Original article

Liver regeneration following experimental major hepatectomy with choledochojejunostomy

T. Takagi, Y. Yokoyama, T. Kokuryo, J. Yamaguchi and M. Nagino

Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, Japan 466-8550

Correspondence to: Dr Y. Yokoyama (e-mail: yyoko@med.nagoya-u.ac.jp)

Background: **Surgical treatment for perihilar cholangiocarcinoma frequently involves hepatectomy and extrahepatic bile duct resection with a choledochojejunostomy (CJ). Cholangitis owing to bilioenteric anastomosis is a common complication. The impact of CJ or regurgitating cholangitis on the liver regeneration process after major hepatectomy is unknown.**

Methods: **Rats underwent 70 per cent hepatectomy (Hx group) or hepatectomy with CJ (Hx + CJ group). The intrahepatic inflammatory response, hepatic regeneration rate, and expression of regeneration-associated genes in the liver and blood were compared between these two groups.**

Results: **Levels of hepatobiliary markers in the blood were significantly higher 4 and 7 days after operation in the Hx + CJ group than the Hx group. Intrahepatic expression of inflammation-associated genes, such as interleukin 6 and tumour necrosis factor α, was also significantly higher in the Hx + CJ group on days 4 and 7. A progressive periportal inflammatory response was identified in the Hx + CJ group by histological examination. The hepatic regeneration rate was significantly lower in the Hx + CJ group than in the Hx group on day 2 (mean(s.d.) 14·2(6·3) *versus* 21·4(2·6) per cent; $P = 0·013$) and day 4 (32·4(5·3) *versus* 41·3(4·4) per cent; $P = 0·004$). Gene expression levels of hepatic regeneration-promoting factors such as hepatocyte growth factor were significantly lower in the Hx + CJ group than the Hx group on day 1.**

Conclusion: **CJ perturbs early liver regeneration after hepatectomy. An excessive inflammatory response in the liver and suppression of liver regeneration-associated factors may play a role.**

Surgical relevance

Patients with perihilar cholangiocarcinoma may need major hepatectomy with extrahepatic bile duct resection and choledochojejunostomy. This carries a substantial risk of postoperative complications including liver failure.

A rat model of partial hepatectomy with choledochojejunostomy was established. The molecular mechanisms underlying liver regeneration, and perturbation of this process by duodenobiliary reflux via the choledochojejunostomy, are described.

The results give insight into the pathophysiological events following major hepatectomy with extrahepatic bile duct resection and choledochojejunostomy. This may help to develop a treatment strategy to reduce postoperative liver failure.

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Introduction

Patients with perihilar cholangiocarcinoma frequently need major surgery including hepatectomy and extrahepatic bile duct resection. The continuity of the biliary tract is often restored by a choledochojejunostomy (CJ). The rate of postoperative complications after this procedure, including liver failure, is high[1]. In one study[2] approximately 70 per cent of patients who underwent hepatobiliary resection with CJ had a positive bile culture. Hence cholangitis resulting from reflux of intestinal contents across the CJ is common, occurring in approximately 10 per cent of patients[3].

One of the major triggers of postoperative liver failure is an insufficient remnant liver volume owing to inadequate liver regeneration following major hepatectomy. The maximum postoperative serum total bilirubin levels, morbidity rate and postoperative hospital stay following

major hepatectomy with extrahepatic bile duct resection are greater in patients with cholangitis than those without[4]. In addition, the presence of segmental cholangitis has been shown to suppress the liver regeneration capacity in a rat experimental model[5].

Liver regeneration is a complex process including well controlled upregulation of the inflammatory cytokines interleukin (IL) 6[6,7] and tumour necrosis factor (TNF) α[8,9], and growth factors hepatocyte growth factor (HGF) and epidermal growth factor (EGF)[10]. It is unclear whether the presence of cholangitis has an adverse impact on the hepatic regeneration process, including perturbation of growth factors and cytokines.

The aim of this study was to assess the effect of cholangitis induced by jejunobiliary reflux on hepatic physiology and regeneration capacity in an animal model.

Methods

Male Wistar rats (Charles River Laboratories, Wilmington, Massachusetts, USA) weighing 250–300 g were purchased from Japan SLC (Nagoya, Japan) and housed in a temperature- and humidity-controlled environment on a constant 12 : 12-h light–dark cycle. Animals had free access to water and food. All experiments were approved by the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine, and followed Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines.

