot Analysis

Total proteins were extracted from the frozen livers in liquid nitrogen according to the previously described method [13, 15]. Briefly, the tissue specimens were homogenized in a lysis buffer (5 mM phosphate buffer with 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1μg/mL leupeptin, 1μg/mL chymostatin pH 7.2) at 0–4°C using a Potter type homogenizer. The concentration of protein samples was measured by the Bradford method [16], using bovine serum albumin as a standard. Protein extracts were mixed with a loading buffer (0.25 M Tris-HCl, 2% SDS, 30% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue pH 6.8) at the rate of 1:1, and then heated for 5 min at 95°C. The sample proteins (10μg) per lane were separated in 10%–20% gradient gels by SDS-PAGE, as described by Laemmli [17]. Proteins were electrophoretically transferred onto polyvinylidine difluoride membranes (Immobilon P; Millipore Corp., Bedford, MA) by the semi-dry method using a blotting apparatus. Then, the membranes were soaked in 5% nonfat dry milk in PBS overnight at 4 °C. The washed membranes were incubated for 2 h with a 1:800 dilution of rabbit anti-Fas (P4; 13), anti-FasL (P5; 13), or anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) antibody at room temperature. After washing in T-PBS three times (10 mM phosphate-buffered saline with 0.1% Tween 20 pH 7.4), the membranes were incubated with a 1:1000 dilution of HRP conjugated goat anti-rabbit IgG F(ab')2 (MBL, Nagoya, Japan) as a second antibody for 1 h. The membranes were washed three times with T-PBS and then were visualized by DAB and H_2O_2 with enhancement of nickel and cobalt for 5 min [14].

Immunohistochemical Staining

Immunohistochemical staining of Fas, FasL, Bcl-2, and Bax were performed according to the method used in the previous paper [18]. The primary antibodies were as follows; the polyclonal antibodies for anti-mouse Fas (diluted 1:200, P4), anti-rat FasL (diluted 1:100, P5), anti-Bcl-2 (diluted 1:200), and anti-Bax (diluted 1:200) derived from rabbits described above [13]. In the case of Bcl-2 staining, the sections were immersed in 0.01 M citrate buffer (pH 6.0), and autoclaved at 121 °C for 10 min [19]. The sections were reacted with 500 μg/mL normal goat IgG in 1% BSA/PBS at room temperature for 1 h, and were incubated with the primary antibody solution overnight for anti-Fas and anti-Bcl-2 antibodies, or for 2 h for anti-FasL and anti-Bax antibodies. All slides were washed with 0.075% Brij 35 in PBS three times, and were incubated with HRP-goat anti-rabbit IgG (1:200) diluted in 1% BSA/PBS at room temperature for 1 h. HRP was added for visualization with DAB and H_2O_2 for Fas and FasL staining. On the other hand, for Bcl-2 and Bax staining, nickel and cobalt enhancement [14] with H_2O_2 and DAB were performed. Normal rabbit serum was used in place of the specific primary antibody as a negative control. For Fas and FasL staining, the sections were counterstained with methyl green. In addition, immunohistochemical staining of T cell receptor was performed to identify the infiltrating cells in the periportal areas, using monoclonal antibody R73 ($\alpha\beta$ T cell receptors; Serotec, Oxford, UK) as the primary antibody, according to the previous paper [20].

Statistical Analysis

The statistical analysis between the two groups was carried out using Mann-Whitney U test. P values < 0.05 were considered to indicate a significant difference. All data are presented as the means ± SD.

RESULTS

Survival Time

The survival time of the allo-FK group rats was significantly prolonged, in comparison to the allo group rats (10.4 ± 1.52 *versus* 42.8 ± 11.8 d, $P < 0.01$).

