**Table 2** Summary of safety during ETV-060 in the lamivudine-refractory long-term treatment cohort

| | *n* (%) ETV-060 Entecavir 1.0 mg *n* = 82 (%) |
|---|---|
| Any adverse event | 82 (100) |
| Clinical adverse events | 78 (95.1) |
| Clinical serious adverse events | 6 (7.3) |
| Grade 3–4 clinical adverse events | 2 (2.4) |
| Most frequent clinical adverse events | |
| Nasopharyngitis | 57 (69.5) |
| Headache | 21 (25.6) |
| Diarrhea | 12 (14.6) |
| Back pain | 8 (9.8) |
| Laboratory adverse events | 77 (93.9) |
| Laboratory serious adverse events | 3 (3.7) |
| Grade 3–4 laboratory adverse events | 15 (18.3) |
| ALT increased | 24 (29.3) |
| ALT flare[a] | 0 |
| Discontinuations due to adverse events | 8 (9.8) |
| Deaths | 0 |

[a] ALT > 2 × baseline and >10 × ULN

Sixteen (16/42) patients in the 1-mg cohort had paired evaluable liver biopsies from three time points: pretreatment (phase II) baseline, week 48, and week 148 total entecavir treatment time (ETV-060, week 96). Of these, 81% (13/16) demonstrated histologic improvement from baseline through week 148. The mean Knodell necroinflammatory score improved from 6.06 at baseline to 1.44 at week 148, and all patients (16/16) exhibited minimal necroinflammation (a Knodell necroinflammatory score of ≤3 points) at week 148 (Fig. 5a). Knodell fibrosis scores improved in 38% (6/16) of patients from baseline through week 148, and the mean Knodell fibrosis score decreased from 2.44 at baseline to 1.94 at week 148 (Fig. 5b). Liver biopsy assessments using the New Inuyama classification system confirmed the results obtained using the Knodell classification system (data not shown).

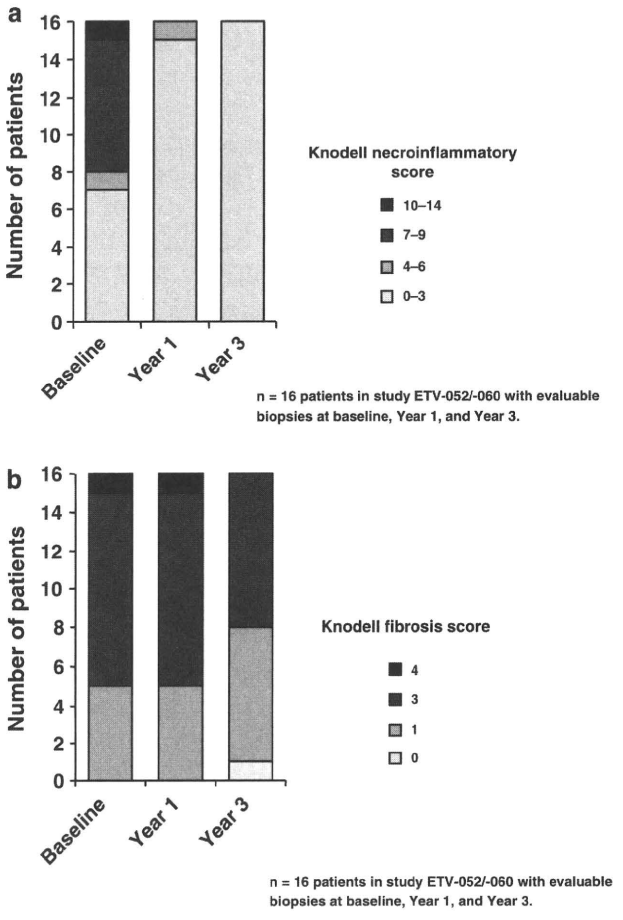


**Fig. 5** Distribution of Knodell necroinflammatory scores (**a**) and Knodell fibrosis scores (**b**) at baseline, year 1 (48 weeks), and year 3 (148 weeks) for the 16 patients who had evaluable liver biopsies at all 3 time points

## Discussion

This report describes the results of 3 years of continuous entecavir therapy in a lamivudine-refractory patient population. All patients in the lamivudine-refractory, long-term treatment cohort had highly elevated levels of HBV DNA with documented lamivudine-resistance mutations at baseline, and 94% were infected with HBV genotype C. This represents a population with potentially poor long-term outcomes. Patients with lamivudine-resistant HBV may have cross-resistance to other antivirals, and genotype C infection is associated with low rates of HBe seroconversion and high rates of liver disease progression [7, 25, 36]. These results show that entecavir therapy for up to 3 years for this population resulted in durable HBV DNA suppression and ALT normalization. More than 50% of patients in the cohort achieved undetectable HBV DNA and almost 90% normalized ALT by year 3. Similar levels of HBV DNA suppression and ALT normalization were observed for the subset of patients who received entecavir 1 mg daily throughout the treatment period. Among patients with liver biopsies from three time points (all of whom received the recommended 1-mg dose of entecavir from phase II baseline), substantial improvements in liver histology were observed: more than 80% of patients demonstrated histologic improvement at year +++3 and slow improvements in fibrosis were observed in 38% of patients.

In previously published results of a multinational clinical trial, entecavir demonstrated potent inhibition of viral

replication in HBeAg-positive, lamivudine-refractory patients infected with a variety of HBV genotypes (A–D) [28, 30]. In that trial, after 48 weeks of treatment with entecavir 1 mg daily, the mean change from baseline in HBV DNA was $-5.11$ $\log_{10}$ copies/mL, and 19% of patients achieved HBV DNA of >300 copies/mL. Among patients who continued to a second year of entecavir therapy, the mean change from baseline in HBV DNA increased to $-5.9$ $\log_{10}$ copies/mL, and 40% of patients achieved HBV DNA of >300 copies/mL. In the current study in Japanese patients, 54% achieved HBV DNA of >400 copies/mL. The higher proportion of Japanese patients suppressing HBV DNA to below the PCR limit of quantification in the current study likely reflects the effect of an additional year of entecavir therapy, as well as the lower baseline HBV DNA (7.69 $\log_{10}$ vs. 9.59 $\log_{10}$ copies/mL in the multinational study). The relatively low rate of HBe seroconversion observed in this study (15%) may be related to infection with genotype C virus. In studies in Japan and elsewhere in Asia, HBV genotype C has been associated with lower seroconversion rates than with other HBV genotypes [7, 36–38].

Achieving and maintaining HBV DNA suppression is a principal goal of CHB therapy [25, 39]. Data from prospective long-term studies have shown that elevated HBV DNA levels are associated with the development of long-term complications including cirrhosis and HCC [12–14]. Other research has correlated durable HBV DNA suppression with improved liver histology among antiviral-treated patients [19, 40]. Liaw et al. [15] showed that lamivudine therapy benefits CHB patients with advanced liver disease by reducing the risk of liver disease progression, including the development of HCC. In the present study, the reduction in hepatic necroinflammation and fibrosis observed in a subset of patients through 3 years, along with the durable virologic suppression observed in the larger cohort, suggests that entecavir helps halt or reverse liver disease progression that can lead to poor long-term outcomes.

The emergence of lamivudine resistance can lead to serious clinical consequences, including elevated levels of HBV DNA, exacerbations of hepatitis, and hepatic decompensation [18, 22, 23, 41]. While early studies of patients with lamivudine-resistant HBV suggested that switching to adefovir was efficacious, subsequent work demonstrated the rapid emergence of adefovir resistance in this patient population [42–44]. The emergence of adefovir resistance in this setting can be associated with viral rebound and hepatic decompensation [45]. Adding adefovir to ongoing lamivudine for patients who have developed lamivudine resistance has been recommended as a strategy to reduce the subsequent emergence of adefovir resistance [25, 46]. This strategy is most efficacious in patients with low HBV DNA levels and requires continued resistance surveillance [47, 48]. Studies evaluating the combination of entecavir with adefovir in lamivudine-resistant patients are currently in progress.

The rate of genotypic resistance to entecavir reported here is consistent with the rate that has been observed in multinational populations of lamivudine-refractory patients [49]. In nucleoside-naive patients, emergence of entecavir resistance is rare because of entecavir's potent viral load reduction and high genetic barrier to resistance [49, 50]. Substitutions at M204 ± L180 were detected at baseline for all patients described in this report and have been shown in previous studies to reduce in vitro susceptibility to entecavir by approximately eightfold [51]. Resistance to entecavir requires the presence of the rtM204V/I lamivudine-resistance substitution plus at least one additional amino acid substitution at rtT184, rtS202, or rtM250. In the current study, for the subset of patients who received entecavir 1 mg daily throughout the treatment period, the cumulative rate of entecavir resistance was 30% through 3 years. This is consistent with the rate observed in the entire lamivudine-refractory, long-term treatment cohort and in multinational studies of lamivudine-refractory patients through 3 years (36%) [49]. Combining entecavir with an antiviral with a different resistance profile, such as tenofovir or adefovir, may result in less frequent resistance emergence.

Entecavir was well tolerated during treatment in study ETV-052, with no discontinuations due to adverse events and three early on-treatment flares that were transient and associated with declining levels of HBV DNA [32]. Throughout the extended treatment period during ETV-060, entecavir continued to be well tolerated with relatively few discontinuations and no ALT flares observed. There were no deaths during the study, and one patient was diagnosed with HCC at week 57 of ETV-060. The extent to which long-term treatment with entecavir may reduce development of HCC in CHB patients remains under investigation.

In summary, these results show that treatment with entecavir for up to 3 years in lamivudine-refractory CHB results in continued benefit beyond the first year, including durable HBV DNA suppression and progressive improvements in liver histology, with a resistance profile consistent with that observed in other studies. Entecavir at the recommended dose of 1 mg daily is an option for patients with lamivudine-refractory CHB. Additional research evaluating the combination of entecavir plus adefovir or tenofovir in this patient population is ongoing.

