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ORIGINAL ARTICLE

Expansion of hepatic progenitor cell in fatty liver graft after living donor liver transplantation

Jai Young Cho,[1] Kyung-Suk Suh,[1] Woo Young Shin,[1] Hae Won Lee,[1] Nam-Joon Yi,[1] Min A Kim,[2] Ja-June Jang[2] and Kuhn Uk Lee[1]

1 Department of Surgery, Seoul National University College of Medicine, Seoul, Korea
2 Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

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cell replication, ductular reaction, fibrosis, hepatic steatosis, liver regeneration.

Correspondence
Kyung-Suk Suh MD, PhD, Department of Surgery, Seoul National University College of Medicine, Seoul National University Hospital, 28 Yongon-dong, Chongno-gu, Seoul, 110-744, Korea. Tel.: 82-2-2072-3789; fax: 82-2-766-3975; e-mail: kssuh@plaza.snu.ac.kr

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Summary

Although it is known that steatotic livers have a reduced ability to regenerate, most individuals with steatosis show generally benign prognosis. We hypothesized that a proliferative blockade in steatotic hepatocytes results in the compensatory expansion of hepatic progenitor cells (HPC) during fatty liver regeneration. Fifty-four cases of living donor liver transplantation (LDLT) with a liver biopsy performed at the postoperative 10th day were examined. HPC were counted by immunofluorescence histochemical dual-staining technique using cytokeratin 7 and Ki-67, and the replicative arrest of hepatocytes was assessed by p21 immunohistochemistry. The degree of ductular proliferation during regeneration 10 days after LDLT correlated both with the degree of steatosis and the number of HPC ($P < 0.001$). There was no difference in the average number of HPC and the replicative arrest index between donors with or without steatosis before LDLT ($P = 0.111$ and $P = 0.062$). However, degree of steatosis correlated with both the expansion of HPC and the replicative arrest index during liver regeneration 10 days after LDLT ($P < 0.001$ and $P < 0.001$, respectively). Moreover, increased replicative arrest was strongly associated with HPC expansion ($P < 0.001$). In conclusion, the compensatory expansion of HPC as a result of impaired hepatocyte replication occurred during steatotic liver regeneration after LDLT.

Introduction

Nonalcoholic fatty liver disease (NAFLD), which represents a spectrum of disorders that include fatty liver alone, nonalcoholic steatohepatitis (NASH), and cryptogenic cirrhosis, is a major cause of morbidity and mortality [1–3]. Hepatic steatosis is the earliest and most common histopathology of NAFLD [4], and has two subtypes, macrovesicular steatosis (MaS), and microvesicular steatosis (MiS) [5]. Of these, MaS is more prevalent and a more important subtype from a clinical standpoint [6,7]. In liver transplantation, grafts with severe steatosis are often associated with primary graft nonfunction, delayed graft function, and postoperative morbidity, particularly after deceased donor liver transplantation [8].

Ever since a reproducible two-thirds partial hepatectomy (PH) was first described [9], liver regeneration in steatotic livers after PH or acute hepatic necrogenic injury has been studied intensively [10,11]. However, the experimental models used have provided conflicting results [12,13]. Although some individuals with steatosis develop cirrhosis, most do not, and a fatty liver persists for years, causing only hepatomegaly and mild increases in the serum aminotransferase values [14]. Moreover, in living donor liver transplantation (LDLT) donors, the early regenerative capacity of the remnant liver with a mild degree of MaS is initially impaired after a PH. However, its long-term regenerative capacity is unimpaired [15]. The mechanisms that limit the liver damage in most patients with hepatic steatosis are poorly understood. One

possible explanation for the generally benign prognosis of fatty liver disease is that the fatty hepatocytes are not injured. An alternative possibility is that most individuals can compensate for the liver damage that accompanies hepatic steatosis [16].