Alteration of the Serum Levels of ALT, AST, and T.Bil

The levels of AST in the allo-FK group tended to be lower than that of the allo group after liver transplantation, but not significantly. On the other hand, there was

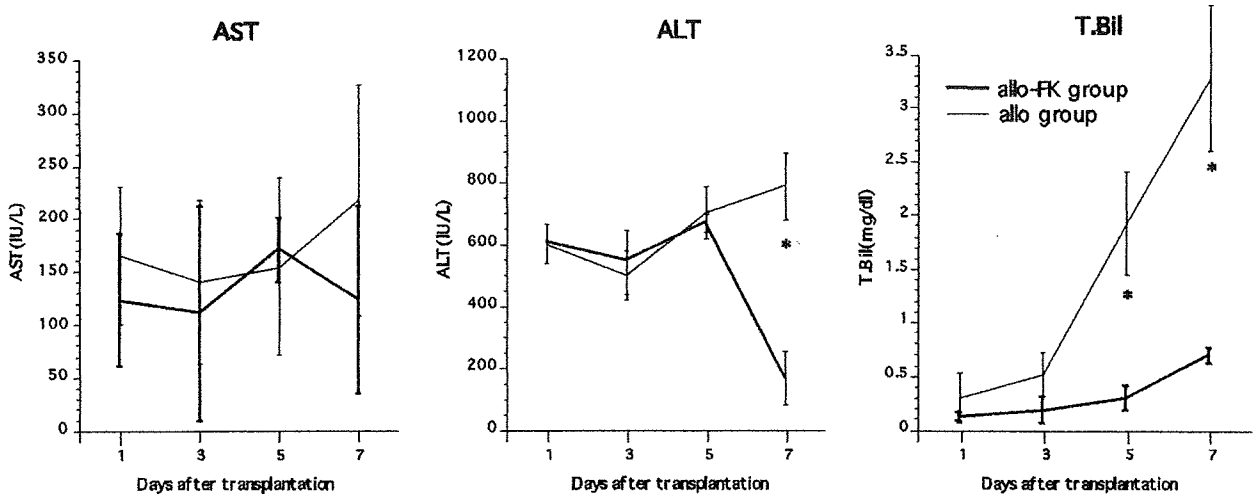


FIG. 1. Alteration of serum levels of AST, ALT, and Total bilirubin (T.Bil) after rat OLT. The levels of AST and T.Bil in the allo-FK group tended to be lower than the allo group throughout after OLT. There was a significant difference on day 7 between the allo group and the allo-FK group in ALT levels after OLT. Data are plotted as the means ± SD. *Statistically significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

a significant difference on day 7 between the allo group and the allo-FK group in levels of ALT (Fig. 1). The elevation in the T.Bil level was suppressed in the allo-FK group after liver transplantation. In the allo group, the T.Bil level significantly increased on day 5 and 7 (Fig. 1).

Histological Assessment of the Grafted Liver

The clearly defined diagnosis criteria according to the Banff schema for grading liver allograft rejection was used to evaluate acute rejection in grafted livers [21], which suggests a global assessment of the grafted liver. The grading of acute rejection into the indeterminate, mild, moderate, and severe categories was based on the overall appearance of the specimens according to the criteria. Portal inflammation, perivenular inflammation, and bile duct damage were the main features of that's criteria. Acute rejection was observed to be more severely advanced in the allo group in comparison to the other groups (Fig. 2). In the allo-FK group, the grade of acute rejection was lower than that of the allo group on day 3, 5, and 7. Meanwhile, there were no cases with acute rejection in the syn group (Fig. 2). In addition, most of the infiltrating cells in the