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Original Article

# Application of a new histological staging and grading system for primary biliary cirrhosis to liver biopsy specimens: Interobserver agreement

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**Recently the authors proposed a new staging and grading system for primary biliary cirrhosis (PBC) that takes into account necroinflammatory activity and histological heterogeneity. Herein is proposed a convenient version of this system. Scores for fibrosis, bile duct loss, and chronic cholestasis were combined for staging: stage 1, total score of 0; stage 2, score 1–3; stage 3, score 4–6; and stage 4, score 7–9. Cholangitis activity (CA) and hepatitis activity (HA) were graded as CA0–3, and HA0–3, respectively. Analysis of interobserver agreement was then conducted. Digital images of 62 needle liver biopsy specimens of PBC were recorded as virtual slides on DVDs that were sent to 28 pathologists, including five located overseas. All participants were able to apply this version in all 62 cases. For staging, kappa was 0.385 (fair agreement) and the concordance rate was 63.9%. For necroinflammatory activity, the kappa and concordance rate were 0.110 (slight agreement) and 36.9% for CA, and 0.197 (slight agreement) and 47% for HA, respectively. In conclusion, this new staging and grading system for PBC seems to be more convenient and practical than those used at present, but more instruction and guidance are recommended for the grading of necroinflammatory activity in practice.**

**Key words:** cholangitis, fibrosis, hepatitis, primary biliary cirrhosis, staging and grading

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Primary biliary cirrhosis (PBC) is an autoimmune liver disease that predominantly affects middle-aged–old women.[1,2] Serologically, anti-mitochondrial antibodies (AMA) are frequently detectable and serum IgM levels are usually elevated in PBC patients.[3] Histologically, intrahepatic small bile ducts (interlobular bile ducts) are selectively affected, presenting characteristic findings such as chronic non-suppurative destructive cholangitis (CNSDC), and the affected bile ducts eventually disappear from the liver and chronic cholestatic features develop gradually.[4–6] At the same time, hepatitis activity (HA) of varying degrees is not infrequently superimposed on the liver. Chronic cholangitis activity (CA) and HA in variable combination may be responsible for progressive hepatocellular damage and fibrosis, and liver cirrhosis and hepatic failure finally develop.[7,8]

For evaluating the progression of PBC, histological staging systems have been proposed by Rubin *et al.*,[9] Scheuer and Lefkowitch,[10] Scheuer, [11] Popper and Schaffner,[12] and Ludwig *et al.*[13] These histological stages reflect the progression of the disease from destruction of the intrahepatic bile ducts or portal inflammation to cirrhosis. Although the classical systems appear simple and seem to be applicable, the staging process itself is subjective. In addition, there are reports that histological changes are heterogeneous in a whole PBC liver, and sampling errors occur in needle liver biopsies of PBC; histological features characterizing different stages can be seen in the same liver biopsy specimen and the staging is not infrequently different in tissue specimens obtained from different parts at the same time.[5,8,14] Furthermore, the grading of necroinflammatory activity to reflect the autoimmune-mediated pathogenesis of PBC is not reflected in these classical staging systems.

Since publication of the latest staging method, that of Ludwig *et al.* in 1978,[13] much progress has been made in clinical areas, particularly in therapeutic fields.[15–18] There are now a number of treatments for PBC such as ursodeoxycholic acid (UDCA) and also combined UDCA and corticosteroid therapy for overlapping syndrome (hepatitic form of PBC).[16,18] The effects of such therapies should be evaluated according to histological aspects, in addition to laboratory and clinical effects.

We recently proposed a new histological staging and grading system of PBC, for the comprehensive analysis of the histological progression of PBC (staging) toward extensive duct loss, chronic cholestasis and cirrhosis, and also of the immune-mediated necroinflammatory activity of small bile duct (chronic cholangitis) and of hepatocytes (interface and lobular hepatitis).[5] The original description, however, is very detailed and practical application seems not easy.[5] Herein we have proposed a practical and convenient version of this new histological staging and grading system.

First, we concisely described the convenient version of our new staging and grading system of PBC. Then, we assessed the system using interobserver agreement among a total of 28 liver pathologists, using 62 needle liver biopsy specimens.

## MATERIALS AND METHODS

### Classification of the intrahepatic biliary tree

The intrahepatic biliary tree is classified into the intrahepatic large and small bile ducts according to their size and distributions in the liver.[6,19] Intrahepatic small bile ducts, which are recognizable on microscopy, run parallel with hepatic arterial branch(es) and portal vein branch within portal tracts. They are classifiable into septal and interlobular bile ducts: the former has its own fibrous wall and its size is >80 μm, while the latter's size is <80 μm. Bile ductules are located at the periphery of portal tracts. In the present study the interlobular bile ducts, which are selectively damaged in PBC, are mainly examined.

### New convenient version for the staging and grading of PBC

#### *Staging*

Three items (fibrosis, bile duct loss and deposition of orcein-positive granules) were used for staging. Fibrosis reflects a progression of chronic liver disease leading to cirrhosis (Fig. 1a) and is used for the histological staging of chronic hepatitis and non-alcoholic steatohepatitis (NASH).[20–22] Bile duct loss is characteristic of PBC and a result of immune-mediated progressive bile damage (Fig. 1b).[4,6,23] Orcein-positive granules are copper-binding proteins in lysosomes and their deposition reflects chronic cholestasis.[5,24] These granules are detectable in the relatively early stages of PBC, and their deposition becomes more severe and extensive with the progression of the disease (Fig. 1c). These three items constitute the basis of this staging system.

The three items are scored as shown in Table 1. For fibrosis (F), a score of 0 means that there is almost no fibrosis or the fibrosis is confined to the portal tracts. A score of 1 means that the fibrosis extends beyond the portal area occasionally with incomplete septal fibrosis; a score of 2, that there is completely connecting septal fibrosis or bridging fibrosis with variable lobular distortion, and a score of 3, cirrhosis (extensive fibrosis with regenerative nodules). For bile duct loss (B), interlobular bile ducts were evaluated in well-formed portal tracts with evident hepatic arterial branches and portal vein branches.[6,19,25] A score of 0 means interlobular bile ducts were discernible in all portal tracts in the specimens. A score of 1 and 2 means that bile duct loss is evident in <1/3 and in 1/3–2/3 of portal tracts, respectively. A score of 3 indicates

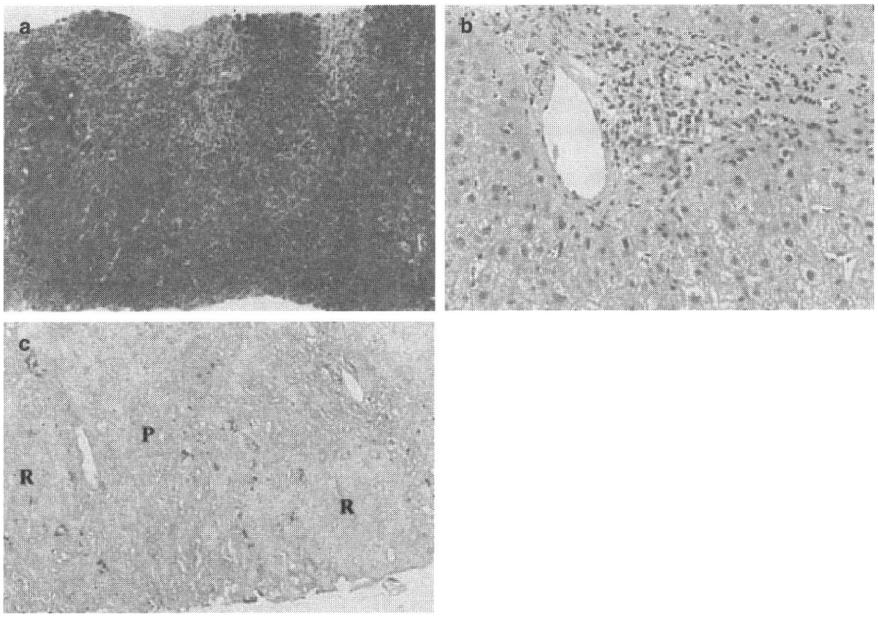


**Figure 1** Three histological items for evaluation of staging of primary biliary cirrhosis. (**a**) Portal tracts are fibrously enlarged with complete fibrous septa formation. Score 2 of fibrosis (Azan-Mallory staining, original magnification ×80. (**b**) Well-formed interlobular bile duct is lost in this portal tract (HE, original magnification ×200). (**c**) Orcein-positive granules are deposited in the periseptal hepatocytes of regenerative nodules (R). Score of 3 for deposition of orcein-positive granules. P, enlarged portal tract. (Orcein stain, original magnification ×200).