In a nondiseased human liver, hepatic progenitor cells (HPC) reside in the bile ductules, and the canals of Hering localized in the portal tract and periportal parenchyma. It has been shown in both animal and human livers that HPC are bipotential cells that can differentiate toward a biliary or the hepatocytic lineage [17,18]. In a normal liver, the replacement of hepatocytes occurs through the replication of other adjacent hepatocytes within the lobules. However, an impairment in this primary pathway leads to the proliferation of HPC, which by default becomes the source of regenerating hepatocytes [19]. Given these facts, this study examined whether or not HPC compensate for impaired hepatocyte replication in steatotic livers during regeneration 10 days after LDLT.

Patients and methods

Patients, clinical, and laboratory data

Fifty-four consecutive adult LDLT recipients, who were diagnosed with hepatitis B virus related end stage liver disease, underwent a liver biopsy on the 10th postoperative day at our institution between December 2003 and April 2005. Informed consent was obtained from each patient by the surgical team, and the Institutional Review Board of Seoul National University Hospital (H-0602-023-167) approved this study protocol. All the donors underwent an intraoperative wedge liver biopsy at the time of the hepatic resection. In the recipients, protocol liver biopsies were performed routinely. All biopsy specimens from the recipients were obtained using a uniform procedure at two different sites using an 18-gauge percutaneous biopsy needle. Information prospectively stored on the database was reviewed.

Immunosuppression was based on a flexible double-drug protocol. The maintenance immunosuppressive agents used during the study period consisted primarily of a calcineurin inhibitor and a corticosteroid. The primary immunosuppressant was tacrolimus in 35 patients and cyclosporine in 19. All patients uniformly received postoperative combination prophylaxis with hepatitis B immune globulin and lamivudine.

Histopathological analysis

Fresh liver sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to delineate their hepatic histology. The sections were analyzed by an experienced hepatopathologist who was blinded to the laboratory parameters and clinical data. The absence of significant pathologic changes, except for hepatic steatosis, in the graft livers obtained at the time of the hepatic resection was confirmed by reviewing all the biopsies. Steatosis in the donor livers before the donor hepatectomy was quantified by determining the percentage of hepatocytes affected according to the type (MaS or MiS), and was scored as either absent (<5% of hepatocytes affected) or present (≥5% of hepatocytes affected) [20]. The level of ductular proliferation in all the protocol biopsy specimens was assessed semiquantitatively on a scale of grade 0 (absent), grade 1 (scattered small ductules at the periphery of the portal areas), grade 2 (prominent small ductular branches, but not extended into the adjacent portal area), or grade 3 (prominent small ductular branches extended into the adjacent portal area). Portal fibrosis was assessed semiquantitatively on grade 0 (none), grade 1 (fibrous portal expansion), grade 2 (bridging fibrosis), or grade 3 (presence of cirrhosis). In addition, all protocol biopsy specimens were also assessed semiquantitatively on a scale of 0 (absent) to 3 (severe) for rejection activity indices, sinusoidal dilatation, hepatocyte ballooning, centrilobular necrosis, centrilobular inflammation, and central vein fibrosis [21].

Immunohistochemical staining and quantification

Immunohistochemical staining for nuclear antigen, such as Ki-67, and proliferating cell nuclear antigen (PCNA) was performed using a monoclonal anti-Ki-67 antibody (clone MIB-1; Dako, Glostrup, Denmark) and a monoclonal anti-PCNA antibody (clone PC10; Amersham, Westbury, NY, USA) for monitoring hepatic regenerative activity. Hepatocellular proliferative activity was assessed by counting Ki-67 or PCNA-positive hepatocyte nuclei in 10 random lobular fields at a magnification of 400×.

