Partial bile duct ligation in mice: A novel model of acute cholestasis

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Background. The standard model for research in cholestasis is the total ligation of the bile duct (tBDL). Because this model causes severe hepatic injury in mice, we developed a novel model of cholestasis using a partial bile duct ligation (pBDL) and evaluate different mechanisms of injury.

Methods. Male C57Bl/6 mice were subjected to sham operation, tBDL, or pBDL. Blood from tail veins was taken repeatedly until day 14 after surgery to assess markers of tissue injury (aspartate aminotransferase [AST]) and cholestasis (bilirubin, alkaline phosphatase [AP]). Also, liver samples were obtained at various time points to determine the histologic injury (hematoxylin and eosin) and tissue repair (Ki67). In addition, the biliary pressure and serum bile acids were evaluated as potential mechanisms of injury.

Results. Both models of cholestasis were equal in terms of bilirubin, AST, and AP serum levels during the first week of the experiment. Although these parameters remained constantly elevated thereafter in the tBDL model, all parameters normalized within the second week after pBDL. Moreover, pBDL resulted in significantly less necrosis formation ($P = .001$) and consequent hepatocyte proliferation ($P = .01$). Most important, serum bile acid levels ($P = .04$) and biliary pressures ($P = .02$) were significantly lower after pBDL than after tBDL and were the best predictors for hepatic necrosis formation.

Conclusion. We established a model of acute cholestasis, which is ideal for research in resolved acute cholestasis (eg, surgery for Klatskin tumors). Moreover, biliary pressure and toxic bile acid serum levels may be better predictors of cholestatic liver injury than standard laboratory parameters. (Surgery 2011;149:445-51.)

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Cholestasis is a well-known risk factor for complications after liver surgery.1 Thus, patients with cholestasis, such as cancer of the extrahepatic bile ducts, are at a greater risk for postoperative liver failure, sepsis, and death.2 Intensive research is required to evaluate the underlying mechanisms of cholestasis and organ injury after liver surgery to prevent these complications. The potential causes of cholestasis are various. Post-hepatic (obstructive) cholestasis is characterized by portal tract expansion, leukocyte infiltration, bile duct and septal proliferation, liver fibrosis, and eventually cirrhosis in humans.3 Patients suffer from jaundice and pruritus, and cholestatic parameters such as bilirubin and alkaline phosphatase (AP) as well as transaminases are usually elevated.

One of the standard animal models for research in cholestasis is the total ligation of the common bile duct (tBDL). This model has been well described by many groups and extensively used in rats.4 During recent years, tBDL has also been increasingly used in mice, because the mouse model offers several advantages over the rat model, of which 1 is the availability of transgenic and knock-out mutants. BDL in mice results in immediate jaundice with elevated bilirubin serum levels and release of transaminases.5 Similar to humans, mice develop bile duct and septal proliferations with leukocyte infiltration and liver fibrosis.5 In contrast with rats and humans, however, mice develop substantial tissue injury with biliary necrosis formation in response to tBDL; but this injury does not proceed to liver cirrhosis.5,6 One explanation for this difference between humans and mice is that a malignant bile duct obstruction requiring liver surgery (eg, tumors) is a slow and continuous process in humans and is usually detected before a total occlusion of the bile duct.

Moreover, the extensive necrosis formation after tBDL in mice might hamper the results and relevance of many animal experiments, because
they might reveal effects of necrosis formation in general rather than specific consequences of cholestasis.

To establish a model that is closer to the human situation, we searched for a new, reproducible model of acute cholestasis which is associated with less cholestatic injury. To this end, we developed a novel technique of partial bile duct ligation (pBDL). The rationale for this model is to maintain a limited bile flow by a subtotal obstruction of the bile duct. Also, we evaluate several potential mechanisms of cholestatic liver injury.

METHODS

Animal experiments. Animal experiments were performed in 8- to 10-week-old male C57Bl/6 mice (Harlan, Horst, The Netherlands). Mice were fed a laboratory diet with water and food ad libitum until use and were kept under constant environmental conditions with a 12-hour light-dark cycle. The respective number of animals per group \((n = 6–10)\) is provided separately for each experiment. Animals received humane care in compliance with guidelines for experimental animals from the Zurich Veterinary Institution.

Operative techniques. All operative interventions were performed under constant isoflurane inhalation. Buprenorphin (0.1-mg/kg body weight) was applied intraperitoneally during anesthesia, and was repeated subcutaneously 8–12 hours later and when clinically indicated.

After median laparotomy, the gallbladder was removed to avoid cholecystitis by ligation of the cystic duct and excision of the gallbladder out of its bed. Then, the bile duct was mobilized at the upper margin of the pancreas. For tBDL, the bile duct was transected between 2 ligations (9-0 nylon; S&T, Neuhausen, Switzerland). For pBDL, a 7-0 curved needle (Φ 0.2 mm, CC Prolene; Ethicon, Warsaw, IN) was placed next to the bile duct, and the bile duct was then ligated around this needle using 9-0 nylon (S&T). After securing the ligation, the needle was removed, and a defined lumen was left to allow some bile flow (Fig 1). At the end, the abdomen was closed by a 6-0 double-layer running suture, and the animal was allowed to wake up on a heating pad.