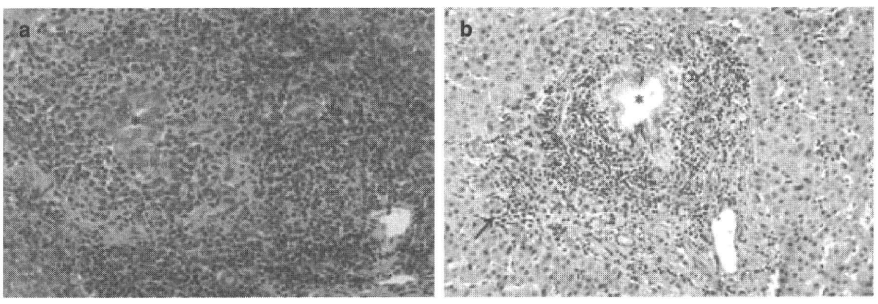


**Figure 2** (**a**) Chronic non-suppurative destructive cholangitis, typical to primary biliary cirrhosis (*) (HE, original magnification ×200). (**b**) Evident chronic cholangitis with mild–moderate periductal lymphocytic infiltration (*). Part of the limiting plate shows interface hepatitis affecting approximately 10 hepatocytes (arrow; HE, original magnification ×200.

that bile ducts were absent in >2/3 of portal tracts. For chronic cholestasis assessed base on the deposition of orcein-positive granules (C), a score of 0 means no deposition in periportal hepatocytes. A score of 1 indicates deposition in <1/3 of periportal hepatocytes of at least one portal tract, and a score of 3, deposition in >2/3 of periportal hepatocytes of all the portal tracts or fibrous septa. Anything between score 1 and score 3 is assigned a score of 2. After each of these items is scored, a total is obtained: a total score of 0 is stage 1 (no or minimum progression), 1–3 is stage 2 (mild progression), 4–6 is stage 3 (moderate progression), and 7–9 is stage 4 (advanced progression) (Table 2; first half ). If orcein staining is not available for the evaluation, the sum of the scores for fibrosis and bile duct loss is also applicable, as shown in Table 2 (second half) .

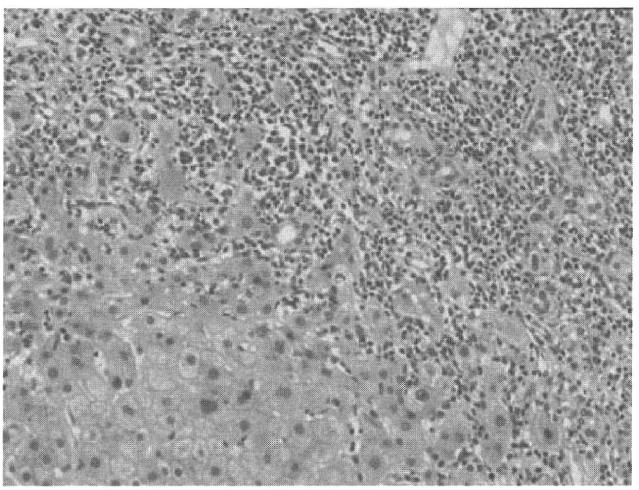

**Figure 3** Interface hepatitis affecting approximately 20 hepatocytes at the interface in primary biliary cirrhosis. Grade 3 hepatitic activity (HE, original magnification ×100).

*Grading of necroinflammatory activity*

Chronic cholangitis including CNSDC and chronic active hepatitis-like change are two essential and representative necroinflammatory and immune-mediated lesions of PBC

**Table 1** Scoring of primary biliary cirrhosis

| | |
|---|---|
| | Scoring of fibrosis |
| Score 0 | No portal fibrosis, or fibrosis limited to portal tracts |
| Score 1 | Portal fibrosis with periportal fibrosis or incomplete septal fibrosis |
| Score 2 | Bridging fibrosis with variable lobular disarray |
| Score 3 | Liver cirrhosis with regenerative nodules and extensive fibrosis |
| | Scoring of bile duct loss |
| Score 0 | No bile duct loss |
| Score 1 | Bile duct loss in <1/3 of portal tracts |
| Score 2 | Bile duct loss in 1/3–2/3 of portal tracts |
| Score 3 | Bile duct loss in >2/3 of portal tracts |
| | Scoring of deposition of orcein-positive granules |
| Score 0 | No deposition of granules |
| Score 1 | Deposition of granules in several periportal hepatocytes in <1/3 of portal tracts |
| Score 2 | Deposition of granules in variable periportal hepatocytes in 1/3–2/3 of portal tracts |
| Score 3 | Deposition of granules in many hepatocytes in >2/3 of portal tracts |

**Table 2** Staging of primary biliary cirrhosis

| Stage | Sum of score: fibrosis, bile duct loss and deposition of orcein-positive granules |
|---|---|
| Stage 1 (no progression) | 0 |
| Stage 2 (mild progression) | 1–3 |
| Stage 3 (moderate progression) | 4–6 |
| Stage 4 (advanced progression) | 7–9 |
| Stage | Sum of score: bile duct loss and fibrosis |
| Stage 1 (no progression) | 0 |
| Stage 2 (mild progression) | 1–2 |
| Stage 3 (moderate progression) | 3–4 |
| Stage 4 (advanced progression) | 5–6 |

(Table 3). In this system, chronic cholangitis including CNSDC typical to PBC (Fig. 2a) and also evident chronic cholangitis with mild periductal lymphoplasmacytic infiltration (Fig. 2b) was categorized into four grades according to the degree and distribution of cholangitis in the liver specimen. In contrast, interface hepatitis and lobular hepatitis were chosen for evaluation of HA (Fig. 2b,3) and their combined activity was categorized into four grades, respectively. Portal inflammation itself was not counted in the evaluation of HA.

*Chronic cholangitis activity.* Grade 0 means absent or ambiguous bile duct damage. Mild biliary epithelial damage can also be found in grade 0. In grades 1–3, evident cholangitis including CNSDC is seen in <1/3, 1/3–2/3, and >2/3 of portal tracts in liver biopsy specimens, respectively (Table 3).

Analysis of interobserver agreement showed that the kappa of CA was low (0.110, slight agreement). This means that more instruction and guidance were recommended, and the grading of CA was revised as follows (Table 4). In grade 3, at least one damaged bile duct showing CNSDC is found in the liver biopsy specimen, irrespective of other types of bile duct damages in the liver specimen. CNSDC shows marked biliary epithelial damaged bile ducts surrounded entirely by marked duct-oriented lymphoplasmacytic infiltration (Fig. 2a). The damaged bile ducts partly or entirely surrounded by epithelioid granuloma (granulomatous cholangitis) are also included. In grade 1, one damaged bile duct showing evident chronic cholangitis is found in the liver biopsy specimen. Evident chronic cholangitis involves damaged bile duct entirely surrounded by mild–moderate, duct-oriented lymphoplasmacytes (Fig. 2b), and this type of cholangitis is also occasionally encountered in chronic viral hepatitis.[26,27] Interlobular bile ducts surrounded by a small number of lymphoplasmacytes or adjacent to lymphoid cell infiltration in the portal tract are not regarded as evident chronic cholangitis. In grade 2, more than two bile ducts showing evident chronic cholangitis are present in the liver specimen, irrespective of other types of bile duct damage.

*Hepatitis activity.* Grade 0 means no interface hepatitis. Grades 1–3 mean the presence of interface hepatitis in <1/3, 1/3–2/3, and >2/3 of portal tracts, respectively. No or minimum lobular hepatitis is found in grade 0, mild–moderate lobular hepatitis may also be found in grade 1 or 2, and moderate lobular hepatitis with occasional zonal necrosis and/or bridging necrosis may also be found in grade 3. The combined activity of interface hepatitis with or without lobular hepatitis is categorized into four grades (Table 2).

Analysis of interobserver agreement showed that the kappa of HA was found to be low (0.197, slight agreement). More instruction and guidance were therefore recommended, and the grading of HA was revised as follows (Table 4).

Grade 0 means no interface hepatitis. Grade 1 and grade 2 mean the presence of interface hepatitis affecting approximately 10 continuous hepatocytes at the interface (Fig. 2a) of one portal tract or fibrous septa, and of more than two portal tracts or fibrous septa in the specimen, respectively. Grade 3 means the presence of interface hepatitis affecting >20 continuous hepatocytes at the limiting plate (Fig. 3) of many portal tracts or fibrous septa in the specimen. Entrapment of hepatocytes in the widened portal tract is also found in grade 3 HA. No or minimum lobular hepatitis is found in grade 0, mild–moderate lobular hepatitis may also be found in grade 1 or 2, and moderate lobular hepatitis in grade 3. Occasional zonal necrosis and/or bridging necrosis is regarded as grade 3.

*Enrollment of observers*

The observers were composed of 28 doctors (KH, MS, AN, TU, KS, FK, TF, KT, MI, KW, MN, HM, HH, MK, HY, JH, SA, TM, HA, AMH, TM, HS, HO, TCC, EU, JHK, YNP, WT). Four of them were pathologists based overseas, while the remainder were Japanese pathologists including internists with a special interest in liver biopsy diagnosis. Two pathologists (YN and YZ) acted as controllers in the present study, and were not enrolled in the panel of observers.

*Case selection and liver specimens*

A total of 62 needle liver biopsy specimens from 62 patients with PBC who fulfilled clinical, serological or histological criteria,[1,2] were evaluated (58 women, four men, aged 45–76 years; mean, 62 years). Patients with known causes of liver disease such as NASH were excluded. None of the PBC patients had serological markers for HCV or HBV. The 62 cases were selected consecutively from the files of Kanazawa University Hospital and Department of Human Pathology, Kanazawa, Japan, covering 1993–2002. These patients were not receiving specific therapy, such as UDCA, corticosteroids or D-penicillamine. More than five portal tracts were identifiable in all of the liver biopsies. All PBC patients were serologically positive for AMA. The specimens were immediately fixed in formalin and embedded in paraffin, and >10 sections 3 μm thick were cut from each block for HE, reticulin and orcein staining. The orcein staining was used to evaluate the deposition of copper-binding proteins in hepatocytes.[24] The availability of histological sections in these cases for detailed histological observations was checked by YZ and YN.

All specimens were scanned to make virtual slides using VASSALO (Claro, Yokohama, Japan). Scanning was performed using a 20× field lens. DVD-ROMs containing all virtual slides were sent to a panel of 28 pathologists with a questionnaire covering the following items. No clinical history or laboratory data were available to the observers.