Immunohistochemical staining for the p21 protein was performed to determine if cell cycle arrest occurs in regenerating steatotic livers [22]. Deparaffinized 5 micron-thick sections were first treated with 0.3% H_2O_2 in methanol for 30 min to eliminate their endogenous peroxidase activity. The sections were then hydrated in a graded series of ethanol (100% – 70%), trypsinized, and stained using the immunoperoxidase technique. Before staining, the level of antigen recovery was enhanced in a pressure cooker. The monoclonal primary antibody, anti-p21 (clone SX115, 1:100 from BD Biosciences Pharmingen, Seoul, Korea) was applied for 2–3 h at room temperature. The secondary antibody was applied and peroxidase staining was performed using a Dako Envision Kit according to the manufacturer's instructions. Only the cells showing distinct and near-total nuclear staining for p21 were considered positive. The number of positive

hepatocytes was expressed as the percentage of the total to give a labeling index (LI) by counting in 10 randomly chosen fields at 400× magnification. A variable amount of nonspecific background cytoplasmic staining was identified in all cases and was not considered when determining the LI.

Immunofluorescence histochemical dual-staining technique

Immunofluorescence histochemical dual-staining technique was performed using monoclonal anti-Cytokeratin-7 antibody and monoclonal anti-Ki-67 antibody to identify the location and number of HPC. The slides were incubated with normal bovine serum for 1 h at room temperature, followed by incubation with goat the anti-human Cytokeratin-7 antibody (clone N-20, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-human Ki-67 antibody (clone MIB-1, 1:200, Dako) for 24 h at 4 °C. These were then washed with PBS and incubated with bovine anti-goat IgG-FITC (1:500), and bovine anti-mouse IgG-TR (1:1000) for 1 h at room temperature. After rinsing in PBS, the slides were observed under a fluorescence microscope (Venox AHBT3; Olympus, Tokyo, Japan) using the appropriate exciter and barrier filters for viewing the FITC (exciter filter BP 450 ± 490; barrier filter LP 520; dichroic mirror FT 510), and TR (exciter filter BP 546 ± 12; barrier filter LP 590; dichroic mirror FT 580) fluorescence alternately.

All the tissue sections were photographed using a PixeLink Colour Digital Camera (Total Turnkey Solutions, Mona Vale, NSW, Australia) according to fluorescence of each alternately. In all the liver specimens, nonoverlapping fields of the entire biopsy were photographed under alternative fluorescence at 100×, and the images were merged together using image viewer software (ACDSee 7.0; L2C Inc., Seoul, Korea). The HPC were identified by counting the cells that had been dual-stained (yellow color in merged image) by both Cytokeratin-7 (green color by FITC) and Ki-67 (red color by TR) in the periportal area of the lobule and expressing these as the number per portal tract.

The definitions used and statistical methods

Degree of steatosis was quantified by determining the percentage of hepatocytes affected according to the type (MaS or MiS) and ductular proliferation was defined as proliferation of small ductules in periportal area. HPC were defined as cells dual-stained by both Cytokeratin-7 and Ki-67 in the periportal area of the lobule. Replicative arrest index was defined as the percentage of the number of positive hepatocytes by p21 staining.

Continuous normally distributed variables are represented as a mean ± SD. A Fisher exact test was used to determine whether the distributions of nonparametric variables in groups differed. The Mann–Whitney *U*-test was used to compare group means and the Pearson correlation coefficient was used to determine correlations between continuous normally distributed variables. Degrees of association between nonparametric variables were assessed using the Spearman correlation. The independent effects of normally distributed variables were assessed by multiple linear regression. A stepwise approach was used to remove nonsignificant variables and determine the most parsimonious model, which included both fixed factors and covariates. Binary logistic regression was used to assess the relative influences of variables on categorical data. All the analyses were carried out using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered significant.