The rats were alternately assigned to one of two experimental groups: 70 per cent partial hepatectomy (Hx group) and 70 per cent partial hepatectomy with CJ (Hx + CJ group) (*Fig. 1a*). All surgical procedures were performed under general anaesthesia by inhalation of isoflurane. A median laparotomy was made, and the liver was freed from its ligaments. The pedicles of the left lateral and median lobes (equivalent to 70 per cent of the liver) were ligated with 3/0 silk sutures and resected. In the Hx + CJ group, the CJ was created by inserting a silicone catheter (internal diameter 0·28 mm, external diameter 0·61 mm; Imamura, Tokyo, Japan) proximally in the common bile duct and distally in the jejunum by applying a purse-string suture with 5/0 polypropylene thread (*Fig. 1b*). The rats were killed on days 1, 2, 4 and 7 after the operation ($n = 6$ on days 1 and 2, $n = 7$ on day 4, and $n = 8$ on day 7 in both groups). Blood and liver samples from the remnant lobes were harvested at each time point.

Blood tests

Levels of endotoxin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin in blood samples were measured using standard laboratory methods (SRL, Tokyo, Japan).

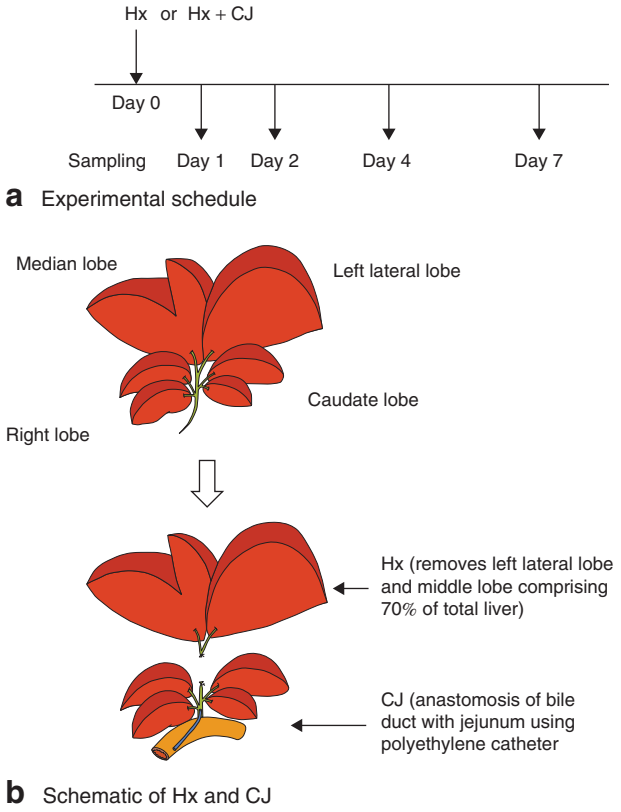


Fig. 1 **a** Experimental schedule ($n = 6$–8 at each time point). **b** Procedure of partial hepatectomy (Hx) and choledochojejunostomy (CJ)

Histological evaluation

Liver tissue samples were fixed immediately in 10 per cent buffered formalin, dehydrated in a graded ethanol series, embedded in paraffin, and then stained with haematoxylin and eosin. The tissue sections from each rat were examined under light microscopy (×20 objective). The examined views were recorded and analysed using cellSens Dimension (Olympus, Tokyo, Japan).

Determination of hepatic mRNA expression

Some 100 mg liver tissue was mixed with 1000 μl phosphate-buffered saline, homogenized and centrifuged at 16 000*g* for 3 min. To measure changes in gene expression in the liver, quantitative real-time reverse transcription (RT)–PCR analysis was performed with an Applied Biosystems Prism™ 7300 sequence detection system (Applied

