

## 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- $\beta$ 1, 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- $\beta$ 1 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- $\beta$ 1 levels compared with splenic artery ligation in DMN-treated cirrhotic rats.

## Keywords

liver cirrhosis, liver regeneration, spleen, splenectomy, splenic arterial ligation, TGF- $\beta$ 1

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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.<sup>1,2</sup> 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,<sup>3</sup> and splenectomy improves the post-operative function of the hepatectomized cirrhotic liver.<sup>4-6</sup> Experiments in cirrhotic rats have demonstrated better hepatic regeneration in splenectomized rats than the sham-operated counterparts.<sup>1,7</sup>

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.<sup>8</sup> In small-for-size grafts for living-donor adult liver transplantation, graft survival improved after the adoption of the splenic artery ligation technique.<sup>9,10</sup> 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 ( $21 \pm 2^\circ\text{C}$ ) 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.<sup>11,12</sup> 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- $\beta 1$  (TGF- $\beta 1$ ). 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- $\beta 1$

The blood samples collected from the portal vein were immediately centrifuged at  $2000 \times g$  for 10 min at  $4^\circ\text{C}$  and stored at  $-80^\circ\text{C}$  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  $\pm$  SD of eight animals.

Plasma TGF- $\beta 1$  was assayed using the Quantikine human TGF- $\beta 1$  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- $\beta 1$  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- $\beta 1$  to liver weight at a given time point to liver weight was calculated. Data reported for each time point represent the mean  $\pm$  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- $\mu$ m 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).<sup>13</sup> Values are presented as mean  $\pm$  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 (%) =  $100 \times \text{liver weight (LW)}/\text{body weight (BW)}$  at a given time point. Each point represents the mean  $\pm$  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 labeling index (LI) represented the percentage of Ki-67-stained hepatocytes per total number of hepatocytes in randomly chosen sections (under 100 $\times$ magnification, 10 fields selected at random from each of 3 rats; a total of 30 fields were examined in each group).<sup>14</sup>

### Statistical analysis

All values were expressed as the mean  $\pm$  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.

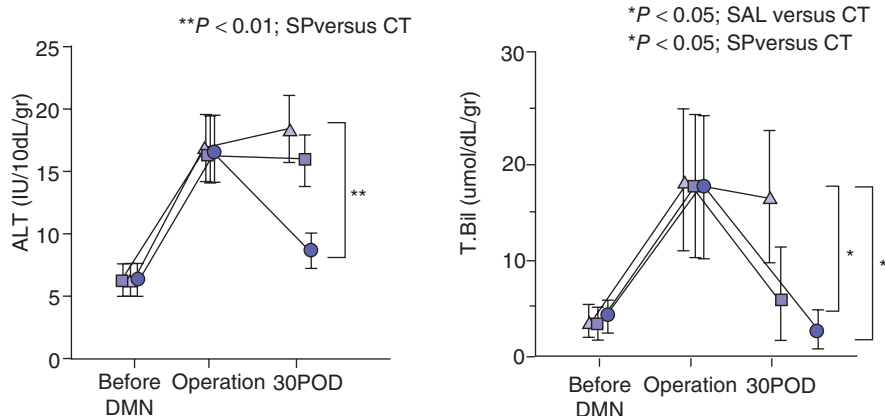
## Results

### 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 ( $7.2 \pm 1.3$   $\mu$ mol/10 dl/g), but not the SAL group ( $17.2 \pm 1.8$ ), than in the CT group ( $20.1 \pm 2.1$ ,  $P < 0.01$ ). 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 ( $3.5 \pm 2.1$   $\mu$ mol/dl/g) and SAL groups ( $6.7 \pm 3.7$ ) were significantly lower than that of the CT group ( $16.4 \pm 4.1$ ) ( $P < 0.05$ , each) (Fig. 1).