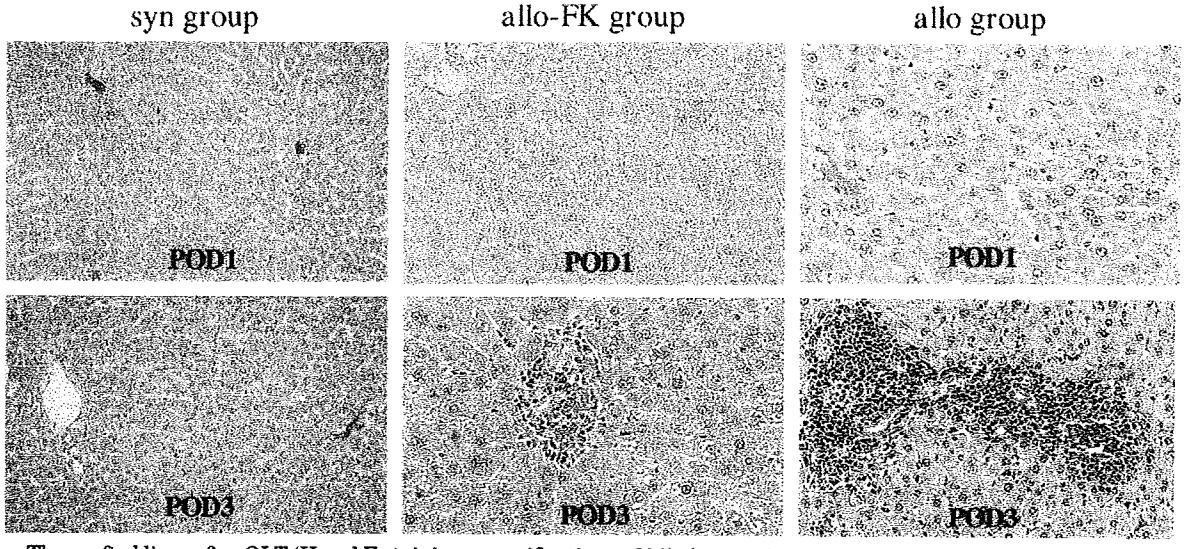


FIG. 2. The grafted liver after OLT (H and E staining; magnification, ×200). Acute rejection was observed to be more severely advanced in the allo group than in the other two groups. In the allo-FK group, the grade of acute rejection was lower than that of the allo group on day 3, 5, and 7. In the syn group, there were no cases with acute rejection.