**Data analysis**

Interobserver agreement was estimated regarding the score of fibrosis, bile duct loss and deposition of copper-binding protein, and that of chronic CA and HA. The former three are regarded to reflect staging and the latter two, necroinflammatory activity.[5] This interobserver agreement was evaluated according to the concordance rate (%) and kappa. Interpretations for kappa have been described previously, that is, <0.00, poor agreement; 0.00–0.20, slight agreement, 0.21–0.40; fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–0.10, almost perfect agreement.[28]

## RESULTS

**Applicability of this version**

All participants were able to apply this version in all 62 needle liver biopsies of PBC.

**Two examples of the staging and grading of PBC**

All of the histopathological items were examined in 62 needle biopsy specimens. The scores of three histological items for staging (fibrosis, bile duct loss and deposition of orcein-positive granules) and scores of grading of cholangitis and hepatitis for 62 cases of PBC evaluated by two pathologists are shown in Table 5. For staging, the majority of the cases examined here were of stage 2, and the majority of fibrosis, bile duct loss and the deposition of copper binding proteins were scored as 0 or 1. For grading, the majority of CA and HA were either grade 1 or 2.

**Interobserver agreement for staging of PBC**

As shown in Table 6, in the assessment of staging as a whole, kappa was 0.385 (fair agreement) and the concordance rate was 63.9%. Among the four stages, kappa was lowest in stage 3. As for the individual items used for the staging, kappa was 0.353 (fair agreement) and the concordance rate was 55.6% for fibrosis. For bile duct loss, kappa was 0.228 (fair agreement) and concordance rate was 44.3%. For deposition of copper-binding protein granules, kappa was 0.409 (moderate agreement) and concordance rate was 67.1%. Among the four scores, kappa was also lowest in score 2 for all three items: 0.090 for bile duct loss, 0.077 for deposition of orcein positive granules, and 0.172 for fibrosis.

**Table 3** Grading of necroinflammatory activities of primary biliary cirrhosis (original)

| | |
|---|---|
| | Cholangitis activitys |
| CA 0 (no activity) | No cholangitis, but mild duct epithelial damage may be present |
| CA 1 (mild activity) | Chronic cholangitis in <1/3 of portal tracts |
| CA 2 (moderate activity) | Chronic cholangitis in 1/3–2/3 of portal tracts |
| CA 3 (marked activity) | Chronic cholangitis in >2/3 of portal tracts |
| | Hepatitis activity |
| HA 0 (no activity) | No interface hepatitis, and no or minimum lobular hepatitis |
| HA 1 (mild activity) | Focal interface hepatitis in a few portal tract(s), and focal necrosis in the parenchyma |
| HA 2 (moderate activity) | Moderate interface hepatitis in several portal tracts, and variable lobular hepatitis |
| HA 3 (marked activity) | Moderate–marked interface hepatitis in many portal tracts, or bridging or zonal necrosis, or both |

CA, cholangitis activity; CNSDC, chronic non-suppurative destructive cholangitis; HA, hepatitis activity.

**Table 4** Grading of necroinflammatory activity of primary biliary cirrhosis (revised after analysis of interobserver agreement)

| | |
|---|---|
| | Cholangitis activity |
| CA 0 (no activity) | No cholangitis, but mild duct epithelial damage may be present |
| CA 1 (mild activity) | One evident chronic cholangitis in the specimen |
| CA 2 (moderate activity) | More than two bile ducts with evident chronic cholangitis |
| CA 3 (marked activity) | At least one CNSDC in the specimen |
| | Hepatitis activity |
| HA 0 (no activity) | No interface hepatitis, and no or minimum lobular hepatitis |
| HA 1 (mild activity) | Interface hepatitis affecting 10 continuous hepatocytes in one portal tract or fibrous septa, and mild–moderate lobular hepatitis |
| HA 2 (moderate activity) | Interface hepatitis affecting 10 continuous hepatocytes in more than two portal tracts or fibrous septa, and mild–moderate lobular hepatitis |
| HA 3 (marked activity) | Interface hepatitis affecting 20 continuous hepatocytes in more than half of the portal tracts, and moderate lobular hepatitis, or bridging or zonal necrosis |

CA, cholangitis activity; CNSDC, chronic non-suppurative destructive cholangitis; HA, hepatitis activity.

**Table 5** Example of staging and grading of 62 needle liver biopsy specimens of PBC by two examiners

| | | | | |
|---|---|---|---|---|
| Examiner A | | | | |
| | Score 0 | Score 1 | Score 2 | Score 3 |
| CA (no. cases) | 10 | 28 | 14 | 10 |
| HA (no. cases) | 9 | 31 | 14 | 8 |
| Staging | Stage 1 | Stage 2 | Stage 3 | Stage 4 |
| No. cases | 7 | 38 | 9 | 8 |
| | Score 0 | Score 1 | Score 2 | Score 3 |
| Bile duct loss (no. cases) | 13 | 23 | 16 | 10 |
| Fibrosis (no. cases) | 26 | 24 | 8 | 4 |
| Deposition of orcein positive granules (no. cases) | 43 | 8 | 3 | 8 |
| Examiner B | | | | |
| | Score 0 | Score 1 | Score 2 | Score 3 |
| CA (no. cases) | 1 | 25 | 25 | 11 |
| HA (no. cases) | 8 | 41 | 11 | 2 |
| Staging | Stage 1 | Stage 2 | Stage 3 | Stage 4 |
| No. cases | 6 | 42 | 6 | 8 |
| | Score 0 | Score 1 | Score 2 | Score 3 |
| Bile duct loss (no. cases) | 11 | 38 | 6 | 7 |
| Fibrosis (no. cases) | 17 | 28 | 11 | 6 |
| Deposition of orcein positive granules (no. cases) | 41 | 12 | 2 | 7 |

CA, cholangitis activity; HA, hepatitis activity; PBC, primary biliary cirrhosis.

### Interobserver agreement for grading of necroinflammatory activity of PBC

As shown in Table 6, in the assessment of CA, kappa was 0.110 (slight agreement) and concordance rate was 36.9%. For the assessment of HA, kappa was 0.197 (slight agreement) and concordance rate was 47.0%. Among the four grades, kappa was lowest in grade 2 in CA and also HA.

## DISCUSSION

In the present study we have proposed a concise version of our new histological staging and grading system for PBC,[5] and this version was intended to apply to needle liver biopsies. We then conducted an analysis of interobserver agreement. The results obtained can be summarized as follows: (i) all participants were able to apply this version in all needle

**Table 6** Interobserver agreement for staging of PBC (kappa)

| Staging | Stage 1 (total score 0) | Stage 2 (1–3) | Stage 3 (4–6) | Stage 4 (7–9) | Total |
|---|---|---|---|---|---|
| | 0.174 | 0.350 | 0.134 | 0.270 | 0.385 |
| Grading (activity) | | | Score | | |
| CA | CA 0 | CA 1 | CA 2 | CA 3 | Total |
| | 0.109 | 0.172 | 0.052 | 0.109 | 0.110 |
| HA | HA 0 | HA 1 | HA 2 | HA 3 | Total |
| | 0.198 | 0.211 | 0.110 | 0.14 | 0.197 |
| Histologic findings used for staging | Score 0 | Score 1 | Score 2 | Score 3 | Total |
| Bile duct loss | 0.225 | 0.202 | 0.090 | 0.189 | 0.228 |
| Deposition of orcein-positive granules | 0.445 | 0.170 | 0.077 | 0.279 | 0.409 |
| Fibrosis | 0.336 | 0.265 | 0.172 | 0.227 | 0.353 |

CA, cholangitis activity; HA, hepatitis activity; PBC, primary biliary cirrhosis.

biopsies of PBC distributed; (ii) Interobserver agreement over the degree of staging was 'fair', and (iii) interobserver agreement for CA and HA was 'slight', less than our expectation.

For the staging of PBC, Scheuer's system is used worldwide.[10,11] In this system, PBC is histologically classified into four stages using characteristic histological features: stage 1 is characterized by florid duct lesions or CNSDC, and in stage 2 there is a characteristic proliferation of bile ductules. Stage 3 is characterized by fibrosis or scarring, and stage 4, by cirrhosis. In our experience, however, these characteristic features occur variably and heterogeneously in the liver during the long course of PBC, and stage 1 and/or stage 2 lesions and stage 3 and/or stage 4 lesions are found in the same liver. In Ludwig's system,[13] the histological features used for the staging of chronic active hepatitis are applied to the staging of PBC: portal hepatitis, periportal interface hepatitis, bridging necrosis or bridging fibrosis, and cirrhosis. Unfortunately, bile ductal lesions and cholestatic changes, which are very important and characteristic features of PBC, are not evaluated at all.

Different from other chronic liver diseases such as chronic hepatitis and NASH,[20–22,29] PBC has at least two features characterizing disease progression: bile duct loss and its consequences and hepatocellular damage and its consequences.[5,8,30–33] These two factors should be included in a staging system for PBC. Accordingly, we adopted three features reflecting the progression of PBC in our system: fibrosis, bile duct loss and the deposition of copper-binding proteins. In PBC, fibrosis reflects continuing hepatocellular damage due to diverse mechanisms such chronic cholestasis and hepatitis-related hepatocellular necrosis, bile duct loss is the result of chronic immune mediated cholangitis, and the deposition of copper binding proteins reflects chronic cholestasis due to bile duct loss.[24] These three lesions are very important pathological features reflecting the progression of PBC.