Results

Clinical, histological, and laboratory data

Of the 54 patients, there were 36 men and 18 women, with a mean patient age of 50.1 ± 7.7 years (range: 34–64; Table 1). The recipients' demographic factors in the MaS-negative (<5% of MaS) and MaS-positive (≥5% of MaS) groups were similar in terms of age, gender, MELD score or immunosuppressive drugs. There was no significant difference found between the donor demographic factors in these two groups in terms of age or gender, but the mean BMI was significantly higher in the donors with MaS ($P = 0.004$). The graft parameters, including the GRWR, graft type, and cold and warm ischemic times were similar in the two groups. The preoperative laboratory test results, including AST, ALT, total bilirubin, ALP, and GGT were similar in both groups ($P > 0.05$).

Prominent ductular proliferation was seen in regenerating steatotic livers

Serum total bilirubin results on the 1st, 3rd, and 7th days post-LT in patients with MaS were significantly higher than those of patients without MaS ($P = 0.025$, 0.038, and 0.021 respectively), but serum total bilirubin results obtained 30, 180, and 365 days post-LT were not different in the two groups. Changes in serum AST, ALT, ALP, and GGT levels on the 1st, 3rd, 7th, 30th, 180th, and 365th days after LDLT were similar in the two groups.

A prominent ductular proliferation was observed in the regenerating steatotic livers. The grade of ductular proliferation in the liver biopsy specimens obtained from the recipients 10 days after LDLT strongly correlated with the degree of MaS in the grafts. Moreover, the grade of

Table 1. Demographic, donor, and graft characteristics of patients with chronic hepatitis B virus who underwent living donor liver transplantation.

	Without MaS (n = 26)	With MaS (n = 28)	P-value
Recipient factors			
Age (years)	49.1 ± 7.0	51.1 ± 8.3	0.340
Gender (male/female)	17/9	19/9	0.847
MELD score	21.7 ± 7.6	24.1 ± 9.2	0.306
Immunosuppression (tacrolimus/cyclosporine)	17/9	18/10	0.580
Donor factors			
Age (years)	29.6 ± 9.5	30.4 ± 8.6	0.741
Gender (male/female)	17/9	24/4	0.081
BMI (kg/m^2)	22.0 ± 2.2	24.4 ± 3.5	0.004
Graft factors			
GRWR (%)	1.06 ± 0.23	1.05 ± 0.21	0.859
Cold ischemic time (min)	81.7 ± 25.1	72.4 ± 30.4	0.241
Warm ischemic time (min)	41.7 ± 10.9	40.6 ± 15.2	0.762

MaS, macrovesicular steatosis; MELD, model for end-stage liver disease; BMI, body mass index; GRWR, graft-to-recipient weight ratio.

ductular proliferation correlated with the number of HPC (r = 0.556; $P < 0.001$) in the liver biopsy specimens obtained from the recipients.

The degree of steatosis was not associated with the degree of portal fibrosis (P = 0.152). However, the degree of portal fibrosis correlated with the number of HPC in the liver biopsy specimens obtained from the recipients (r = 0.305; P = 0.050). In addition, the number of HPC in the liver biopsy specimens obtained from the recipients did not correlate with rejection activity index (P = 0.449), sinusoidal dilatation (P = 0.172), hepatocyte ballooning (P = 0.382), centrilobular necrosis (P = 0.920), centrilobular inflammation (P = 0.963), and central vein fibrosis (P = 0.664).

Expansion of HPC in patients with MaS during liver regeneration are directly associated with hepatic steatosis

The number of HPC per portal tract in the liver biopsy specimens obtained from the donors before the donor hepatectomy ranged from 0 to 11.2, with a mean of 4.3 ± 1.7. There were no difference in the average number of HPC per portal tract between the donors with (4.6 ± 1.8) or without MaS (4.0 ± 1.6; P = 0.111). An expansion of HPC was observed in the regenerating steatotic livers. The number of HPC in the liver biopsy specimens obtained from the recipients 10 days after the LDLT in the patients with MaS (13.8 ± 8.1) was significantly higher than in those without (5.4 ± 3.5; $P < 0.001$; Fig. 1). The degree of MaS was significantly associated with the number of HPC in the liver biopsy specimens obtained from the recipients (r = 0.852; $P < 0.001$).

