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Original Communications

Inhibition of Toll-like receptor 4 ameliorates experimental postischemic injury in the cholestatic liver through inhibition of high-mobility group box protein b1 (HMGB1) signaling

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ABSTRACT

Background. The objective of this study was to elucidate whether the inhibition of Toll-like receptor 4 attenuates liver injury ischemia/reperfusion in the cholestatic liver.

Method. Rats were assigned into sham, bile duct ligation, sham ischemia/reperfusion (ischemia/reperfusion after laparotomy), and bile duct ligation ischemia/reperfusion (ischemia/reperfusion after bile duct ligation) groups. In some rats, TAK-242, an inhibitor of Toll-like receptor 4, was administered 15 minutes before ischemia/reperfusion. We measured intrahepatic Toll-like receptor 4 expression, serum hepatic marker expression, liver necrosis, gene expression of inflammation-associated factors, and serum high-mobility group box protein b1 levels.

Results. Intrahepatic Toll-like receptor 4 expression was significantly greater in the bile duct ligation group than in the sham group. Toll-like receptor 4 expression was further increased after ischemia/reperfusion in bile duct ligation ischemia/reperfusion groups. The levels of serum hepatic markers were significantly greater in both the sham ischemia/reperfusion and bile duct ligation ischemia/reperfusion groups than in the groups without ischemia/reperfusion. Liver necrosis was greater in the bile duct ligation group than in the sham group and was further increased in the bile duct ligation ischemia/reperfusion group. Genomic expression of inflammation-associated factors was also significantly greater in the bile duct ligation ischemia/reperfusion group than in the sham group. Serum high-mobility groups box protein b1 levels were greater in the bile duct ligation ischemia/reperfusion group than in the sham group (28.1 ng/ml versus 9.2 ng/ml, $P = .011$) and the bile duct ligation group (28.1 ng/ml versus 10.6 ng/ml, $P = .017$). These changes in the bile duct ligation ischemia/reperfusion group were significantly attenuated by preconditioning with TAK242.

Conclusions. Toll-like receptor 4 inhibition has a potential to minimize severe injury after ischemia/reperfusion in the cholestatic liver through inhibition of high-mobility groups box protein b1.

Introduction

Periods of vascular inflow occlusion are used often to minimize intraoperative blood loss during liver resection. This ischemia/reperfusion (I/R) process, however, induces liver injury and may sometimes lead to postoperative liver failure.[1] Therefore, minimizing I/R-induced liver injury is crucial to safely performing liver surgery.

Patients with perihilar cholangiocarcinoma generally have biliary obstruction and often need to undergo major hepatectomy with extrahepatic bile duct resection. In the past, some authors demonstrated that major liver resection without biliary drainage is safe in most patients with biliary obstruction.[2-5] Although these studies were important, few surgeons in either Western or Eastern medical centers agreed with their opinions. It is still unclear whether major liver resection can be safely performed in patients with biliary obstruction without preoperative biliary drainage. To answer this question, the estimation of liver injury after I/R under the condition with biliary obstruction should be elucidated, because an intermittent I/R is an important procedure in major liver resections. It is also important to develop an effective preventive method to decrease the liver injury that occurs during I/R in the bile duct obstructed liver.

A previous report demonstrated upregulation of Toll-like receptor 4 (TLR4) in the liver and increased sensitivity to endotoxin after bile duct ligation (BDL) in rats.[6] Other reports showed that liver injury

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after I/R involves the activation of TLR4 signaling in nonparenchymal cells.[7-9] These results indicate that TLR4 plays a crucial role during the stress of liver resection in the presence of hepatic I/R in the liver with biliary obstruction.

Two major activation patterns of TLR4, such as pathogen-associated molecular-pattern molecules (PAMPs) and damage-associated molecular pattern-molecules (DAMPs), have been reported.[10] TLR4 activation through PAMPs is mediated by endotoxin; whereas that through DAMPs is mediated by humoral mediators such as high mobility-box groups protein b1 (HMGB1) released from the damaged tissue. Therefore, it can be hypothesized that TLR4 and HMGB1 play an important role in liver injury after I/R in the biliary obstructed liver. This hypothesis has not yet been investigated.