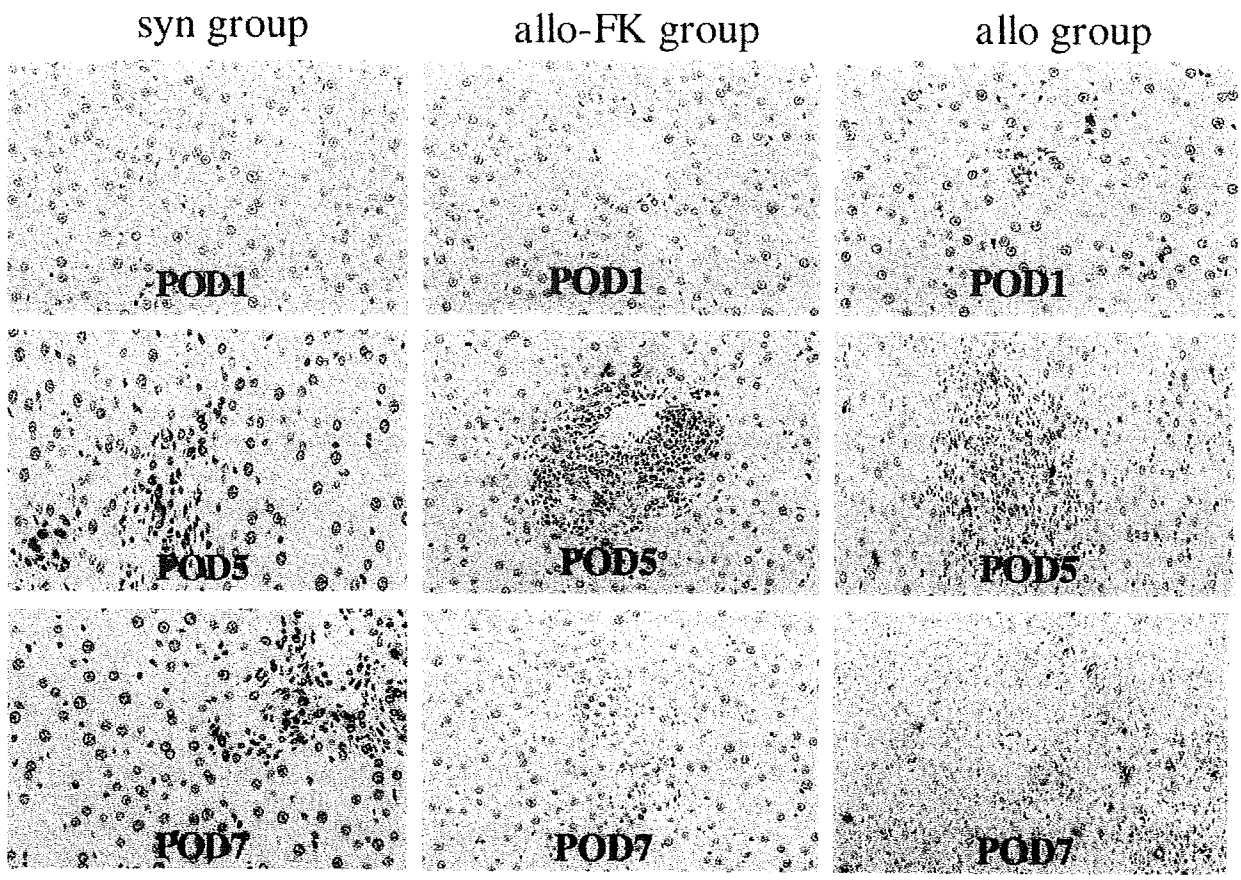


FIG. 3. Apoptotic cells identified in the allo-FK, the allo, and the syn group using TUNEL staining of allografted livers on postoperative days (POD) 1, 5, and 7 (magnification, ×200). The number of TUNEL positive lymphocytes on POD 5 in the allo-FK group was greater than that in the allo group after liver transplantation. TUNEL positive hepatocytes were detected mainly in the periportal areas in both the allo-FK and the allo group and their number in the allo group was greater than that in the allo-FK group. The number of TUNEL positive hepatocytes in the syn group was smaller than in the other groups after OLT.

periportal areas (zone 1) were T lymphocytes, as assessed by T cell receptor staining (data not shown).

Apoptosis in the Grafted Liver

TUNEL positive cells, which included hepatocytes, infiltrating cells, and sinusoidal endothelial cells, were observed in all post-transplant groups. There was no significant change in the number of TUNEL positive infiltrating T lymphocytes during the post-transplant period in the syn group. TUNEL positive T lymphocytes were observed to be markedly increased in the other groups. The AI of infiltrating T lymphocytes in the periportal areas (zone 1) reached a maximum on day 3–5 in the allo-FK group, while there was no significant change in the allo group (Figs. 3 and 4a). On the other hand, hepatocyte apoptosis increased and maximized on day 7 in the allo group, while the number of hepatocyte apoptosis was less in the allo-FK group than that in the allo group (Fig. 4b). TUNEL positive hepatocytes were mainly located in the periportal areas (zone 1) in both groups (Fig. 3). In the syn group, the number of TUNEL positive hepatocytes was smaller than in the other groups after OLT, while it was not significantly changed over time (Fig. 3).