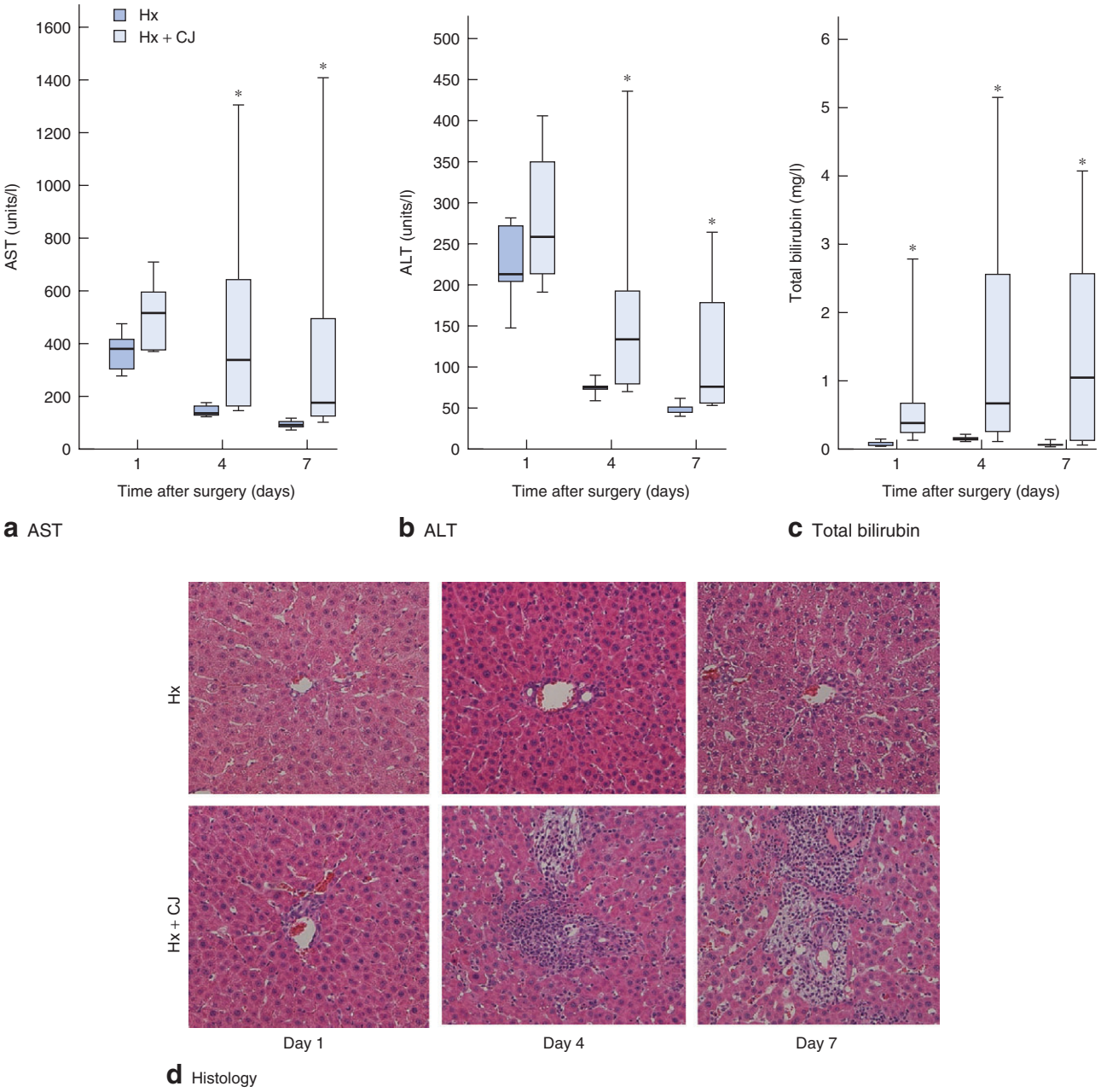


Fig. 2 Serum levels of **a** aspartate aminotransferase (AST), **b** alanine aminotransferase (ALT) and **c** total bilirubin on days 1, 4 and 7 after partial hepatectomy (Hx) or partial hepatectomy and choledochojejunostomy (Hx + CJ). Median values (line within box), i.q.r. (box) and range (error bars) are shown. *$P < 0{\cdot}050$ *versus* Hx (**a**,**b** *t* test, **c** Mann–Whitney *U* test). **d** Histology of the liver on days 1, 4 and 7 after Hx or Hx + CJ. The micrographs depict representative haematoxylin and eosin staining of paraffin-embedded liver sections (original magnification × 200)