TAK-242 is a small-molecule inhibitor of TLR4 that interferes with TLR4-mediated signals without inhibiting directly agonist binding to TLR4.[11,12] We demonstrated previously that the blocking TLR4 signaling by pretreatment with TAK-242 attenuates effectively the liver damage to the same degree as that observed with biliary drainage in rats with BDL and subsequent intraportal endotoxin infusion.[13] These results imply that pretreatment with TAK-242 may also be beneficial in the liver with biliary obstruction when exposed to I/R stress.

The aim of this study was to elucidate the mechanism of liver injury after I/R in BDL rats and to verify the hypothesis that preconditioning with TAK-242 (inhibition of TLR4 signaling) suppresses liver injury induced by I/R in cholestatic rat liver without biliary drainage.

Methods

Animals

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

Experimental design

Rats were assigned into sham, BDL, sham I/R, and BDL I/R groups (n = 6–12 in each group). All operative procedures were performed with the rats placed under general anesthesia with inhaled isoflurane. In the BDL and the BDL I/R groups, an abdominal midline incision was made, and the common bile duct was ligated with 5/0 polypropylene (ETHICON, Cincinnati, OH). In the sham and the sham I/R groups, the common bile duct was freed from the surrounding tissues without ligation. In the BDL I/R group, the abdomen was reopened through the previous incision 7 days after BDL operation, and the hepatoduodenal ligament was clamped for 30 minutes. A period of 30 minutes was chosen because this duration of liver ischemia induces substantial liver damage without any mortality. Thereafter, the liver was reperfused for 4 hours. In the sham I/R group, the same procedure as BDL I/R group was completed 7 days after the sham operation. In contrast, in the sham and BDL groups, only laparotomy and mobilization of the hepatoduodenal ligament were performed 7 days after the initial operation. Four hours after the I/R operation or sham operation, blood samples were collected, and liver tissue samples were removed for analysis. Four hours after the I/R was selected because the increase in liver markers peaked at this time in a preliminary study. In some of the rats in the sham I/R group (n = 6) and the BDL I/R group (n = 6), TAK-242 (1 mg/kg) dissolved in phosphate buffered saline (PBS) (1 mg/ml) was administered via the penile vein 15 minutes before clamping the hepatoduodenal ligament. The same volume of PBS (1 ml/kg) was used as a vehicle. The dose of TAK-242 was determined based on our previous study using BDL rats.[13]

Blood tests

Serum levels of endotoxin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using standard laboratory methods (SRL, Tokyo, Japan).

Histologic evaluation

The liver tissue samples were immersed immediately in 10% buffered formalin and stored overnight. The samples were then dehydrated in a graded ethanol series and embedded in paraffin. We mounted 6-μm-thick sections on glass slides and stained with hematoxylin and eosin. The tissue sections were examined under light microscopy to estimate the extent of liver necrosis in 5 randomly selected, low-powered (×10 objective) fields of view for each rat. The examined views were recorded and analyzed using cellSens Dimension (Olympus, Tokyo, Japan). Other sections were subjected to immunohistochemistry to detect the expression of TLR4. Before staining, paraffin sections were heated to 65°C for 5 minutes in a paraffin oven and were blocked with 1% nonfat milk. The automated slide preparation system Discovery XT (Ventana Medical Systems, Tucson, AZ) was used for immunostaining. The staining procedure was carried out according to the manufacturer's protocol. The anti-TLR4 antibody (ab30667; Abcam, Cambridge, UK) was diluted in Discovery Ab diluent (Ventana Medical Systems).

Enzyme-linked immunosorbent assay

Serum HMGB1 levels were determined using sandwich, enzyme-linked immunosorbent assay methods. The assay was performed according to the manufacturer's protocol (Shino-Test, Tokyo, Japan).