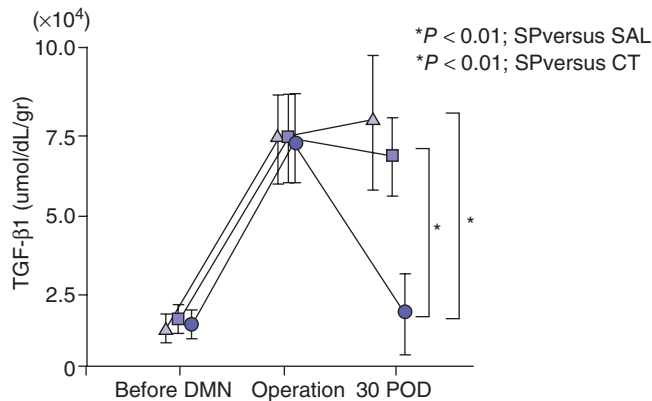
### Effects of a splenectomy and splenic artery ligation on plasma TGF- $\beta$ 1 concentration in the portal vein

The mean plasma TGF- $\beta$ 1 level in the portal vein at 30 POD was significantly lower in the SP group ( $2.1 \pm 1.9$   $\mu$ mol/dl/g) than in



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

the CT group ( $8.2 \pm 2.6$ ) and SAL group ( $7.2 \pm 1.8$ ) ( $P < 0.01$ , 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- $\beta$ 1 in the portal vein. Sham operation (CT) group ( $\Delta$ ), splenic artery ligation (SAL) group ( $\square$ ), splenectomy (SP) group ( $\bullet$ ) at 30 days after the operation (POD). Data are mean  $\pm$  SD of eight rats. Statistical analysis was performed using Student's *t*-test.

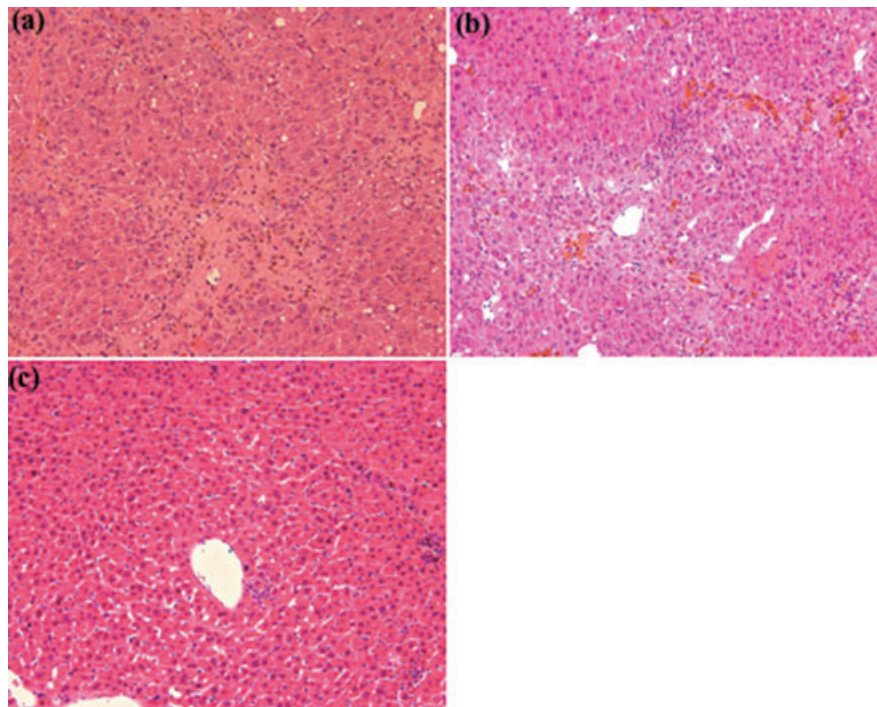
### Effects of a splenectomy and splenic artery ligation on histopathology of the liver

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  $\times 100$ ). 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  $10.7 \pm 3.2\%$ ,  $10.5 \pm 3.7\%$ ,  $8.6 \pm 2.1\%$ , and  $3.1 \pm 2.2\%$ , 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