Biosystems, Foster City, California, USA). Briefly, liver samples were harvested at each time point (days 1, 2, 4 and 7 after surgery) and total RNA was isolated from liver tissues using an RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. TaqMan® gene expression assays (Applied Biosystems) for IL-6, TNF-α, toll-like receptor (TLR) 4, HGF, EGF, α-smooth muscle actin (α-SMA), and 18S rRNA (endogenous control) were purchased as a probe and primer set (IL-6, Rn00561420_m1; TNF-α, Rn01525860_g1;

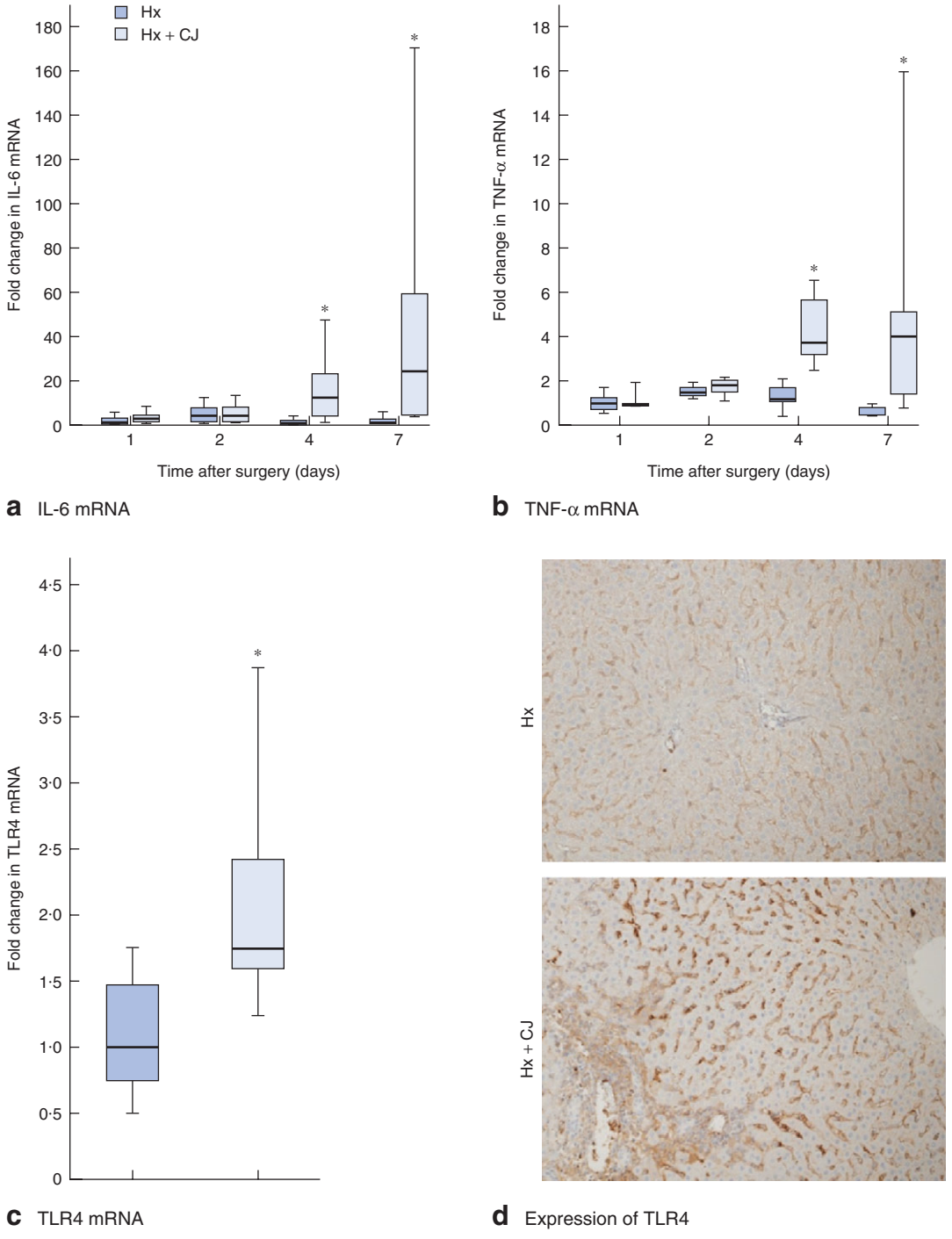
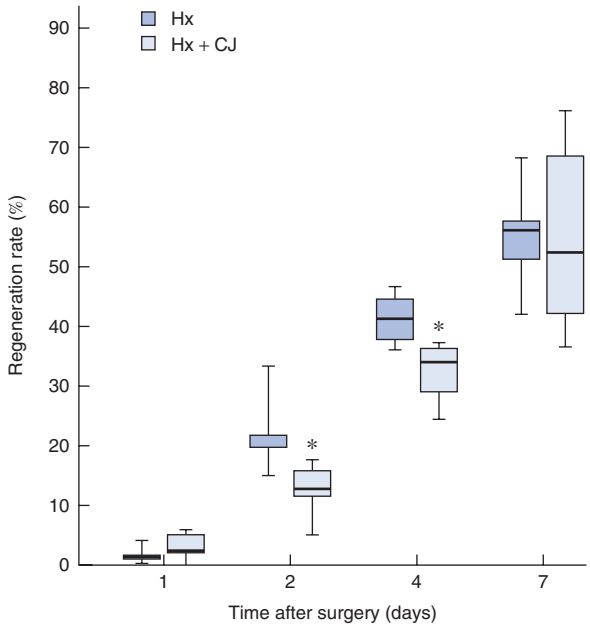
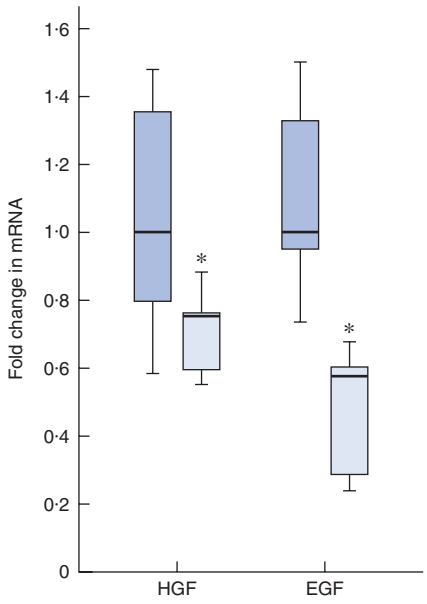