Biochemistry. After pBDL and tBDL, serum was collected from the tail vein at indicated time points, and bilirubin, aspartate aminotransferase (AST), and AP were measured in serum using the Ektachem system (Johnson & Johnson, New Brunswick, NJ). Total bile acids in the serum were measured by high-performance liquid chromatography as described previously.

Histology and immunohistochemistry. Tissues were immersion fixed in 4% buffered formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin or Sirius red, using standard histologic techniques. Ki67 staining was used to quantify hepatocyte proliferation. Tissue sections were incubated with primary anti-Ki67 antibody (monoclonal rabbit clone SP6, 1:100;
NeoMarkers, Fremont, CA) and immunoreactivity was detected with the DAB Map kit (Ventana, Tucson, AZ) using the Ventana Discovery automated staining system. Sections were counterstained with hematoxylin. The percentage of Ki67 positive hepatocytes to visible hepatocytes was determined in seven representative visual fields (original magnification, × 40).

**Determination of hepatic necrosis.** Hepatic necrosis was determined on hematoxylin and eosin slides in 10 high-power fields (original magnification, × 10). Necrosis formation was measured by morphometry using a semi-computed system (Saisam, France) and expressed as proportion of hepatic necrosis to normal liver tissue (hepatic replacement area).

**Measurement of biliary pressure.** For these experiments, the gallbladder was not removed to measure the pressure in the biliary system via the gallbladder. An 1.8-French, saline-filled tube was connected to the sensor of the Servomed (Hellige, Freiburg, Germany) machine and directly inserted into the gallbladder. To avoid bile leakage, the tip of the tube was sharply prepared and the quickly inserted into the extended gallbladder. The pressure was then measured after a short period of equilibration.

**Statistical analysis.** All values are expressed as means ± standard deviation (SD). The Student t test was used for continuous variables to test whether differences between 2 groups were different. For categorical data the χ²-test was used. P < .05 was considered significant.

**RESULTS**

**Does pBDL cause cholestasis?** We first evaluated, whether the partial obstruction of the bile duct causes cholestasis by measuring bilirubin and AP levels in the serum (n = 10). These parameters were serially determined on days 3, 5, 7, 10, and 14 in the same mice and compared with mice that were subjected to tBDL.

Total BDL resulted in an immediate increase in bilirubin and AP which remained constant during 14 days. Bilirubin and AP serum levels within the first 5 days after pBDL did not differ from tBDL (Fig 2). Thereafter, cholestatic parameters decreased and reached normal values within 10 days after pBDL.

**Is the long-term outcome different between tBDL and pBDL?** Body weight was serially assessed during the 14 days experiment in the same mice (n = 10), because rodents loose weight in response to obstructive jaundice. Mice lost a significant proportion of their body weight after tBDL, which persisted during the entire duration of the experiment. The body weight after pBDL did not differ from tBDL during the first week of cholestasis. However, mice gained weight in the second week after pBDL and reached 95% of their initial body weight within 14 days (P = .04).

**Does pBDL result in less hepatic injury?** We assumed that the partial ligation of the bile duct would result in a milder form of cholestasis, and by this in less hepatic tissue injury. As outlined, AP and bilirubin serum levels did not differ between both models in the first week of the experiment. Therefore, we evaluated whether the tissue injury of obstructive jaundice would also be comparable. We serially determined the AST release (n = 10) in the serum on days 1, 3, 5, 7, 10, and 14, and assessed the hepatic replacement area of hepatic necrosis (n = 6) in both models at different time points.

The AST release was comparable between both models during the first week of the experiment. Although the AST values remained elevated during the entire experiment after tBDL, they normalized in the pBDL group, during the second week of the experiment (Fig 3, A).
Hepatic necrosis formation peaked on day 3 after tBDL. Despite the equality of laboratory parameters for cholestasis and hepatocyte injury, the mean hepatic replacement area for necrosis was significantly smaller after pBDL compared with tBDL on day 3 (5% vs 16%; P = .001). Although hepatic necrosis was basically absent 14 days after pBDL, some necrosis was present until day 14 after tBDL (Fig 3, B).

**Does liver regeneration correlate with tissue injury?** Because the liver regenerates after tissue loss and injury, we next evaluated whether the observed cholestatic liver injury also induced DNA synthesis. Therefore, we used immunohistochemistry for the cell-cycle protein Ki67 to quantify the amount of DNA synthesis (n = 6).

Indeed, we observed significant DNA synthesis from day 3 on, which peaked on day 5 after tBDL. Thereafter, a low proliferative activity persisted until day 14 after tBDL. The DNA synthesis after pBDL was significantly lower. The peak of DNA synthesis on day 3 was absent after pBDL, and we did not observe DNA synthesis later on (Fig 4).