The degree of fibrosis seems to constitute a basis for the staging of various chronic liver diseases such as chronic hepatitis and NASH.[20–22,29] In PBC, however, there are many reports that histological changes including fibrosis are heterogeneous in a whole liver, and sampling errors have been noted in staging using needle liver biopsies. Use of multiple and heterogeneous histological features for disease progression might prevent or reduce sampling errors in the histological evaluation of PBC. Although the kappa of the new staging version proposed here was 0.385 (fair agreement), it seems plausible that this version combining three histological items to minimize the sampling errors inherent in PBC liver histology is superior to other staging systems reported so far, and this version reflects both the progression of bile duct destruction and the progression of fibrosis to cirrhosis resulting from hepatocellular damage due to chronic CA and HA. By becoming more familiar with this new version of staging, the kappa of staging would become higher, although it was lower than that for deposition of orcein-positive granules (0.409) and was comparable to that for fibrosis (0.353) in the present study.

In the evaluation of chronic progressive liver diseases, the grade of necroinflammatory activity inherent to these diseases should be carefully assessed. The concept of necroinflammatory activity reflecting the autoimmune-mediated pathology of PBC is, however, lacking or insufficient in classical staging systems available.[9–14] In PBC, CA, reflecting bile duct damage, and HA, reflecting hepatocellular damage, are regarded as fundamental immunopathological and necroinflammatory processes. CA was evaluated according to the degree and character of chronic cholangitis, and the degree and extent of two histological features, interface hepatitis and lobular hepatitis, were assessed for the grading (activity) of HA. It was found in this analysis of interobserver agreement that kappa was unexpectedly low in CA (0.110) and also in HA (0.197). This suggests that the assessment process of necroinflammatory activity may be different in different institutions and also among pathologists. We therefore added more instruction and guidance for the grading of necroinflammatory activity of CA and HA, and in Table 2 (revised after analysis of interobserver agreement). Although this revision would be more easily applicable in the assessment of grading

of CA and HA, more practice and experience are mandatory to polish this staging and grading system.

In conclusion, this new staging and grading system of PBC can be applied to routine histological sections of liver biopsies. Although it may be a little burdensome for pathologists, we believe that this method will provide more objective information from liver biopsy specimens of PBC to clinicians, and this grading and staging system could be applicable to the evaluation of therapeutic approaches in PBC.

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ORIGINAL ARTICLE

# Comparison of liver regeneration after a splenectomy and splenic artery ligation in a dimethylnitrosamine-induced cirrhotic rat model

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## Abstract

**Aim:** A splenectomy and splenic artery ligation accelerate liver regeneration and improve liver function after a hepatectomy. However, there are no studies that directly compared the effects of a splenectomy and splenic artery ligation. In the present study, we compared the effects of a splenectomy and splenic artery ligation in cirrhotic rats.

**Methods:** Dimethylnitrosamine (DMN) was administered intraperitoneally for 4 weeks to induce cirrhosis. The rats were divided into three groups: sham operation (CT group), splenic artery ligation (SAL group) and splenectomy (SP group). Liver functions [alanine aminotransferase (ALT) and total bilirubin (T. Bil)], plasma TGF-β1, histopathological changes, extent of liver fibrosis (fibrotic rate) and regeneration [Ki-67 labelling index(LI)] were investigated in each group.

**Results:** ALT and T. Bil levels were significantly lower in the SP group than the CT and SAL groups. TGF-β1 levels were significantly lower in the SP group than in the CT and SAL groups. The fibrotic rate was significantly lower in the SP group than in the CT and SAL groups. The Ki-67 labelling index was significantly higher in the SP group than in the CT and SAL groups.

**Discussion:** A Splenectomy significantly improved liver regeneration with reduction of plasma TGF-β1 levels compared with splenic artery ligation in DMN-treated cirrhotic rats.

## Keywords

liver cirrhosis, liver regeneration, spleen, splenectomy, splenic arterial ligation, TGF-β1

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## Introduction

Functional deterioration of the liver is a common problem in patients with cirrhosis associated with hepatocellular carcinoma (HCC) especially those with a background of viral hepatitis B or C. Liver regeneration in patients with cirrhosis is a promising way to improve the functional status of the liver. Experimental studies have reported that splenomegaly inhibits liver regeneration in liver cirrhosis.[1,2] After a partial hepatectomy in cirrhotic patients with splenomegaly, the remnant liver tends to regenerate to a smaller volume than in patients free of splenomegaly,[3] and splenectomy improves the post-operative function of the hepatectomized cirrhotic liver.[4–6] Experiments in cirrhotic rats have demonstrated better hepatic regeneration in splenectomized rats than the sham-operated counterparts.[1,7]

Previous reports also described improvement of liver function after splenic artery ligation with splenic preservation. In HCC patients with liver cirrhosis, splenic artery ligation reduced the incidence of post-operative hepatopathy.[8] In small-for-size grafts for living-donor adult liver transplantation, graft survival improved after the adoption of the splenic artery ligation technique.[9,10] Previous studies have showed that a splenectomy accelerates liver regeneration compared with sham-operated cirrhotic rats. On the other hand, splenic artery ligation in either liver transplantation or hepatectomy has also been reported to significantly improve post-operative liver function. Although there are no reports indicating improvement of liver regeneration after splenic artery ligation, it is nevertheless presumed that splenic artery ligation could improve liver regeneration.

To our knowledge, there are no reports that directly compared the effects of splenectomy and splenic artery ligation on the liver function and regeneration in patients with cirrhosis. The present experimental study was designed to address this issue using a cirrhotic rat model.

## Materials and methods

### Animals

Sixty-four male Wister rats weighing 250–350 g (Kyudo; Kumamoto, Japan) were used in all experiments. They were housed in wire-topped metal cages in a temperature-controlled room (21 ± 2°C) and constant light-dark cycle (12 h light, 12 h dark). All rats received food and water ad libitum throughout the study and the bedding in the cages was changed once a week. The animals were fasted for 12 h before the operation. All rats were anaesthetized with intraperitoneal injection of Nembutal at 50 mg/kg body weight (Tokyo, Japan). All experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kurume University.

### Study design

Eight rats were used as controls and sacrificed for the collection of tissues and portal blood [before injection of dimethylnitrosamine (DMN) (Wako, Osaka, Japan)]. The other 56 rats were treated with DMN to induce persistent liver fibrosis, which closely resembles human cirrhosis, as confirmed in previous studies.[11,12] DMN (1% dissolved in saline) was injected intraperitoneally at a dose of 1 ml/kg body weight, three times a week (on 3 consecutive days with 4 days off per week) for 4 weeks. After the last DMN injection, eight rats were sacrificed for the collection of tissue and portal blood specimens (before operation, $n = 8$). Ten days after the last DMN injection, surgical exploration of the remaining rats showed granular liver surface, massive splenomegaly, ascites and collateral venous circulation around the spleen. The remaining 48 rats were randomly divided in three groups: the sham operation group (CT group, $n = 16$), the splenic artery ligation group (SAL group, $n = 16$) and the splenectomy group (SP group, $n = 16$). Out of the 48 rats, 24 (8 of each group) were sacrificed during the operation for the collection of specimens. The remaining 24 rats (8 for each group) were sacrificed 30 days after the operation (30 POD). All rats were sacrificed by an overdose of anaesthetic agent. At sacrifice, portal blood was collected for liver function tests and measurement of transforming growth factor-β1 (TGF-β1). The whole liver lobes and body were weighed at the indicated time points. The liver and the spleen were excised for histopathology and immunohistochemistry. Each organ was divided into thin slices and fixed with phosphate-buffered 10% neutral formalin. The fixed sections were used to estimate liver fibrosis and regeneration.

### Operative procedures

For splenectomy, the abdomen was opened through a midline incision. The spleen was mobilized to the centre of the operative field after dissecting the surrounding ligaments. The hilar vessels were ligated with 3-0 silk suture. The spleen was removed and the abdominal incision was closed. For splenic artery ligation, the abdomen was opened and the splenic artery was dissected and carefully isolated from the splenic vein. Then it was ligated about 1.5 cm proximal to the bifurcation of the hilar vessels, at two adjacent locations, with 4-0 silk and the abdomen was closed. The sham operation was performed exactly as for the splenectomy and splenic artery ligation, but the last two procedures were not performed.

### Liver function tests and measurement of TGF-β1

The blood samples collected from the portal vein were immediately centrifuged at 2000× *g* for 10 min at 4°C and stored at –80°C until use for liver function tests. Serum alanine aminotransferase (ALT) and total bilirubin (T. Bil) levels were estimated. The ratios of ALT and T. Bil to liver weight at a given time point were calculated. Data reported for each time point represent the mean ± SD of eight animals.

Plasma TGF-β1 was assayed using the Quantikine human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, sandwich ELISA was set in a 96-well microtitre plate to estimate TGF-β1 levels and a monoclonal antibody was used for the first antibody analysis. The colour intensity was analysed quantitatively by measuring absorbance at 450 nm with a micro-plate reader E-max (Molecular Devices, Sunnyvale, CA, USA). The concentration in the test sample was determined based on a standard curve prepared with samples of known concentrations. The ratio of TGF-β1 to liver weight at a given time point to liver weight was calculated. Data reported for each time point represent the mean ± SD of eight animals.

### Assessment of histopathological changes

The liver specimens were fixed in phosphate-buffered 10% neutral formalin solution, embedded in paraffin, and then were serially cut into thin slices and placed on glass slides. After deparaffinization, the sections were stained with hematoxylin and eosin for histopathological examination. The specimens were examined by a pathologist who was blinded to the animal groups and tissue sampling. Histopathological assessment was performed based on the degeneration of hepatocytes, hepatic necrosis and infiltration of inflammatory cells such as neutrophils, lymphocytes and macrophages. Histopathological changes were graded as follow: mild (slight inflammatory cells infiltration in the tissue), moderate (moderate degeneration of hepatocytes and /or moderate inflammatory cell infiltration) and severe (focal necrosis and /or severe degeneration of hepatocytes with inflammatory cell infiltration). The adjacent category model was applied to the categorical data.