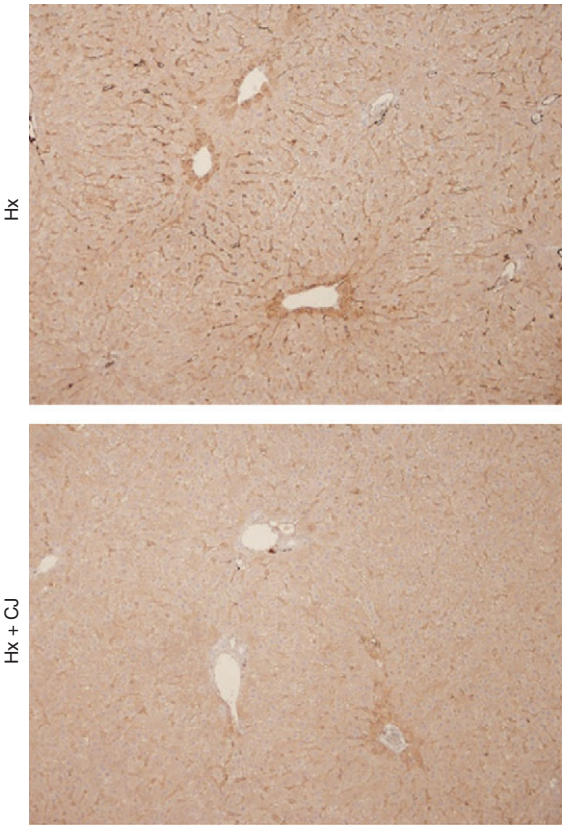
Fig. 3 Gene expression of **a** interleukin (IL) 6 and **b** tumour necrosis factor (TNF) α in the remnant liver lobes on days 1, 2, 4 and 7 after partial hepatectomy (Hx) or partial hepatectomy and choledochojejunostomy (Hx + CJ). **c** Gene expression of toll-like receptor (TLR) 4 in the liver on day 4 after surgery. The median expression level in the Hx group on day 1 was set to 1·0, and other data were adjusted to this baseline. Median values (line within box), i.q.r. (box) and range (error bars) are shown. *$P < 0{\cdot}050$ *versus* Hx (**a**,**c** Student's *t* test, **b** Mann–Whitney *U* test). **d** Representative micrographs of immunohistochemical staining for TLR4 in liver tissue on day 4 after Hx or Hx + CJ (haematoxylin counterstain, original magnification × 200)