Quantitative real-time polymerase chain reaction

To validate gene expression in the liver, quantitative real-time polymerase chain reaction (PCR) analysis was performed with a Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA,). Total ribonucleic acid (RNA) was isolated from whole liver using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary deoxyribonucleic acid (cDNA) was generated from total RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each reaction was performed in a 10-μl mixture that included the TaqMan universal PCR master mix according to the manufacturer's instructions (Applied Biosystems). The expression of TLR4 (TLR4 [assay identification no. Rn00569848_m1; Applied Biosystems]), inflammatory cytokines (interleukin [IL]-1β [assay identification no. Rn00580432_m1; Applied Biosystems] and IL-6 [assay identification no. Rn99999011_m1; Applied Biosystems]), calcium-independent nitric oxide synthase 9inducible nitric oxide synthase [iNOS] [assay identification no. Rn00561646_m1; Applied Biosystems]), and a monocyte chemoattractant chemokine (C-C motif chemokine ligand [CCL] 2 [assay identification no. Rn00580555_m1; Applied Biosystems]) were determined by comparative quantitative real-time PCR using the Prism 7300 sequence detection systems. 18S ribosomal ribonucleic acid (rRNA) (assay identification no. Hs99999901_s1) was used as an endogenous control. The reaction mixture was denatured with a 10-minute cycle at 95°C and incubated for 40 cycles (denaturation for 15 seconds at 95°C, annealing and extension for 1 minute at 60°C). The amplification data

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were analyzed with Prism sequence detection software v 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 the median values were used for quantification.

Statistical analysis

Significant differences among multiple groups were analyzed using a one-way ANOVA followed by the Dunnett procedure; whereas those between 2 groups were analyzed using Student *t* test. When the criteria for parametric testing were violated, the appropriate nonparametric Mann-Whitney *U*-test was used. Correlations between the 2 factors were calculated by Pearson rank correlation. All results are presented as the means ± SE.

Results

TLR4 expression in the liver

Genetic expression of TLR4 in the liver was significantly higher in the BDL group than in the sham group (Fig 1, *A*). The addition of I/R further increased the expression of TLR4 in both the sham I/R and BDL I/R groups. Immunostaining revealed excessive TLR4 expression along the sinusoid in the BDL I/R group compared with that of the other groups (Fig 1, *B*).

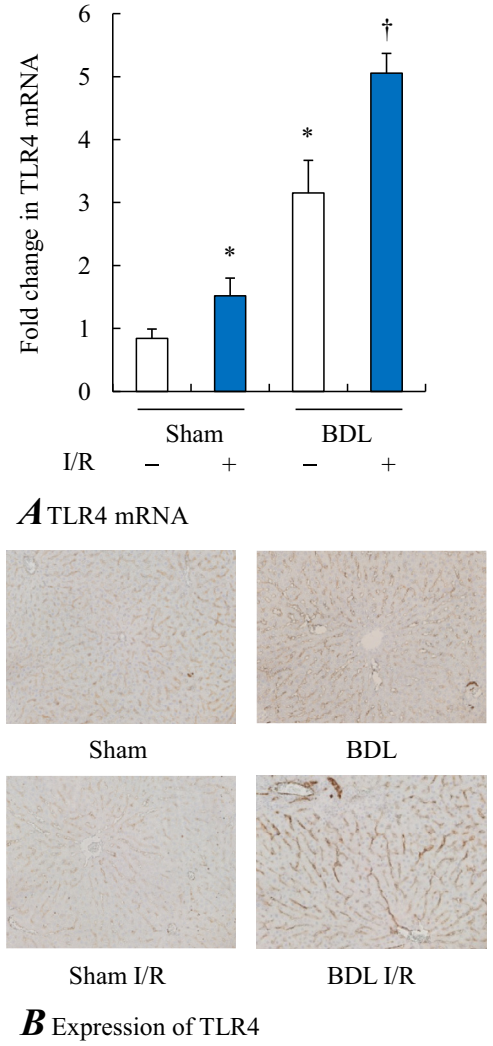

Fig. 1. Expression of TLR4 in the sham and BDL livers with and without I/R determined by (*A*) RT-PCR and (*B*) immunohistochemistry. *$P < .05$ versus sham; †$P < .05$ versus BDL.