No difference in hepatocytes undergoing division was observed between patients with or without MaS. The mean numbers of positively stained hepatocytes in 10 high power fields were 45.3 ± 17.7 in patients without MaS and 52.4 ± 12.6 in patients with MaS according to PCNA antibody staining (P = 0.313), and 25.7 ± 14.2 in patients without MaS and 25.4 ± 10.4 in patients with MaS according to Ki-67 antibody staining (P = 0.953).

Univariate analysis revealed the degree of MaS (r = 0.852; $P < 0.001$) and MiS (r = 0.417; $P < 0.001$), MELD score (r = 0.259; P = 0.040), and GRWR (r = 0.259; P = 0.044) to be significantly associated with the number of HPC. Multivariate analysis revealed the degree of MaS (OR = 2.338; $P < 0.001$) and GRWR (OR = 0.627; $P < 0.001$) to be independently associated with the number of HPC (Table 2).

Hepatocyte replicative arrest was associated with expansion of hpc

The replicative arrest index was similar in the donors without MaS (4.6 ± 1.0) and with MaS (5.2 ± 1.5; P = 0.062). The number of hepatocytes expressing p21 during liver regeneration in the liver biopsy specimens obtained from the recipients 10 days after LDLT in the patients with MaS (11.7 ± 7.3) was significantly higher than in those without MaS (4.8 ± 1.2; $P < 0.001$; Fig. 2). The degree of MaS correlated with the replicative arrest index in the liver biopsy specimens obtained from the recipients (r = 0.905; $P < 0.001$; Fig. 3). Univariate analysis revealed the donor BMI (r = 0.0.301; P = 0.015), the degree of MaS (r = 0.905; $P < 0.001$), and MiS (r = 0.528; $P < 0.001$), and MELD score (r = 0.309; P = 0.013) to be significantly associated with the number of hepatocytes expressing p21 in the liver biopsy specimens obtained from the recipients. Multivariate analysis showed that the degree of MaS alone remained independently associated with the replicative arrest index