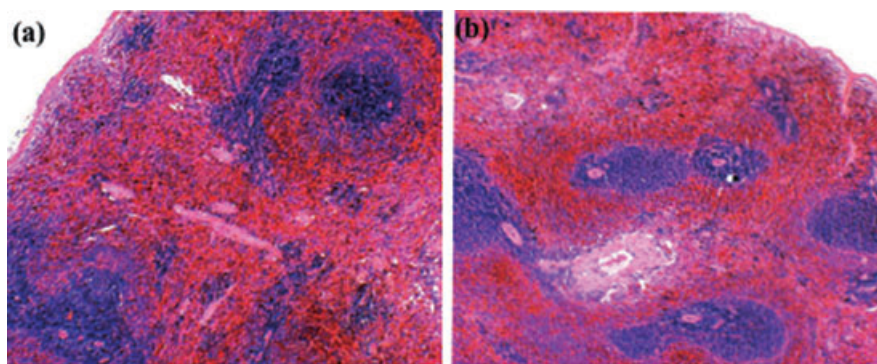
The mean liver regeneration rates before DMN injection, before the operation and 30 POD in the CT, SAL and SP groups were  $1.4 \pm 0.1\%$ ,  $2.7 \pm 0.1\%$ ,  $2.6 \pm 0.1\%$ ,  $2.7 \pm 0.2\%$  and  $4.6 \pm 0.2\%$ , respectively. The liver regeneration rate was significantly higher in the SP group 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  $6.1 \pm 2.3\%$ ,  $6.3 \pm 2.5\%$ ,  $5.1 \pm 1.6\%$  and  $10.9 \pm 4.1\%$ , respectively.

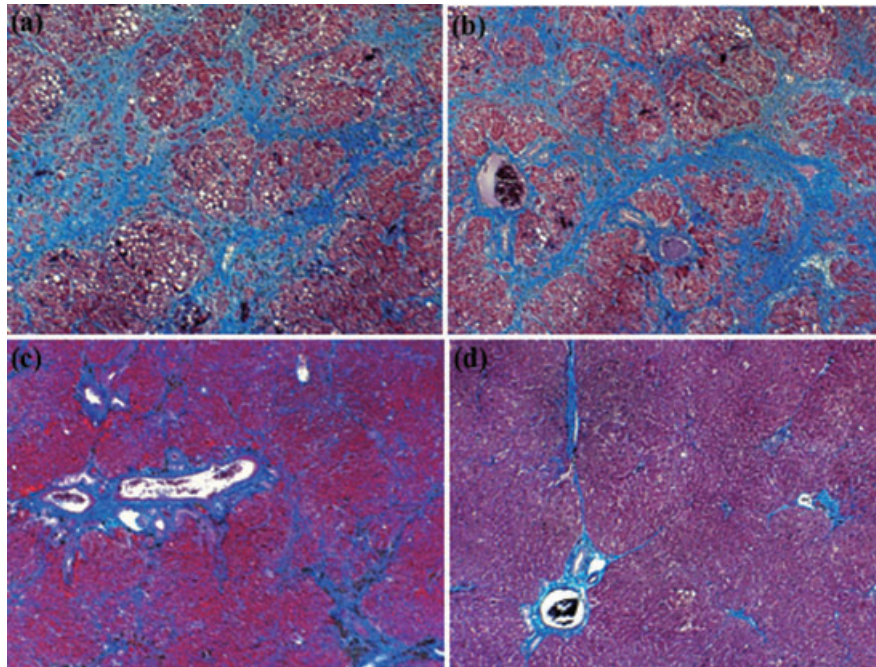
The Ki-67 LI was not significantly different between before the operation and 30 POD in the CT group, but was significantly higher between the SP group and CT and SAL groups (SP vs. CT-  $P < 0.01$ , SP vs. SAL-  $P < 0.01$ ). The Ki-67 LI was not significantly different between the SAL and CT groups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