a Regeneration rate



b Hepatic regeneration promoter mRNAs



c Expression of HGF

Fig. 4 a Percentage of newly regenerated liver weight (hepatic regeneration rate) on days 1, 2, 4 and 7 after partial hepatectomy (Hx) or partial hepatectomy and choledochojejunostomy (Hx + CJ). **b** Gene expression of the hepatic regeneration promoters hepatocyte growth factor (HGF) and epidermal growth factor (EGF) in the remnant liver lobes on day 1. The relative expression in the Hx group was set to 1·0, and other data were adjusted to this baseline. Median values (line within box), i.q.r. (box) and range (error bars) are shown. *$P < 0{\cdot}050$ *versus* Hx (Student's *t* test). **c** Immunohistochemical staining for HGF in remnant liver tissue on day 1 after Hx or Hx + CJ (haematoxylin counterstain, original magnification × 100)

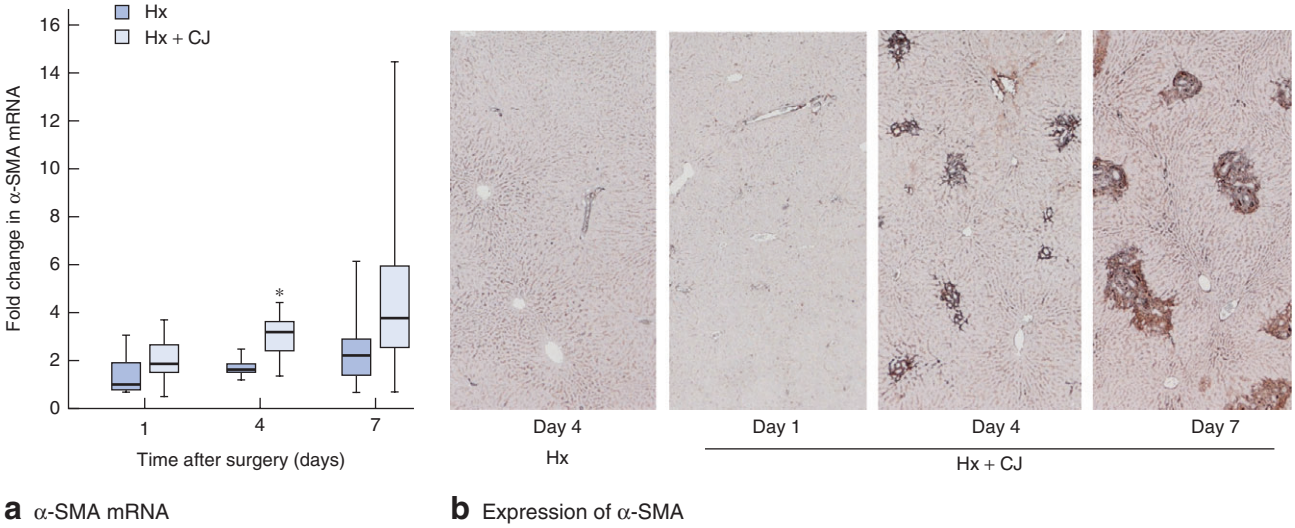


Fig. 5 **a** Gene expression of α-smooth muscle actin (SMA) in the remnant liver lobes on day 1, 4 and 7 after partial hepatectomy (Hx) or partial hepatectomy and choledochojejunostomy (Hx + CJ). The median expression level for α-SMA in the Hx group on day 1 was set to 1·0, and other data were adjusted to this baseline. Median values (line within box), i.q.r. (box) and range (error bars) are shown. *$P < 0{\cdot}050$ *versus* Hx (Student's *t* test). **b** Immunohistochemical staining for α-SMA in remnant liver tissue after Hx or Hx + CJ (haematoxylin counterstain, original magnification × 40)