### Assessment of liver fibrosis

The formalin-fixed liver tissue was embedded in paraffin and cut into 4-μm thick sections. Sections were stained with Azan-Mallory stain to estimate the degree of liver fibrosis. The degree of fibrosis was morphometrically defined as the ratio of randomly selected connective tissue to the whole area of the liver (10 fields selected at random from each of 3 rats; a total of 30 fields for each group were examined), using an image analysis technique (NIH image software package) (Scion Corporation, Frederick, MD, USA).[13] Values are presented as mean ± SD.

### Assessment of liver regeneration

Changes in body weight were measured in all groups before DMN injection, before the operation, during the operation and 30 days after the operation. After euthanasia, the liver was dissected out and weighed. The ratio of liver weight at a given time point to body weight was calculated. The liver regeneration rate (%) was calculated using the following equation. Liver regeneration rate (%) = 100 × liver weight (LW)/body weight (BW) at a given time point. Each point represents the mean ± SD of eight animals.

Using immunohistochemistry, Ki-67-stain was to evaluate liver regeneration after the operation. The liver sections were deparaffinized with xylene and immersed in absolute and 95% ethanol for 15 s each. After washing in phosphate-buffered saline (PBS), the sections were incubated in 3% hydrogen peroxide solution for inactivation of endogenous tissue peroxidase, and subsequently in 5% skimmed milk for blocking non-specific immunoreactions. The sections were then autoclaved in citrate buffer (pH 6.0) at 95°C for 35 min. The sections were again incubated with anti-rat secondary antibody (LSAB2 kit; Dako, Tokyo, Japan) diluted 1 : 50 with PBS at room temperature for 30 min. After washing in PBS, the sections were incubated with a streptavidin-biotin staining kit (Histofine SAB-PO kit, Nichirei, Tokyo) at room temperature for 30 min. Immunocomplexes were visualized by 3.3′-diaminobenzidine tetrahydrochloride (Dako) and then the sections were counterstained with hematoxylin. The Ki-67 labelling index (LI) represented the percentage of Ki-67-stained hepatocytes per total number of hepatocytes in randomly chosen sections (under 100×magnification, 10 fields selected at random from each of 3 rats; a total of 30 fields were examined in each group).[14]

### Statistical analysis

All values were expressed as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using the Student's *t*-test. Polytomous logistic regression was performed to compare histological changes. A *P*-value less than 0.05 was considered statistically significant.

## Results

### Effects of a splenectomy and splenic artery ligation on serum ALT and T. Bil levels

The mean serum ALT level at 30 POD was significantly lower in the SP group (7.2 ± 1.3 umol/10 dl/g), but not the SAL group (17.2 ± 1.8), than in the CT group (20.1 ± 2.1, $P < 0.01$). Although the level in the SAL group tended to be lower than in the CT group, the difference was not significant ($P = 0.240$). Serum T. Bil levels in the SP (3.5 ± 2.1 umol/dl/g) and SAL groups (6.7 ± 3.7) were significantly lower than that of the CT group (16.4 ± 4.1) ($P < 0.05$, each) (Fig. 1).

### Effects of a splenectomy and splenic artery ligation on plasma TGF-β1 concentration in the portal vein

The mean plasma TGF-β1 level in the portal vein at 30 POD was significantly lower in the SP group (2.1 ± 1.9 umol/dl/g) than in

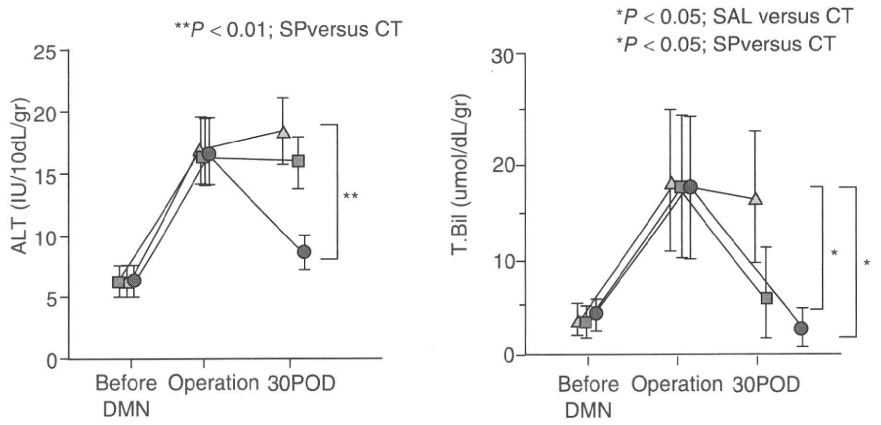


**Figure 1** Serum levels of alanine aminotransferase (ALT) (left), total bilirubin (T. Bil) (right). Sham operation (CT) group (△), splenic artery ligation (SAL) group (□), splenectomy (SP) group (●) at 30 days after the operation (POD). Data are mean ± SD of eight rats. Statistical analysis was performed using Student's *t*-test.

the CT group (8.2 ± 2.6) and SAL group (7.2 ± 1.8) ($P < 0.01$, each) (Fig. 2). Although the level in the SAL group tended to be lower than in the CT group, the difference was not significant ($P = 0.282$).

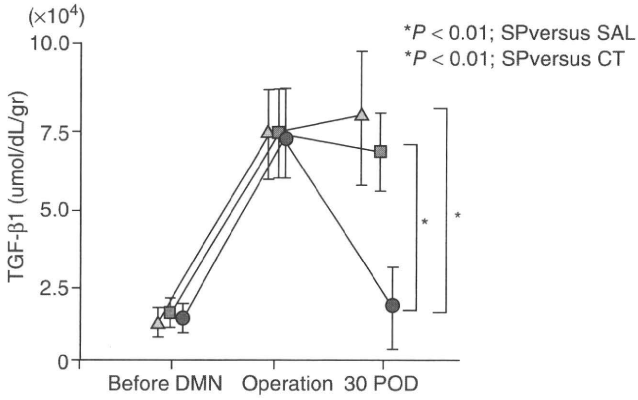


**Figure 2** Plasma concentrations of TGF-β1 in the portal vein. Sham operation (CT) group (△), splenic artery ligation (SAL) group (□), splenectomy (SP) group (●) at 30 days after the operation (POD). Data are mean ± SD of eight rats. Statistical analysis was performed using Student's *t*-test.

## Effects of a splenectomy and splenic artery ligation on histopathology of the liver

At 30 POD, the liver tissue of the CT group showed centrizonal necrosis and severe degeneration of hepatocytes with marked inflammatory cell infiltration (Fig. 3a). On the other hand, moderate inflammatory cell infiltration and degeneration of hepatocytes were found in the SAL group (Fig. 3b). In contrast, the SP group showed only slight inflammatory cell infiltrations (Fig. 3c).

Table 1 summarizes the histopathological findings at 30 days POD. These findings were significantly different between the CT and SP groups ($P < 0.001$), and also between the SP and SAL groups ($P < 0.05$), but marginally different between the CT and SAL groups ($P = 0.075$).

## Effects of splenic artery ligation on histopathology of the spleen

Gross examinations showed severe atrophic changes in the SAL group at 30 POD. Microscopy revealed slight passive congestion in the red pulp (Fig. 4a). None of the sections of the SAL group showed infarction or necrosis (Fig. 4a). On the other hand, no congestion was observed in the spleen of normal rats (Fig. 4b).

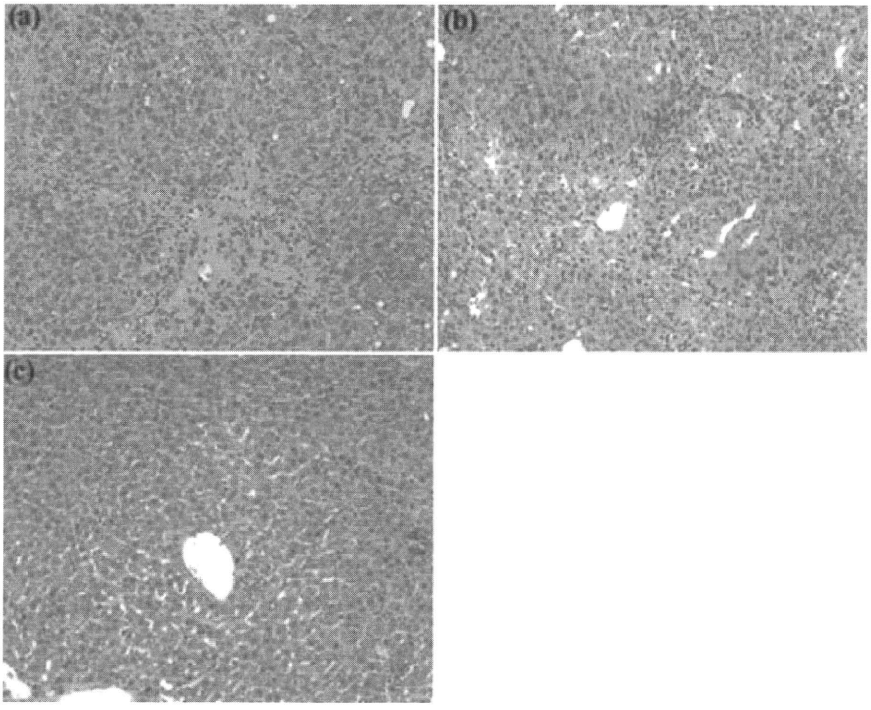


**Figure 3** Histopathology of the liver at 30 days after the operation in the three groups (hematoxylin-eosin staining, original magnification×100). In the sham operation (CT) group, centrizonal necrosis was associated with severe inflammatory cell infiltration, consisting of neutrophils, lymphocytes and haemosiderin-laden macrophages. Degenerative hepatocytes in zone 2 showed marked hydropic swelling (a). In the splenic artery ligation (SAL) group, focal necrosis and degenerative hepatocytes were found in zone 3 with moderate degree of inflammatory cell infiltration (b). In the splenectomy (SP) group, the hepatic cord was straight and the parenchyma showed only mild inflammatory cell infiltration but no focal necrosis (c).