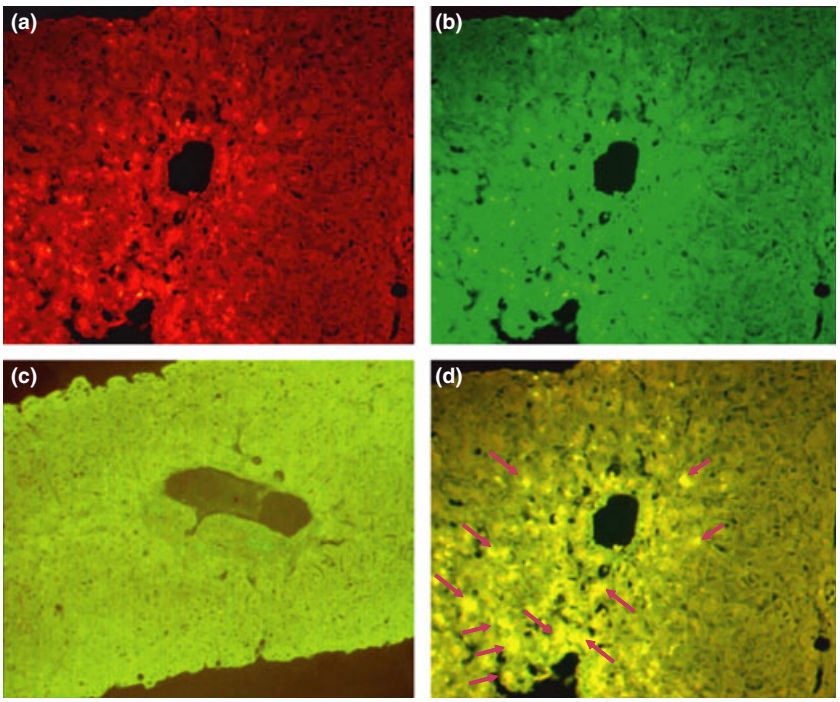

Figure 1 Immunofluorescence histochemical dual-staining for hepatic progenitor cells (HPC). Captured images for immunofluorescence histochemical staining using monoclonal anti-Ki-67 antibody (a) and monoclonal anti-Cytokeratin-7 antibody (b) were merged together. There was no positively stained HPC in the liver biopsy specimens obtained in a postoperative graft liver without steatosis (c). However, prominent HPC (red arrow) were stained mainly in the periportal areas in a postoperative graft liver with steatosis (d).

(OR = 2.175; $P < 0.001$; Table 2). Moreover, there was a correlation between increased replicative arrest and higher HPC numbers ($r = 0.834$; $P < 0.001$; Fig. 4).

However, there was no difference in postoperative complication rate between two groups ($P = 0.661$). There was no difference in 3-year patient survival (88.0% in patients without MaS vs. 77.0% in those with MaS; $P = 0.400$) and graft survival (96.0% vs. 85.2%; $P = 0.132$).

Discussion

This study demonstrates that mild steatosis is independently associated with both an increase in the number of

Table 2. Correlation of hepatic progenitor cell numbers and hepatocyte replicative arrest with clinical variables.

	Hepatic progenitor cells		Hepatocyte replicative arrest	
	P-value	Adjusted *P*-value*	*P*-value	Adjusted *P*-value*
BMI	0.144	NS	0.015	NS
MaS	<0.001	<0.001	<0.001	<0.001
MiS	<0.001	NS	<0.001	NS
Donor gender	0.194	NS	0.087	NS
Donor age	0.790	NS	0.256	NS
Recipient gender	0.736	NS	0.497	NS
Recipient age	0.877	NS	0.602	NS
MELD score	0.040	NS	0.013	NS
GRWR	0.044	<0.001	0.667	NS
CIT	0.137	NS	0.543	NS
WIT	0.626	NS	0.471	NS
Type of graft	0.743	NS	0.842	NS

BMI, body mass index; MaS, macrovesicular steatosis; MiS, microvesicular steatosis; MELD, model for end-stage liver disease; GRWR, graft-to-recipient weight ratio; CIT, cold ischemic time; WIT, warm ischemic time.

*Adjusted for age, gender, and MELD score in recipients, age, gender, and BMI in donors, and degree of MaS, degree of MiS, GRWR, type of graft, cold ischemic time, and warm ischemic time in grafts.