Western Blot Analysis for Fas, FasL, Bcl-2, and Bax in the Grafted Liver

Bands of the appropriate size were visualized, respectively, at 45 kD (Fas; 13), 31 kD (FasL; [13]), 25 kD (Bcl-2; [22, 23]), and 21 kD (Bax; [22, 23]). The expression of Fas in the allo group was more intense than that in the allo-FK group on day 1 and 3 after liver transplantation. Bcl-2 was strongly expressed in the allo-FK group during the post-transplant days, but the expression was slightly decreased on day 7. The expression of Bcl-2 in the allo group was less intense than that in the allo-FK group at any time-point after liver transplantation. However, the expression level of Bcl-2 in the allo group was temporarily increased just on day 3. On the other hand, the expression of FasL and

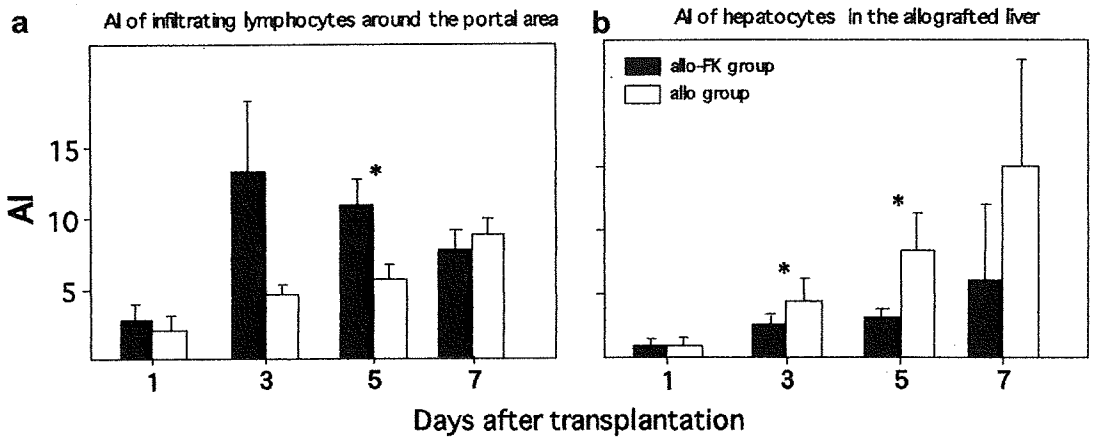


FIG. 4. The apoptotic index (AI) in allografted liver transplantation. The AI was calculated as the number of TUNEL positive lymphocytes per 1000 lymphocytes (a) and hepatocytes (b) likewise in the allo-FK group (closed columns), and the allo group (open columns). Data are plotted as the means ± SD. *Statistically significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

Bax were almost constant and there was essentially no significant difference in the intensity between the allo group and the allo-FK (Fig. 5).

Immunohistochemical Staining of Fas and FasL

Fas positive hepatocytes as well as Fas positive infiltrating lymphocytes were detected mainly in the periportal areas (zone 1) in the allo and the allo-FK group post-transplantation. The immunohistochemical reaction for Fas positive hepatocytes was stronger in the allo group than that in the allo-FK group on day 3, 5, and 7, while there was little difference between the allo group and the allo-FK group on day 1. In the syn group, Fas positive hepatocytes were also observed in all samples, while the expression levels of Fas remained the same throughout the course (data not shown).

Immunohistochemical Staining of Bcl-2 and Bax

Figure 6 shows that most of the immunohistochemical results were consistent with that of the Western blot analysis. Bcl-2 positive hepatocytes were not found in the pericaval area (zone 3) in the normal LEW rats (Fig. 6). Bcl-2 positive hepatocytes in the syn group were mainly detected in pericaval area (zone 3). The