### Effects of a splenectomy and splenic artery ligation on liver fibrosis

Azan-Mallory staining of the liver before the operation and at 30 POD showed severe fibrosis and inflammatory cell infiltration within the fibrous septa in the CT group (Fig. 5a–b). On the other hand, liver fibrosis was less extensive in the SAL group than in the CT group (Fig. 5c). Although bridging fibrosis was seen in the SP group, it was markedly less compared with the SAL group (Fig. 5d).The mean fibrotic rate of the liver before the operation and at 30 POD in the CT, SAL and SP groups were 10.7 ± 3.2%, 10.5 ± 3.7%, 8.6 ± 2.1%, and 3.1 ± 2.2%, respectively. The mean fibrotic rate of the liver was not significantly different between before the operation and CT group at 30 POD ($P = 0.524$), but was significantly lower between the SP group and CT and SAL groups (SP vs. CT $P < 0.001$, SP vs. SAL $P < 0.01$). The mean fibrotic rate was significantly lower in the SAL group than in the CT groups ($P < 0.01$) (Fig. 6).

**Table 1** Histopathological findings at 30 days after the operation

| | Histopathological damage | | |
|---|---|---|---|
| | **Mild** | **Moderate** | **Severe** |
| CT group | – | 2/8 | 6/8 |
| SAL group | 2/8 | 4/8 | 2/8 |
| SP group | 7/8 | 1/8 | – |

The sham operation (CT) group and the splenectomy (SP) group are significantly different (Exact: $P = 0.00011$, Asymptotic: $P = 0.003$), the CT group and the splenic artery ligation (SAL) group tended to be different (Exact: $P = 0.053$, Asymptotic: $P = 0.075$), and the SP group and the SAL group were significantly different (Exact: $P = 0.036$, Asymptotic: $P = 0.029$). Histopathological changes were graded as follow: mild (slight inflammatory cell infiltration), moderate (moderate degeneration of hepatocytes and /or moderate inflammatory cell infiltration) and severe (focal necrosis and /or severe degeneration of hepatocytes with inflammatory cell infiltration). The adjacent category model was applied to the categorical data ($n = 8$).

### Effects of a splenectomy and splenic artery ligation on liver regeneration and Ki-67 LI

The mean liver regeneration rates before DMN injection, before the operation and 30 POD in the CT, SAL and SP groups were 1.4 ± 0.1%, 2.7 ± 0.1%, 2.6 ± 0.1%, 2.7 ± 0.2% and 4.6 ± 0.2%, respectively. The liver regeneration rate was significantly higher in the SP group than in the CT and SAL groups at 30 POD (SP vs. CT $P < 0.01$, SP vs. SAL $P < 0.01$), but not between the CT and SAL groups at 30 POD ($P = 0.523$) (Fig. 7).

Ki-67 positive hepatocytes were rarely seen before the operation (data not shown)in the CT and SAL groups (Fig. 8a–b). Immunohistochemistry revealed enhanced liver regeneration in the 30 POD SP group as evidenced by the large number of Ki-67-positive hepatocytes (Fig. 8c). The mean Ki-67 LI before the operation and 30 POD in the CT, SAL and SP groups were 6.1 ± 2.3%, 6.3 ± 2.5%, 5.1 ± 1.6% and 10.9 ± 4.1%, respectively.

The Ki-67 LI was not significantly different between before the operation and 30 POD in the CT group, but was significantly higher between the SP group and CT and SAL groups (SP vs. CT- $P < 0.01$, SP vs. SAL- $P < 0.01$). The Ki-67 LI was not significantly different between the SAL and CT groups ($P = 0.265$) (Fig. 9).

## Discussion

Cirrhosis can be experimentally induced in animal models by the administration of $CCl_4$ and thioacetamide (TAA). However, the reported reproducibility of $CCl_4$-induced cirrhosis is low and associated with high mortality.[13] Furthermore, induction of cirrhosis by TAA injections requires repeated injections over a long period of time and the outcome is associated with hypersplenism.[1] In our study, injection of rats with 1% DMN over a 4-week period resulted in chronic liver fibrosis with pathological changes closely resembling those of human liver cirrhosis.[11,12] No significant improvement of fibrosis was observed in the sham-operated rats at 40 days after completion of DMN administration, indicating that the cirrhotic changes in this model are irreversible even after cessation of DMN injections.

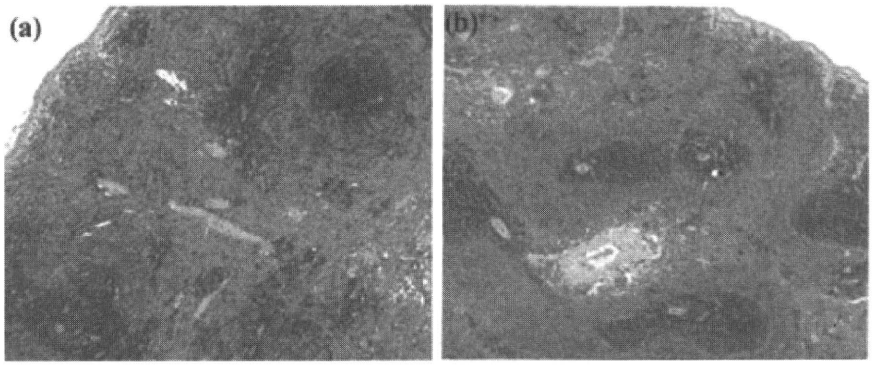


**Figure 4** Histopathology of the spleen after splenic artery ligation (hematoxylin-eosin staining, original magnification ×40). At 30 days after the operation, rats of the splenic artery ligation (SAL) group showed mild congestion in the red pulp (a) compared with before dimethylnitrosamine (DMN) injection (b). No splenic infarction or necrosis was observed in the SAL group (a).

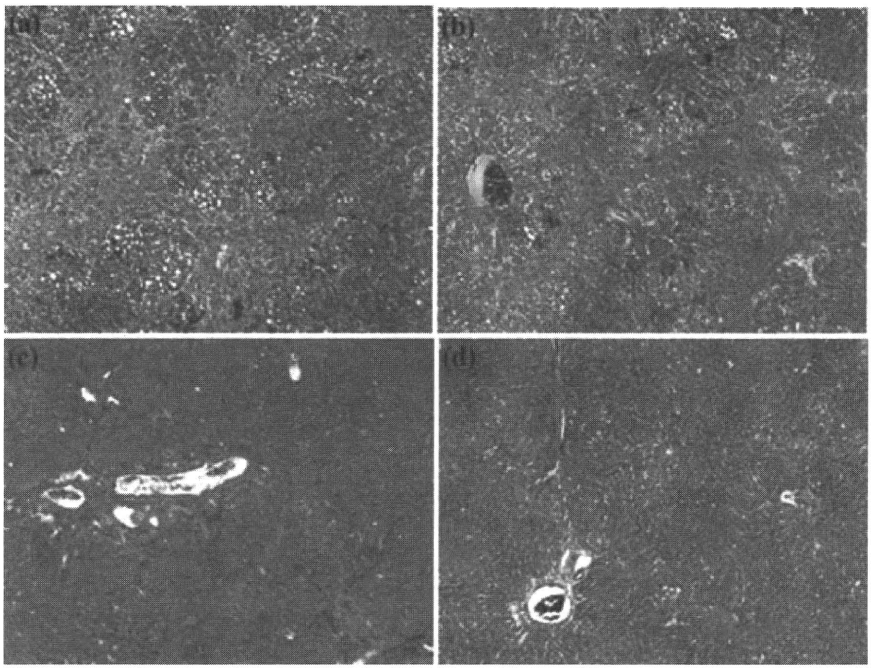

**Figure 5** Histopathology of the liver after a splenectomy and splenic artery ligation (Azan-Mallory staining, original magnification ×40). Severe fibrosis was noted in rats before the operation (a) and at 30 days after the operation in the sham operation (CT) group (b), with no significant difference between the two groups. The fibrosis was less extensive in the splenic artery ligation (SAL) group compared with the CT group at 30 days after the operation (c). Although bridging fibrosis was noted in the splenectomy (SP) group, the fibrosis was markedly lower than in the SAL group (d). Ten fields selected at random from each of three rats; a total of 30 fields were examined in each group.

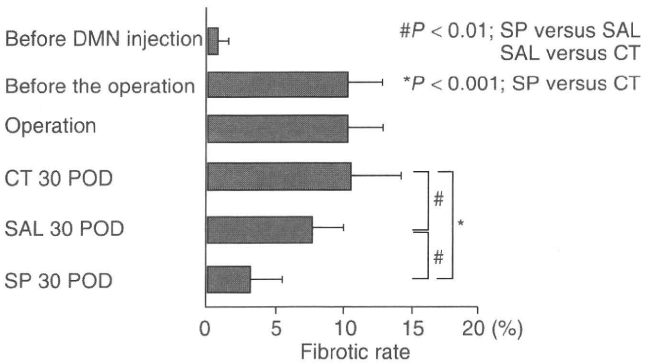


**Figure 6** Assessment of liver fibrosis at 30 days after the operation (POD). The severity of fibrosis was defined morphometrically as the ratio of connective tissue to the whole area of the liver (Ten fields selected at random from each of 3 rats; a total of 30 fields were examined in each group), using image analysis techniques. Values are mean ± SD. For abbreviations, see Fig. 1.