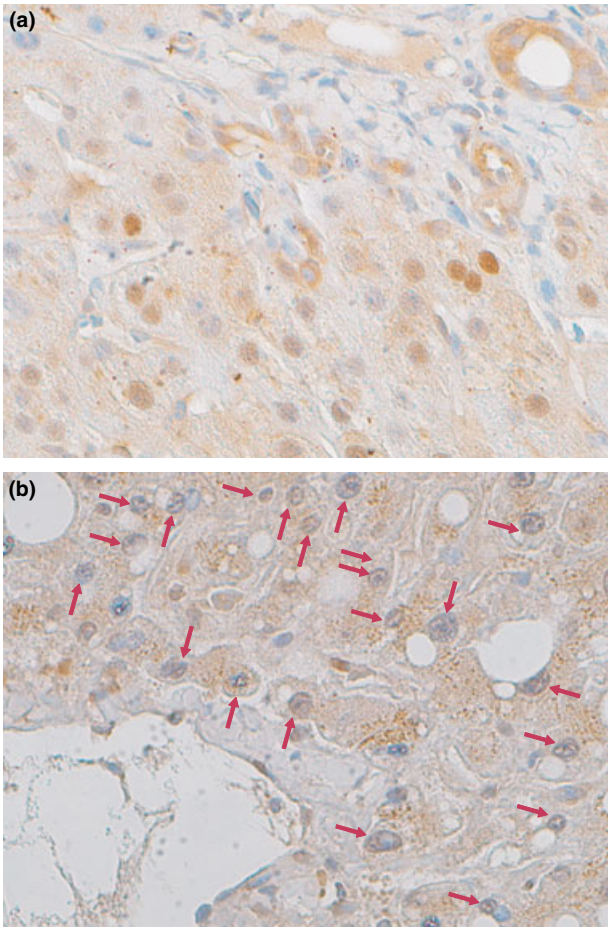

Figure 2 Immunohistochemical staining for p21. Hepatocytes expressing p21 (red arrow) during liver regeneration in the patients without macrovesicular steatosis (a) was significantly lower than those with macrovesicular steatosis (b; $P < 0.001$). Magnification 400×.

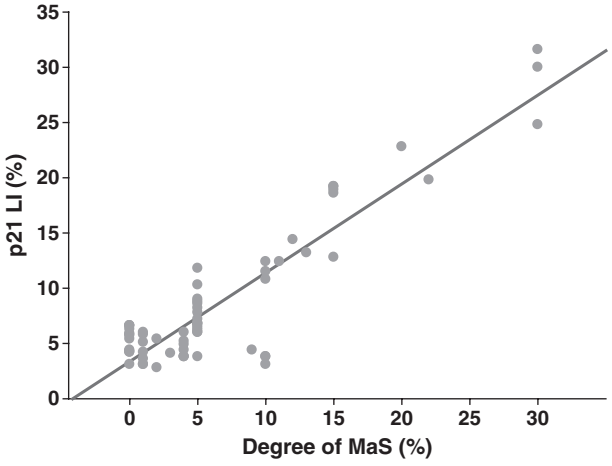

Figure 3 Correlation between the degree of macrovesicular steatosis and p21 index in the liver biopsy specimens obtained from the recipients ($r = 0.905$; $P < 0.001$).

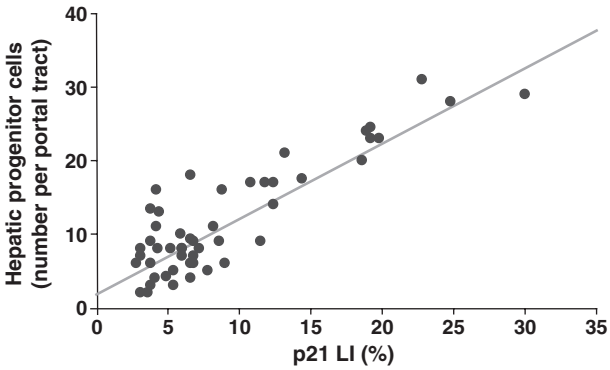

Figure 4 The correlation between increased hepatocyte replicative arrest measured by p21 staining and the higher number of hepatic progenitor cells in the liver biopsy specimens obtained from the recipients ($r = 0.834$; $P < 0.001$).

HPC and ductular proliferation during liver regeneration. The significant relationship between the number of HPC and the number of hepatocytes in replication arrest supports the hypothesis that compensatory volume restoration during regeneration after LDLT using mild steatotic livers is achieved by an expansion of HPC as a consequence of the altered hepatocyte proliferation, which is exacerbated by steatosis. To our knowledge, this is the first study to describe the potential mechanism for compensatory regeneration by HPC after PH in human subjects, in a relatively unique setting of LDLT where the relatively controlled environment of standardized cold and warm ischemia time can be minimized as a significant variable.