## Discussion

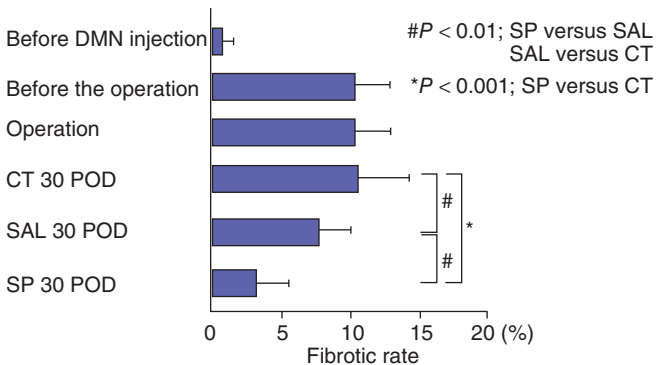
Cirrhosis can be experimentally induced in animal models by the administration of  $\text{CCl}_4$  and thioacetamide (TAA). However, the reported reproducibility of  $\text{CCl}_4$ -induced cirrhosis is low and associated with high mortality.<sup>13</sup> Furthermore, induction of cirrhosis by TAA injections requires repeated injections over a long period of time and the outcome is associated with hypersplenism.<sup>1</sup> 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.<sup>11,12</sup> 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  $\times 40$ ). 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).

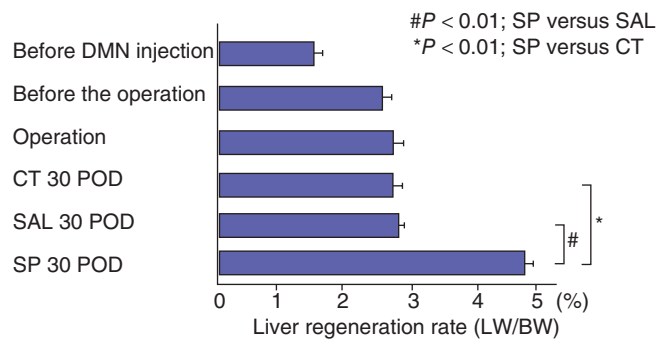


**Figure 5** Histopathology of the liver after a splenectomy and splenic artery ligation (Azan-Mallory staining, original magnification  $\times 40$ ). Severe fibrosis was noted in rats before the operation (a) and at 30 days after the operation in the sham operation (CT) group (b), with no significant 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.



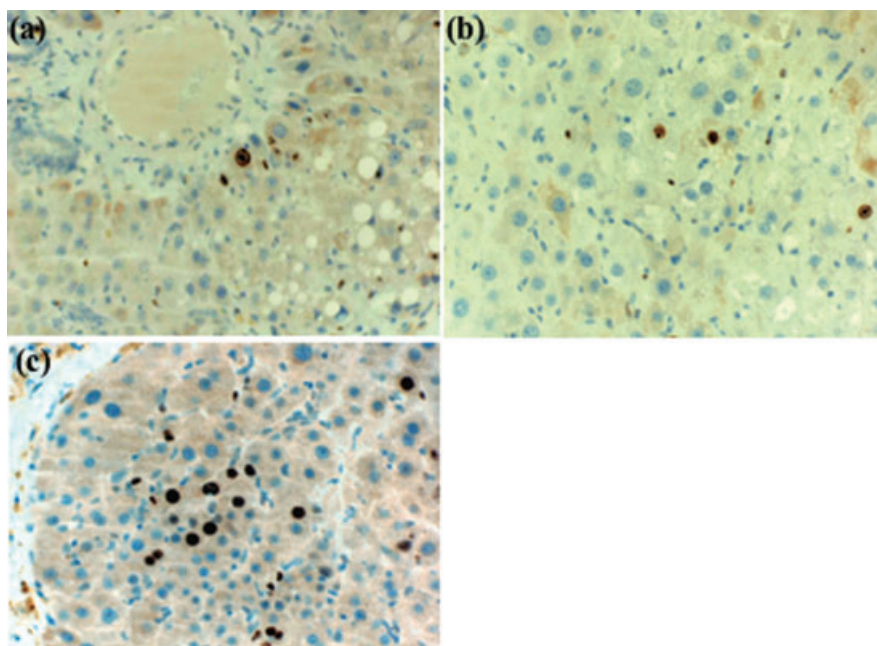
**Figure 6** Assessment of liver fibrosis at 30 days after the operation (POD). The severity of fibrosis was defined morphometrically as the ratio of connective tissue to the whole area of the liver (Ten fields selected at random from each of 3 rats; a total of 30 fields were examined in each group),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