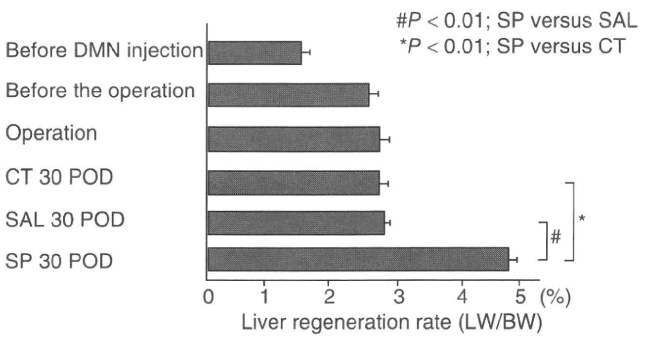


**Figure 7** Assessment of liver regeneration at day 30 after the operation (POD). Bars represent mean ± SD data of eight animals per group. Statistical analysis using Student's *t*-test.

Several experimental studies have shown that the spleen plays an inhibitory role in hepatic liver regeneration.[1,2] There is evidence that humoral factors produced by the splenic tissue are carried to the liver through the portal circulation where they inhibit liver regeneration.[15,16] For example, spleen-derived TGF-β1 has been reported to play a central role in inhibiting the growth of hepatocytes in animals.[1,17,18] TGF-β1 is reported to inhibit liver regeneration by facilitating tissue fibrosis in the liver.[14,19] It also acts directly on hepatocytes by inhibiting cell proliferation and inducing apoptosis.[20,21] Any injury to the liver induces transformation of hepatic stellate cells (HSC) to myofibroblast-like cells (activated HSC), which produce extracellular matrix (ECM) proteins.[14] In this process, the production and accumulation of ECM proteins are regulated by TGF-β1.[19,22] Higher concentrations of TGF-β1 induces ECM production with resultant liver fibrosis, whereas suppression of TGF-β1 induces ECM degradation which

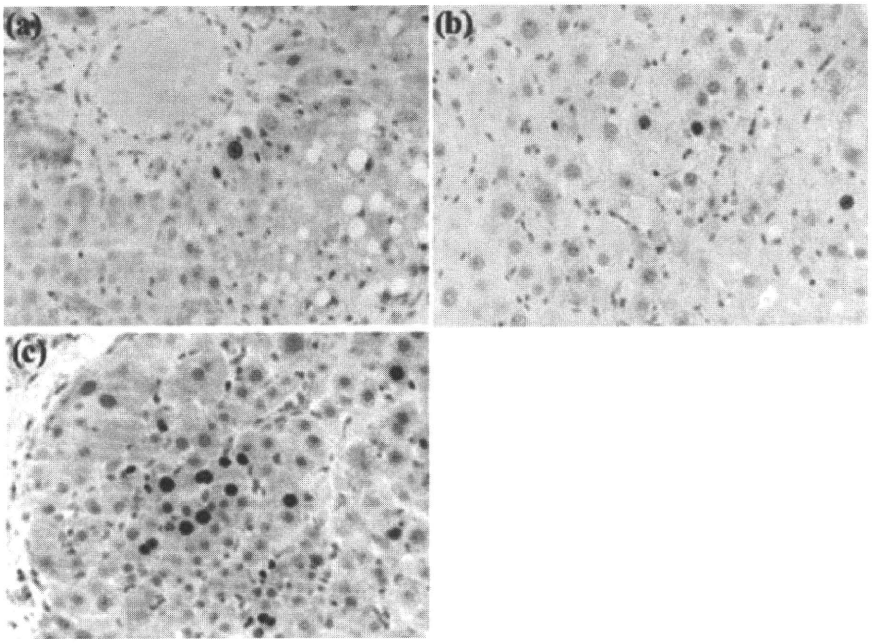


**Figure 8** Immunohistochemical analysis of hepatocyte proliferation in dimethylnitrosamine (DMN)-treated cirrhotic rat liver (original magnification ×200). Note the more abundant Ki-67-positive hepatocytes in the splenectomy (SP) group (c) than the splenic artery ligation (SAL) group (b) and the sham operation (CT) group (a). Ten fields selected at random from each of three rats; total of 30 fields were examined in each group.

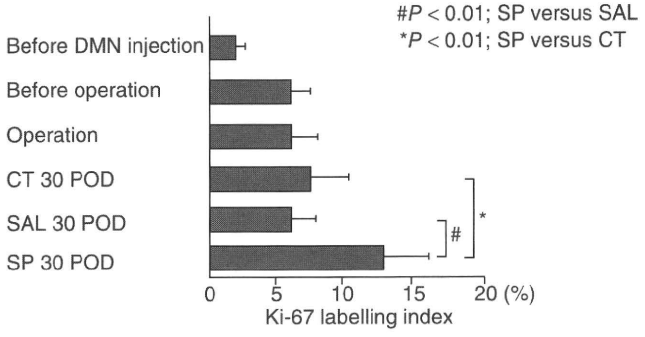


**Figure 9** Effects of a splenectomy and splenic artery ligation on liver regeneration at 30 days after the operation (POD) using the Ki-67 labelling index (LI). The Ki-67 LI (proportion of hepatocytes with nuclei positively stained for Ki-67) was expressed as a percentage for all hepatocytes (morphologically determined) in randomly chosen sections (Ten fields selected at random from each of 3 rats; total of 30 fields were examined in each group.). Values are mean ± SD.

further results in decreased liver fibrosis and improved liver regeneration.[14,23] The level of spleen-derived TGF-β1 is considered a determinant factor of ECM production or degradation.[1] On the other hand, activated HSC themselves produce TGF-β1, which exerts an autocrine effect on hepatocytes inducing growth inhibition.[24–26]

In the present study, histopathological examination of the liver in the splenectomy group showed slight hepatic fibrosis with a significant decrease in plasma TGF-β1 and a significant increase in the Ki-67 LI, compared with the sham operation group. These results support the previously described notion that splenic TGF-β1 plays an important role in the facilitation of liver fibrosis as well as inhibiting the regeneration of the damaged liver. Akahoshi *et al.* postulated that the humoral factor, which originates from the spleen of cirrhotic rat, is TGF-β1 and it is released into the portal circulation.[1] Ueda *et al.*[18] showed strong TGF-β1 expression in the macrophages of the spleen in a liver-injury rat model, and concluded that TGF-β1 was produced and secreted by the spleen and removal of the spleen enhanced proliferation of hepatocytes. To the best of our knowledge, this is the first report describing changes in the TGF-β1 level in the portal vein after splenic artery ligation in the cirrhotic rat model. Indeed, there are no reports that directly compared the effects of splenectomy and splenic artery ligation on liver regeneration in a rat model of liver cirrhosis.

Our study showed significantly low plasma TGF-β1 levels in the portal vein at 30 days after the splenectomy than in the splenic artery ligation group. While a large number of Ki-67-positive hepatocytes were observed after the splenectomy, they were seldom seen after splenic artery ligation at 30 POD. These results suggest that splenic artery ligation neither reduces plasma TGF-β1 levels nor influences liver regeneration. Indeed, the histopathology of the spleen at 30 days after splenic artery ligation showed only mild congestion, and infarction or necrosis were not observed. Previous studies also reported the presence of

functional splenic tissue after splenic artery ligation in the methyl-cellulose-induced hypersplenism rat model[27] and recovery of splenic blood flow even after ligation of the splenic hilar artery because of the collateral venous circulation.[28] Considered together, the above studies and the results of the present experiments imply that splenic artery ligation does not completely abolish splenic viability, which may allow continued production of TGF-β1 in the spleen and its resultant release into the portal vein.

The fibrotic rate in the SP group was significantly lower than in the SAL group. While the SAL group improved liver fibrosis in comparison with the sham operation rats, the extent of improvement was not equal to that observed after a splenectomy, probably because the plasma TGF-β1 level in the SAL group was not significantly lower than that of the CT group. This result suggests that splenic artery ligation with preservation of some splenic tissue did not relate to the reduction of fibrosis. In hepatic inflammation, the severity of the inflammatory process correlates with the extent fibrogenesis.[29] Our findings may explain the significantly small area of fibrosis in the liver of rats of the SAL group that showed no reduction in plasma concentrations of TGF-β1 compared with the CT group.

Both ALT and T. Bil significantly improved in the splenectomized animals. Reduced fibrosis in the Disse's space improves ALT and T. Bil,[14,22] as severe fibrosis in the Disse's space may block the exchange of molecules between the sinusoidal spaces and hepatocytes.[19] In our study, splenic artery ligation tended to improve liver function, similar to a splenectomy. Splenic artery ligation also reduced the severity of histopathological liver damage, such as degenerative hepatocytes, moderate inflammatory cell infiltration and hepatic necrosis. It also decreased the fibrotic rate of the live compared with the CT group. Our findings suggest that the reduction in the extent of liver damage and liver fibrosis was probably the underlying mechanism of splenic artery ligation-induced improvement of liver function. The present study also showed that compared with splenic artery ligation, a splenectomy resulted in better improvement of liver damage, marked reduction of inflammatory cell infiltration and extent of fibrosis. These positive effects of a splenectomy may be related to the improvement of liver function. It is noteworthy that other spleen-derived factors also impair liver regeneration in cirrhotic liver, such as HGF activator-inhibitor (HAI)[30] and endotheline-1 (ET-1).[31] Further experimental and clinical studies are needed to elucidate the importance of TGF-β1 expression.

In the present study, a splenectomy resulted in significant improvement of liver regeneration and lessening of liver fibrosis compared with splenic artery ligation. The most important difference between splenic artery ligation and a splenectomy is considered to be the reduction in the TGF-β1 level, which inhibits hepatocyte regeneration and promotes fibrosis. Our results highlight the benefits of a splenectomy in improvement of liver regeneration in patients with cirrhosis. Prospective clinical studies are required to validate these findings.

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**Conflicts of interest**

None declared.

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