Recently, increasing evidence suggests that hepatic steatosis is more vulnerable to the factors that lead to inflammation and fibrosis [14]. Co-existent steatosis might exacerbate the liver injury when another liver disease is present [23]. As the healthy livers typically regenerate and recover completely from acute inflammation [11], the normal regenerative response to injury might be impaired in steatotic livers. HPC expansion is also most likely to be absent or minimal during liver regeneration after PH for primary or secondary liver tumors, as oval cell expansion does not occur during liver regeneration after the surgical removal of up to 2/3 of a rat liver [24]. It was recently suggested that impaired hepatocyte replication in steatotic livers promotes the expansion of HPC in patients with chronic hepatitis C [16,25] or NASH [26]. The replacement of hepatocytes lost from the normal hepatic parenchyma is known to occur by the replication of mature hepatocytes [20]. The inhibition of this replication by drugs [19], alcohol [22], steatosis [27], or viral infection [28] promotes the expansion of a secondary replicative pathway through the bipotential HPC [19].

Previous studies have also noted an association between the ductular reaction/HPC and fibrosis [25,26,29]. In the present study, a prominent ductular proliferation, which was strongly correlated with the number of HPC, was observed in the regenerating steatotic livers. Moreover, the degree of portal fibrosis correlated with the number of HPC in the liver biopsy specimens obtained from the recipients. Liver regeneration is accompanied by a complex remodeling of hepatic tissue and a concomitant transient breakdown of the lobular architecture [30,31]. This complex process is impaired after PH [32] and results in transient cholestasis with diminished overall bile flow and increased serum bile acid levels after PH in rats [33]. We previously reported that hepatic steatosis is associated with intrahepatic cholestasis and transient hyperbilirubinemia during regeneration, though hepatocellular proliferation was found to be unaffected by the presence of mild steatosis [34]. Taken together, these findings may suggest that impaired regeneration of steatotic liver may be compensated by replication of HPC.

The hepatic regenerative capacity is often assessed experimentally by monitoring the response to PH [11]. Liver regeneration after a two-thirds PH is inhibited in obese, diabetic Zucker fa/fa rats with fatty livers [13], which supports the concept that the liver's ability to regenerate is lower in NAFLD. However, the mechanisms that impair liver regeneration in fatty livers after PH in human subjects have not been identified. Other groups have already demonstrated that p21 is a key inhibitor of the G1 to S phase progression in hepatocytes. For example, liver regeneration is impaired in p21 transgenic mice [35]. However, a previous study has clearly shown that p21 expression by immunohistochemistry can be detected in hepatocytes during the normal cell cycle [36]. Therefore, it would be of importance to determine whether the HPC were actually proliferating and arrived from immature cells, or represent hepatocytes that have undergone ductular metaplasia. In this study, no difference was observed in the mean number of hepatocytes undergoing division between patients with or without MaS. This study also showed that no HPC expansion or no replicative arrest occurred in otherwise healthy fatty liver donors before PH. However, multivariate analysis showed hepatic steatosis to be independently associated with an expansion of HPC, and increased replicative arrest to be associated with HPC expansion during liver regeneration after LDLT. Moreover, GRWR was independently associated with expansion of HPC. This suggests that HPC expansion compensates for reduced liver mass in the fatty grafts in the setting of LDLT.

As HPC can differentiate into hepatocytes [19], they provide a ready source of hepatocytes to replace dying liver cells. On the other hand, evidence for HPC accumulation in fatty livers strongly supports the importance of an alternative mechanism for preserving the functional liver mass, i.e. increased HPC differentiation. Additional experiments that block HPC expansion in fatty livers will be needed to validate this concept. Nevertheless, these results identify HPC as a novel target for therapeutic manipulation in fatty liver disease and emphasize the need for research to characterize the specific signals that promote the accumulation and differentiation of these cells in injured livers.

In summary, hepatic steatosis is associated with an increase in both the number of HPC and the extent of ductular proliferation. The relationship between the increased replicative arrest of hepatocytes and an increase in the number of HPC is consistent with the hypothesis that HPC compensate for impaired hepatocyte replication in steatotic livers during regeneration after LDLT.

Authorship

JYC: wrote the paper. K-SS and KUL: designed research/study. WYS: collected data. HWL and N-JY: performed research/study. MAK and J-JJ: analyzed data

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