Blood tests

The serum levels of AST and ALT significantly increased after I/R in both the sham I/R and BDL I/R groups compared with the groups without I/R. In the sham I/R group, there were no significant differences in the AST and ALT levels between the vehicle treatment group and the TAK-242 treatment group. By contrast, in the BDL I/R group, these levels were significantly less in the TAK-242 treatment group when compared with that of the vehicle treatment group (Fig 2, *A* and 2, *B*). The hepatic expression of TLR4 showed a correlation with the serum AST levels (correlation coefficient, 0.575, $P < .001$). The serum endotoxin level was almost undetectable in all groups regardless of TAK-242 administration (data not presented), indicating that endotoxin did not play an important role in this model.

Histologic evaluation

Histologic changes were examined in the livers from the sham, sham I/R, sham I/R with TAK-242 treatment, BDL, BDL I/R, and BDL I/R with TAK242 treatment groups (Fig 2, *C* and 2, *D*). In both the sham and sham I/R groups, no necrotic areas were observed. Some necrotic areas in the liver were observed in the BDL group, and these necrotic areas were observed mainly in the periportal area (Zone 1) (Fig 2, *C*). The percentage of necrotic areas was significantly greater in the BDL I/R group than in the BDL group. This change was attenuated by treatment with TAK-242 (Fig 2, *C* and 2, *D*). The hepatic expression of TLR4 showed a correlation with the percentage of necrotic areas in the liver (correlation coefficient, 0.555, $P < .001$).

Genetic expression of inflammation associated factors

The gene expression levels of inflammation associated factors, such as IL-1β, IL-6, iNOS, and CCL2 in the liver, were all greater in the BDL group than in the sham group. These levels were further increased in the BDL I/R group and were different from the levels in the sham group. Notably, these changes were substantially attenuated by TAK-242 treatment (Fig 3, *A*–*D*).

Enzyme-linked immunosorbent assay for HMGB1

The serum level of HMGB1 was significantly greater in the BDL I/R group with vehicle treatment than in the sham and BDL groups, and notably, the serum HMGB1 level was significantly attenuated in the BDL I/R group with TAK242 treatment (Fig 4). Correlations were found between the serum levels of HMGB1 and serum AST levels (correlation coefficient, 0.422, $P < .001$), as well as necrotic area in the liver (correlation coefficient 0.235, $P = .019$).

Discussion

In the case of perihilar cholangiocarcinoma, most patients develop biliary obstruction, and a major hepatectomy with extrahepatic bile duct resection is the standard operative procedure for this disease entity. In general, preoperative biliary drainage to improve liver function is commonly performed before resection. Some reports, however, have indicated that major liver resection can be safely performed without biliary drainage.[2-5] The safety of liver resection under the condition of biliary obstruction is an important clinical issue. One of the triggers of post-hepatectomy liver injury is repeated I/R, which is often used during hepatic parenchymal dissection to decrease intraoperative blood loss and possibly to precondition the liver to the