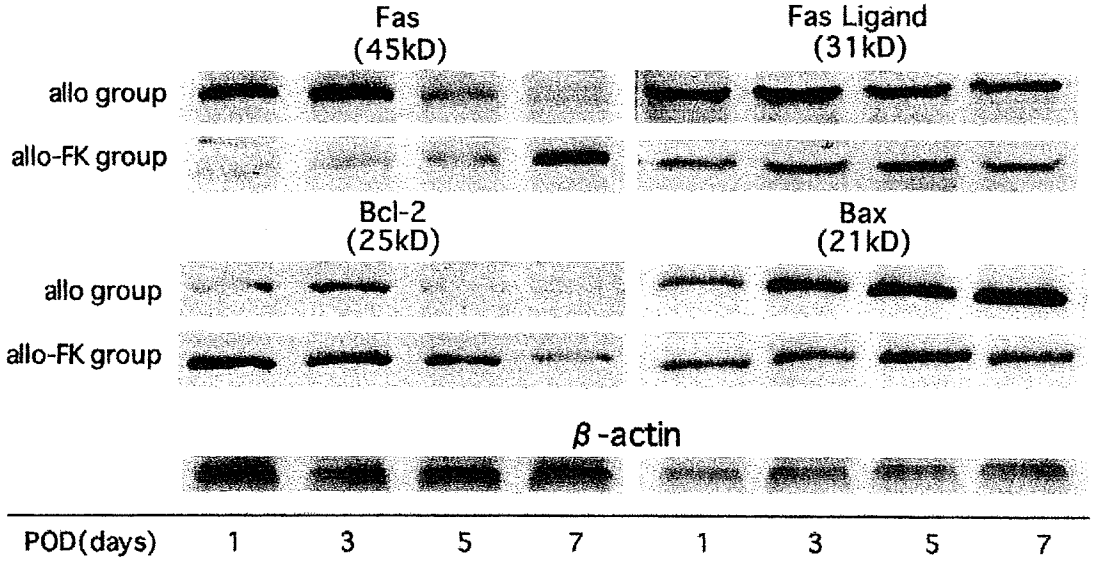


FIG. 5. A Western blot analysis of Fas, Fas-ligand, Bcl-2, and Bax in allografted liver. The expression of Fas in the allo group was more intense than that in the allo-FK group on day 1 and 3 after OLT. The expression of Bcl-2 in the allo group was less intense than that in the allo-FK group throughout posttransplantation. There was no significant difference in the expression of Fas-ligand and Bax between the two groups. Protein samples (10 ug) from allografted livers were separated by SDS-PAGE (10%–20% gradient gels) and subjected to an immunoblot analysis as described.