TLR4, Rn00569848_m1; HGF, Rn00566673_m1; EGF, Rn00563336_m1; α-SMA, Rn01759925_g1; 18S rRNA, Hs99999901_s1). The reaction mixture was denatured with one 10-min cycle at 95°C and incubated for 40 cycles (denaturing for 15 s at 95°C, annealing and extending for 1 min at 60°C). The amplification data were analysed with Prism™ sequence detection software version 1·4 (Applied Biosystems). In each experiment, the relative expression of the gene of interest was normalized with respect to the 18S control using standard curves prepared for each gene and median values were used for quantification. The median values of the liver samples from the Hx group on day 1 were set as onefold induction, and other data were adjusted to that baseline.

Evaluation of hepatic regeneration after hepatectomy

The restitution of liver weight was determined as the percentage of regenerated liver mass and calculated as[11]: hepatic regeneration rate (%) = 100 × [C – (A – B)]/A, in which A is the estimated total liver weight at the time of partial hepatectomy, B is the excised liver weight, and C is the weight of regenerated liver at the time of sampling.

Immunohistochemistry

To detect expression of TLR4, HGF and α-SMA in the liver by immunohistochemistry, the automated slide preparation system Discovery® XT (Ventana Medical Systems, Tucson, Arizona) was used. Before staining, paraffin sections were heated at 65°C for 30 min in a paraffin oven and blocked with 1 per cent non-fat milk. The staining procedure was carried out according to the manufacturer's protocol (Ventana Medical Systems). Anti-TLR4 antibodies (ab30667; Abcam, Cambridge, UK), anti-HGF antibodies (ab83760; Abcam) or anti-α-SMA antibodies (M0851; Dako, Glostrup, Denmark) were diluted in Discovery® Ab diluent (Ventana Medical Systems).

Statistical analysis

After confirming normal distribution of data with a Kolmogorov–Smirnov test, Student's *t* test was used to evaluate significant differences between the two groups. When criteria for parametric testing were violated, the non-parametric Mann–Whitney *U* test was used. $P < 0{\cdot}050$ was considered a significant difference. Analyses were performed with the statistical package SPSS® version 22.0 (IBM, Armonk, New York, USA).

Results

No rat died before the planned date of death in either group. Serum AST, ALT and total bilirubin levels on days 4 and 7 after surgery were significantly higher in the Hx + CJ group compared with the Hx group (*Fig. 2a–c*).

An increase in neutrophil infiltration was observed around the periportal area after surgery in the Hx + CJ group (*Fig. 2d*).

Inflammation-associated factors

Levels of intrahepatic IL-6 and TNF-α mRNA expression in the Hx + CJ group were significantly higher than in the Hx group on days 4 and 7 after surgery (*Fig. 3a*,*b*). The blood endotoxin levels in the Hx group were all negative, but those in the Hx + CJ group were positive in three of seven rats on day 4 after surgery. The higher expression of TLR4, a representative endotoxin receptor, in the Hx + CJ group was confirmed by RT–PCR and immunohistochemistry on day 4 (*Fig. 3c*,*d*). The expression of TLR4 was dominant around the periportal area and along the sinusoids.

Hepatic regeneration

Hepatic regeneration in the Hx + CJ group was significantly less than in the Hx group on day 2 (14·2(6·3) *versus* 21·4(2·6) per cent; $P = 0{\cdot}013$) and day 4 (32·4(5·3) *versus* 41·3(4·4) per cent; $P = 0{\cdot}004$) after surgery, although there was no significant difference between the two groups on days 1 and 7 (*Fig. 4a*). Moreover, expression levels of mRNA for the hepatic regeneration promoters HGF and EGF on day 1 after surgery were significantly lower in the Hx + CJ group than in the Hx group (*Fig. 4b*). The attenuated expression of HGF protein was confirmed by immunohistochemistry (*Fig. 4c*).

Expression of α-smooth muscle actin

In the Hx + CJ group, expression of α-SMA mRNA was significantly higher than in the Hx group on day 4 after surgery (*Fig. 5a*). Expression of α-SMA was seen along the sinusoids and the area of inflammatory cell infiltration was markedly increased on days 4 and 7 after surgery (*Fig. 5b*). These changes were not observed in the Hx group.