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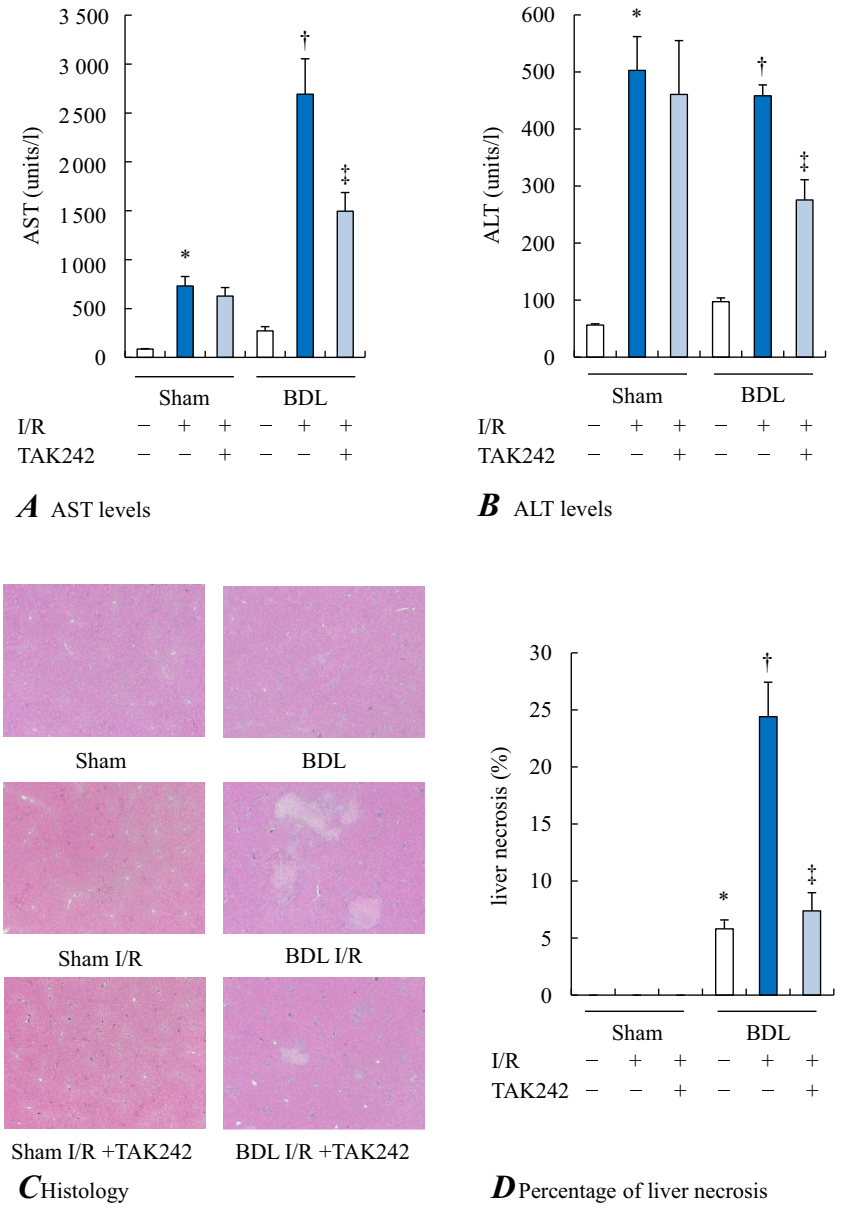

Fig. 2. (*A*) Serum AST and (*B*) ALT levels, (*C*) histology, and (*D*) percentage of liver necrotic area at 4 hr after laparotomy or I/R in both the sham and BDL rats. In some rats, TAK-242, an inhibitor of TLR4, or vehicle (PBS) were used before I/R. $^{*}P < .05$ versus sham; $^{\dagger}P < .05$ versus BDL; $^{\ddagger}P < .05$ versus BDL I/R.

ischemia. Therefore, we established a model combining BDL and I/R stresses to test whether major hepatectomy can be performed safely in patients with biliary obstruction but without preoperative biliary drainage.

Numerous previous reports indicated a crucial role for TLR4 during liver injury under various pathologic conditions, such as biliary obstruction, I/R, alcoholic liver injury, and steatohepatitis.[6,7,14,15] It is well accepted that TLR4 is upregulated in the liver after hepatic I/R.[7] Additionally, we demonstrated previously that TLR4 in the liver is upregulated after biliary obstruction in rat BDL model.[13] These results imply that the combination of the 2 stresses, such as BDL and I/R, further upregulate TLR4 expression and exacerbate liver damage. As expected, the results in this study demonstrated excessive expression of TLR4 in the liver after BDL and subsequent I/R with severe increase in serum levels of liver enzymes. These observations have not been reported previously in either animal models or humans.

In rats with BDL, the serum levels of AST and ALT were significantly increased afterI/R. Although no necrotic areas were observed in the livers of the Sham I/R group, there were excessive necrotic areas in the BDL I/R group, suggesting strongly that the cholestatic liver is more susceptible to I/R stress than is the non-cholestatic liver. The severe liver injury observed in the BDL I/R group coincided with greater expression of TLR4 in the liver, as well as greater serum HMGB1 levels. A strong correlation was found between the levels of AST and the expression of TLR4. The levels of AST also showed a correlation with the expression of HMGB1, again indicating an important role for TLR4 signaling and HMGB1 release into the blood as mechanisms of liver injury after I/R in the cholestatic liver.