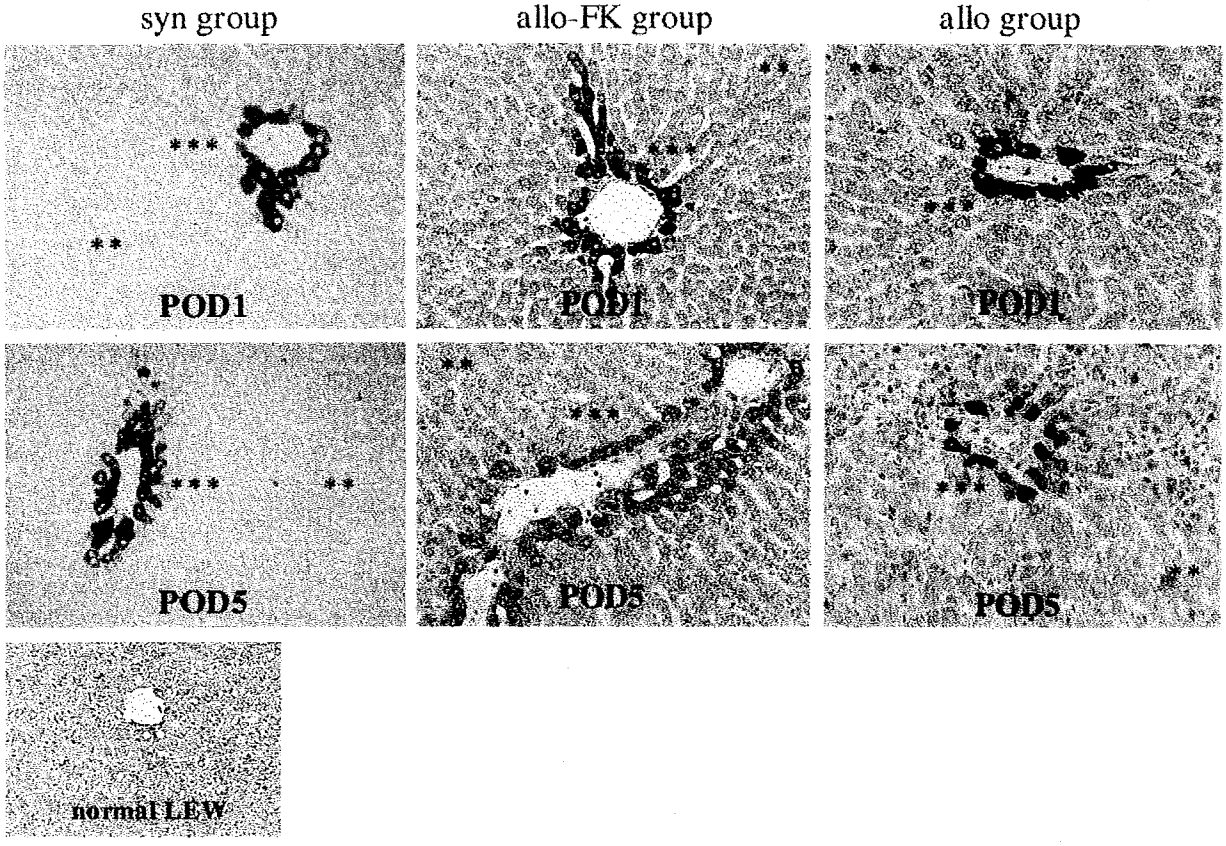


FIG. 6. Immunohistochemical staining of Bcl-2 in representative grafted liver sections on postoperative days (POD) 1 and 5 (magnification, ×200). The immunoreactivity of Bcl-2 in the allo-FK group was stronger than that in the allo group after liver transplantation on POD 1, 3, and 5. Bcl-2 positive hepatocytes in the allo-FK group were more frequently detected than those in the allo group mainly in pericaval area (zone 3), in comparison to the periportal area (zone 1). In normal LEW rats, Bcl-2 positive hepatocytes were not found in the pericaval area (lower left); **zone 2; ***zone 3.

expression level of Bcl-2 in the allo group was lower than that in the allo-FK group on day 1, 3, and 5 after OLT. However, there was little difference between the groups only on day 3 in the immunoreactivity of Bcl-2. Interestingly, the expression level of Bcl-2 in the syn group was lower than that in the allo-FK group on day 1 after OLT (Fig. 6). On the other hand, Bcl-2 positive hepatocytes were detected more frequently in the pericaval area (zone 3) in the allo-FK group in comparison to the allo group (Fig. 6). Bax positive hepatocytes were distributed evenly in grafted livers in the syn group. The expression of Bax was always detected in the hepatocytes of both the allo and the allo-FK groups, and there was no difference in the expression among the three groups (data not shown).

DISCUSSION

The present study investigated the effects of FK on the grafted liver in the acute rejection phase after allogeneic liver transplantation in rats, and successfully demonstrated a significant decrease in the number of infiltrating lymphocytes and damaged hepatocytes in the FK treated grafts. Moreover, the results showed that FK acts on infiltrating T lymphocytes and rescues hepatocytes from apoptosis. The present results reinforce the efficacy of FK as an immunosuppressant in liver transplantation.

It was suggested that FK could thus have dual action on both infiltrating T lymphocytes and hepatocytes in our model by mediating apoptosis. In solid organ transplantation, activated T lymphocytes decrease after suppression of IL-2 production [11, 24], but the relationship between FK and T lymphocyte apoptosis has not been sufficiently clarified *in vivo*. The present study clearly demonstrated that apoptotic T lymphocytes were abundant in the periportal area (zone 1) in the allo-FK group in accordance with the inhibitory effect of FK on IL-2 expression. On the other hand, FK reduces neutrophil accumulation, including the serum cytokine levels, while attenuating the I/R injury of the liver [25–27]. In addition, since FK in itself can protect hepatocytes from apoptosis induced by ischemia-reperfusion