Discussion

After CJ, jejunobiliary reflux occurs which can lead to cholangitis[3]. It has been demonstrated that liver regeneration is suppressed secondary to cholangitis in a rat segmental cholangitis model[5]. The suppressive effects of cholangitis on liver regeneration following portal vein embolization have also been studied in the clinical setting[4]. However, the mechanism underlying the inhibitory effect of CJ on the liver regeneration process has not been investigated. In the present study, a rat model of Hx with CJ, which simulates the surgical procedure of major hepatectomy with extrahepatic bile duct resection and CJ, was established to elucidate the molecular mechanisms involved in liver regeneration.

An intrahepatic inflammatory reaction was seen in the Hx + CJ group, but not in the Hx group. Levels of hepatic enzymes and the expression of inflammation-associated cytokines were significantly higher in the Hx + CJ group. These results indicate that the inflammatory response and accompanying hepatic damage is more severe when CJ is added to Hx. Interestingly, the intrahepatic expression of TLR4, a major endotoxin receptor, was upregulated following CJ. These changes correlated with histological changes in the periportal area (increased inflammatory cell infiltration) following CJ. Previous studies[12,13] on rats have demonstrated an upregulation of TLR4 in the liver in response to *Escherichia coli* or lipopolysaccharide injection via the bile duct. The upregulated TLR4 was associated with excessive activation of nuclear factor κB and inflammatory cytokine production. It was speculated that the upregulation of TLR4 was also induced by CJ, most likely as a result of regurgitation of intestinal bacteria, and this change may have been partly responsible for the liver injury observed in the Hx + CJ group.

It is well known that liver regeneration requires production of inflammatory cytokines such as IL-6[6,7] and TNF-α[8,9]. In contrast, it has been shown in animal models of sepsis that the presence of systemic infection (which may induce high levels of IL-6 and TNF-α production) delays liver regeneration[14,15]. The complexity of liver regeneration may require well orchestrated cytokine production. The presence of local cholangitis may be more potent and drastic than the stimulus of hepatectomy in inducing inflammatory cytokine production, as indicated in this study (*Fig. 3a*,*b*)[16]. An excessive local inflammatory response induced by the CJ may disturb the harmony of the cytokine production that is necessary for liver regeneration. Local cholangitis may also lead to endotoxaemia and subsequent systemic inflammation probably through biliosinusoidal shunting. Although serum endotoxin levels were raised on day 4 after surgery in the Hx + CJ group in the present study, white blood cell counts and serum C-reactive protein levels were no different between the two groups (data not shown). Therefore, it is speculated that local inflammation in the liver caused by enterobiliary regurgitation (rather than systemic inflammation) is the major factor leading to deterioration of liver regeneration in the Hx + CJ model.

It is known widely that endotoxin activates hepatic stellate cells, and the activated hepatic stellate cells produce α-SMA[17]. Activated hepatic stellate cells have

also been shown to lose the ability to produce HGF[18,19], a major liver regeneration-promoting factor[10]. In the present study, there was significantly lower expression of liver regeneration-promoting genes (HGF and EGF mRNA) and greater expression of α-SMA in the Hx + CJ group compared with the Hx group. These observations may in part explain the mechanism of impaired hepatic regeneration in the Hx + CJ group.

It is unclear whether the rat model used in this study accurately simulates the situation in humans. A major hepatectomy with extrahepatic bile duct resection and CJ is usually performed using the Roux-en-Y anastomosis technique. Here, a silicone tube with a small diameter was used for CJ; this was inserted into the jejunum with a purse-string suture. Dislocation of the silicone catheter or obstruction of the jejunum at the purse-string suture site could be a potential flaw in this model. The patency of the silicone tube was another concern, and because of this the observation time was limited to 7 days. However, in a preliminary study using a model in which CJ alone was performed without hepatectomy, the silicone tube and jejunum were patent at least on day 7 after surgery. The incidence or severity of cholangitis may also differ between the rat model and humans. Further elucidation of the precise mechanism of adverse effects of local cholangitis on liver regeneration may be helpful in developing a treatment strategy to improve liver regeneration capacity under such circumstances.

Disclosure

The authors declare no conflict of interest.

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