Kloek et al[16] demonstrated that postischemic injury in the cholestatic rat liver was attenuated by biliary decompression. In some clinical conditions, however, it is difficult to decompress the obstructed bile duct, and surgeons have no choice but to perform liver

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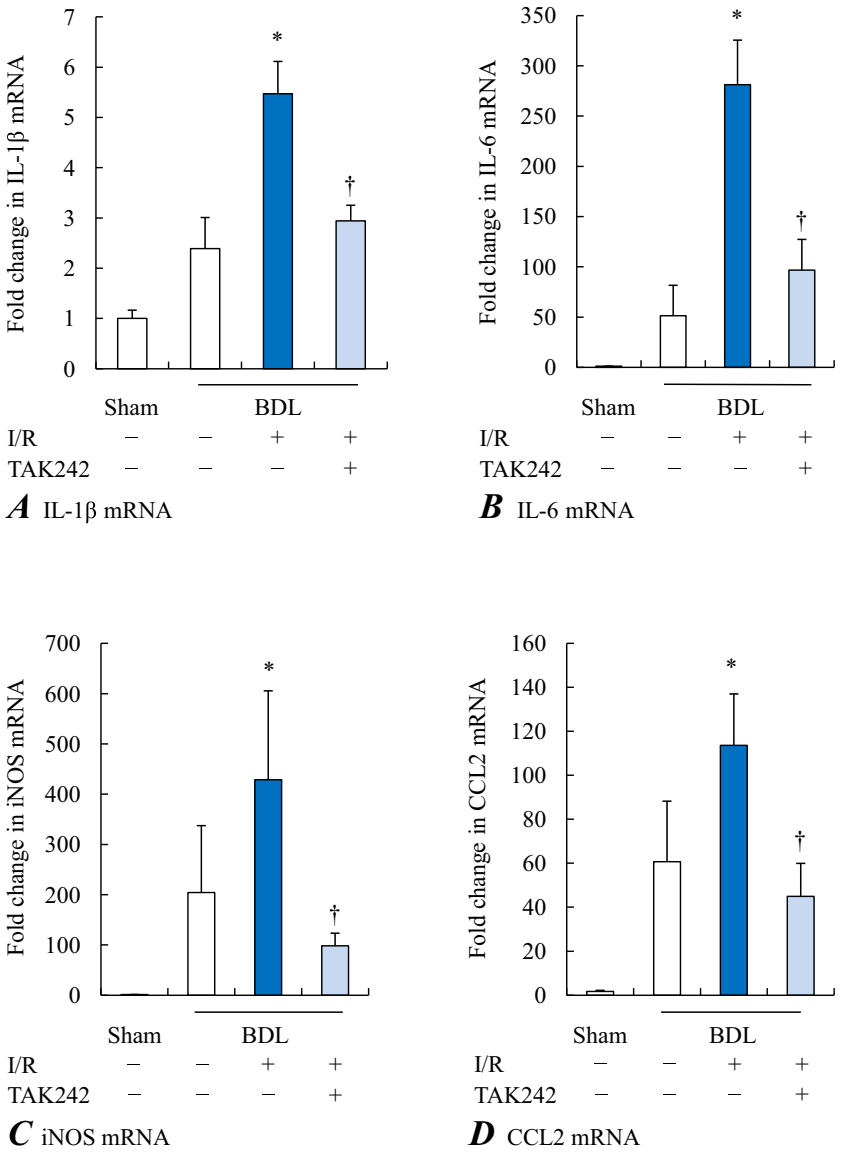

Fig. 3. (*A*) Hepatic gene expression of IL-1β, (*B*) IL-6, (*C*) iNOS, and (*D*) CCL2 in the sham, BDL, BDL I/R, and BDL I/R with TAK-242 treatment groups. *$P < .05$ versus sham; †$P < .05$ versus BDL I/R.