Several experimental studies have shown that the spleen plays an inhibitory role in hepatic liver regeneration.<sup>1,2</sup> 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.<sup>15,16</sup> For example, spleen-derived TGF- $\beta 1$  has been reported to play a central role in inhibiting the growth of

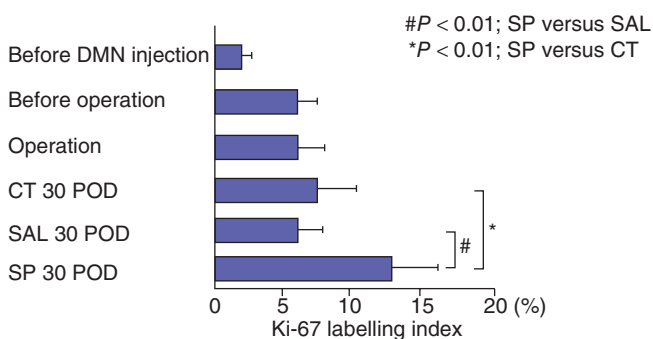


**Figure 7** Assessment of liver regeneration at day 30 after the operation (POD). Bars represent mean  $\pm$  SD data of eight animals per group. Statistical analysis using Student's *t*-test.

hepatocytes in animals.<sup>1,17,18</sup> TGF- $\beta 1$  is reported to inhibit liver regeneration by facilitating tissue fibrosis in the liver.<sup>14,19</sup> It also acts directly on hepatocytes by inhibiting cell proliferation and inducing apoptosis.<sup>20,21</sup> Any injury to the liver induces transformation of hepatic stellate cells (HSC) to myofibroblast-like cells (activated HSC), which produce extracellular matrix (ECM) proteins.<sup>14</sup> In this process, the production and accumulation of ECM proteins are regulated by TGF- $\beta 1$ .<sup>19,22</sup> Higher concentrations of TGF- $\beta 1$  induces ECM production with resultant liver fibrosis, whereas suppression of TGF- $\beta 1$  induces ECM degradation which



**Figure 8** Immunohistochemical analysis of hepatocyte proliferation in dimethylnitrosamine (DMN)-treated cirrhotic rat liver (original magnification  $\times 200$ ). 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  $\pm$  SD.

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

In the present study, histopathological examination of the liver in the splenectomy group showed slight hepatic fibrosis with a

significant decrease in plasma TGF- $\beta$ 1 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- $\beta$ 1 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- $\beta$ 1 and it is released into the portal circulation.<sup>1</sup> Ueda *et al.*<sup>18</sup> showed strong TGF- $\beta$ 1 expression in the macrophages of the spleen in a liver-injury rat model, and concluded that TGF- $\beta$ 1 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- $\beta$ 1 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- $\beta$ 1 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- $\beta$ 1 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<sup>27</sup> and recovery of splenic blood flow even after ligation of the splenic hilar artery because of the collateral venous circulation.<sup>28</sup> 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- $\beta$ 1 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- $\beta$ 1 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.<sup>29</sup> 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- $\beta$ 1 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,<sup>14,22</sup> as severe fibrosis in the Disse's space may block the exchange of molecules between the sinusoidal spaces and hepatocytes.<sup>19</sup> 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)<sup>30</sup> and endothelin-1 (ET-1).<sup>31</sup> Further experimental and clinical studies are needed to elucidate the importance of TGF- $\beta$ 1 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- $\beta$ 1 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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