resection without biliary drainage. In this situation, it is important to minimize the liver damage after hepatectomy. We hypothesized that the inhibition of TLR4 signaling in the cholestatic rat liver leads to the attenuation of liver injury after I/R. As expected, the serum levels of liver enzymes, necrotic areas in the liver, and the expression of several inflammation-associated factors, such as IL-1β, IL-6, iNOS, and CCL2, were significantly attenuated by treatment with a TLR4 inhibitor (TAK-242). TAK-242 was used previously in a randomized controlled trial targeting a total of 274 patients with severe sepsis.[17] In this trial, although there was no clinical benefit of TAK-242 in these patients with severe sepsis, treatment with TAK-242 was well tolerated, causing no major side effects, and thus the safety of TAK-242 has already been shown. Based on the observations in this study, TAK-242 can be proposed as a preconditioning drug to attenuate liver injury after liver resection, especially in cases of biliary obstruction.

One previous study demonstrated that pharmacologic inhibition of TLR4 with eritoran, another inhibitor of TLR4, was protective in a mouse model of liver I/R.[9] In this I/R model, the blood supply to the left and median lobes of the liver were occluded (70% segmental hepatic ischemia) for 60 minutes, and the necrosis was observed in > 10% of the liver area after I/R. In the clinical setting, however, it is uncommon to perform partial ischemia while performing liver resection. Moreover, 60 minutes of ischemia is extremely long and it is not acceptable to perform this procedure in the human liver. In contrast, in our study, the hepatoduodenal ligament was clamped (100% hepatic ischemia) for 30 minutes which simulated more closely the procedure used during liver resection in humans.[18] With this model, no necrotic areas were observed after I/R in the sham group. Although TLR4 was upregulated in the liver after I/R, inhibition of TLR4 signaling did not attenuate the liver injury, probably because the innate liver injury was not severe with only 30 minutes of ischemia; however, as indicated in the BDL I/R group in which TLR4 was highly upregulated, the inhibition of TLR4 was effective in preventing liver injury, indicating that TLR4 signaling can play a crucial role in liver injury after I/R. The results obtained in this study are clinically relevant and indicate the therapeutic potential of a TLR4 inhibitor in preventing liver injury after I/R in cholestatic liver.

TLR4 is activated through 2 of the major molecular pathways involving PAMPs and DAMPs.[10] PAMPs is mediated by endotoxin;

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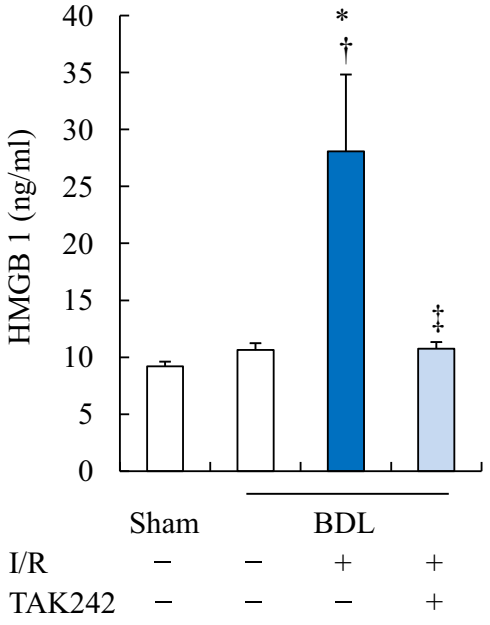

Fig. 4. Serum HMGB1 levels in the sham, BDL, BDL I/R, and BDL I/R with TAK-242 treatment groups. *$P < .05$ versus sham; †$P < .05$ versus BDL; ‡$P < .05$ versus BDL I/R.

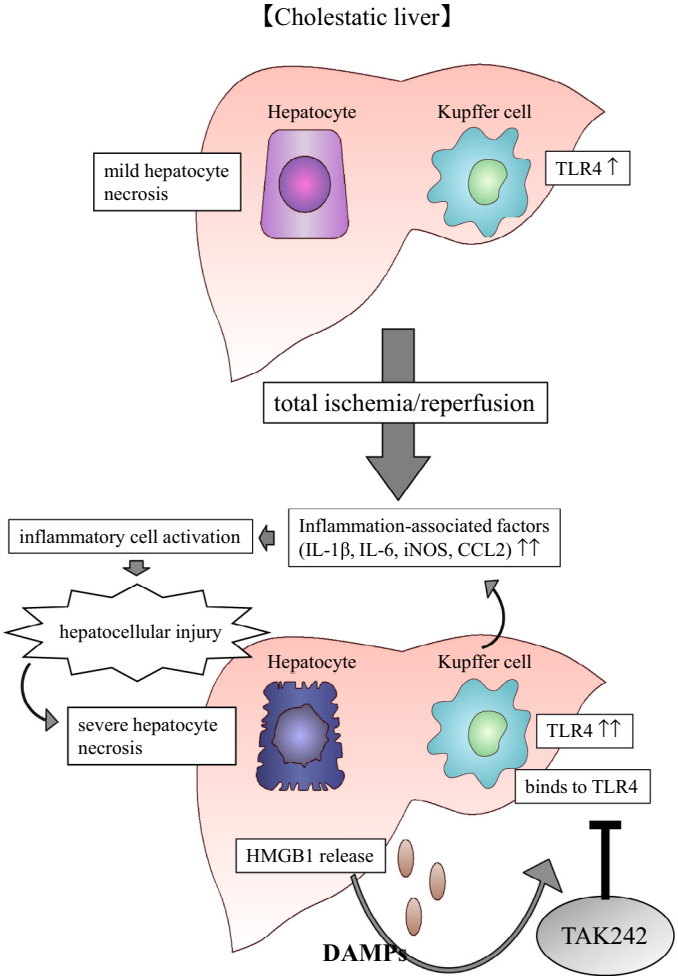

Fig. 5. Hypothetic schema for the mechanism of liver injury induced by BDL and subsequent I/R and the beneficial effects of TLR4 inhibitor (TAK-242) in preventing liver injury. DAMPs, damage associated molecular pattern molecules.

whereas the pathway through DAMPs is mediated by humoral mediators such as HMGB1 released from the damaged tissue. In our study, the level of serum endotoxin was undetectable at 4 hours after I/R (data not shown). In contrast, high levels of HMGB1 (the major mediator of DAMPs) were detected in the sera of the BDL I/R group. These results imply that the severe liver injury induced by I/R in the BDL group was mediated through DAMPs rather than via PAMPs. Interestingly, the serum levels of HMGB1 were significantly lower in the BDL I/R group after TAK-242 treatment. We, therefore, hypothesize that the inhibition of TLR4 by TAK-242 may prevent these types of severe inflammatory responses and may break the vicious cycle of liver injury induced by I/R in the cholestatic liver (Fig 5).

This study has several limitations. Although the dose and timing of TAK-242 administration was determined by the preliminary study in a previous report,[13] it is still unclear whether the parameters used in this study are appropriate in the human model. It is unclear whether our model with 7 days cholestasis mimics accurately the biliary obstruction seen in humans with cholangiocarcinoma, primarily because in humans the biliary obstruction may be of greater duration. Additionally, our model did not test the safety of major hepatectomy under the conditions of BDL and I/R. The protocol of I/R used in this study includes only the 1 time of 30 minutes of ischemia and subsequent reperfusion. Also, we did not study the effects of repeating I/R, which is performed commonly in a clinical setting. Moreover, only male rats were used in this study. Numerous reports have shown sexual dimorphism in liver physiology under various pathologic conditions.[19] In fact, 1 experimental study has shown that young male rats were prone to a greater degree of biochemical liver injury in response to cholestasis than female rats.[20] The impact of sex on the liver injury after BDL and I/R should be investigated in a future study. For the clinical application of TAK-242 in patients who undergo hepatectomy under the condition of biliary obstruction, the issues discussed earlier should be further investigated.

In conclusions, the combination of BDL and I/R induced a severe liver injury and an excessive increase in hepatic TLR4 gene expression as well as serum inflammation-associated factors including HMGB1. The inhibition of TLR4 signaling by TAK-242 attenuated effectively the BDL and I/R-induced liver injury. Preconditioning by a TLR4 inhibitor may have a therapeutic potential in patients who require a major hepatectomy with the condition of biliary obstruction.

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