**Does pBDL cause histologic long-term changes of cholestasis?** Chronic cholestasis results in liver fibrosis and ductular proliferation with lymphocyte infiltrates in mice. Therefore, we evaluated these changes in mice at day 14 (n = 10) after tBDL and pBDL. The most prominent findings 14 days after tBDL were bridging fibrosis and bile duct proliferation. Furthermore, the portal fields revealed a mixed infiltrate of neutrophils and lymphocytes. In contrast, histology revealed only mild histologic changes 14 days after pBDL (Fig 5). We did not observe hepatic necrosis, and only little ductular proliferation but no bridging fibrosis were evident.

**What is the mechanism of hepatic necrosis formation in cholestatic livers?** Although both models of cholestasis caused a similar degree of cholestasis according to serum levels of bilirubin, AST, and AP, the morphologic tissue injury was significantly more severe after tBDL. Because the remnant lumen after pBDL allows minimal bile flow, we hypothesized that the pressure in the biliary tree would be lower after pBDL. Consequently, the regurgitation of bile acids into the serum should be lower after pBDL. To explore these hypotheses, we measured the biliary pressure and serum bile acid concentration in both models (n = 6) on the day of maximal histologic injury (day 3). To assess the course of both parameters, we also determined the biliary pressure and the serum bile acid concentrations 1 day after pBDL or tBDL.

We observed increased biliary pressures in both models from day 1 on. Although the biliary pressure decreased on day 3 of the experiment after pBDL, it further increased after tBDL, and was higher after tBDL than after pBDL on day 3 (P = .02; Fig 6, A). Furthermore, bile acid serum concentrations were significantly elevated in both models, and we observed a constant increase of the serum bile acid levels in both models until day 3, when bile acids were significantly higher after tBDL than after pBDL (P = .04; Fig 6, B).
DISCUSSION

The standard model for research in cholestasis is the tBDL model in rats and mice, in which the common bile duct is transected between a double ligation. In mice, this model reveals a biphasic course. The first phase is characterized by extensive hepatic necrosis and neutrophil infiltrates followed by cholangiocyte and hepatocyte proliferation. The second phase is characterized by tissue fibrosis and lymphocyte infiltrates. Between days 5 and 10 after tBDL, both phases of acute (phase 1) and chronic (phase 2) cholestasis overlap. Although the tBDL model is highly reproducible, it is hampered by several issues: The extensive tissue injury within the first 3 days negatively influence on animal experiments because tissue samples may not resemble specific consequences of cholestatic liver tissue but liver necrosis in general. Furthermore, this model does not result in liver cirrhosis in mice, which is a classic feature of chronic cholestasis in humans and rats. Another model for cholestasis research in mice is the selective ligation of bile duct branches. The advantage of this model is that the cholestatic injury only occurs in the ligated lobes without a major systemic effect. However, the

Fig 5. Liver histology 14 days after tBDL (A, C, E) and pBDL (B, D, F). Hematoxylin and eosin staining shows only minimal tissue necrosis 14 days after tBDL, which is not detectable after pBDL (A, B; original magnification, × 2.5). Also, Sirius red staining demonstrates extensive bridging fibrosis after 14 days after tBDL (B; original magnification, × 2.5). Upon greater magnification of portal fields, mixed portal infiltrates of neutrophils and lymphocytes (E; original magnification, × 10) are visible after tBDL. Also, an extensive ductular proliferation is visible after tBDL (E; original magnification, × 2.5). In contrast, the histologic architecture was basically normal 14 days after pBDL (B, D; original magnification, × 2.5) with only little leukocyte infiltrates and biliary reaction (F; original magnification, × 10). (Color version of figure is available online.)
changes within the affected lobe do not differ from the tBDL model. To overcome these shortcomings of the tBDL model, we developed a model of (incomplete) pBDL.

Although the standard laboratory markers of cholestasis failed to demonstrate a difference between the 2 models of cholestasis during the acute phase, the pBDL model was consistent with the anticipated advantage of less tissue injury. This correlated well with the pressure in the biliary system and serum levels of bile acids on day 3, the time point of maximal tissue injury. In contrast with the tBDL model, all laboratory parameters and histologic changes normalized during the second week after pBDL with only slight changes remaining.

We tested several techniques of pBDL, including internal stents and different external spacers. The implantation of a stent into the common bile duct to achieve an internal reduction of the bile duct lumen is feasible but technically very demanding, limited by the availability of appropriate tubes and associated with delayed obstruction. The ligation of the common bile duct around a spacer as proposed herein is technically easy and highly reproducible. Moreover, needles with various predefined diameters are available. After testing needles with different diameters, we found an 0.2-mm diameter to be most appropriate.

Because the insertion of a tube into the common bile duct would automatically cause a complete occlusion, we measured the biliary pressure in both models indirectly through the gallbladder assuming equivalent pressures in the common bile duct and the gallbladder. This assumption seems to be correct because the biliary pressure in the tBDL group was similar to values reported in the literature. Furthermore, we determined the total bile acid concentrations in the serum using a standardized and validated technique owing to the persistent changes in the bile acid concentrations in response to tBDL. Both parameters were comparably elevated on day 1 of both models, and further increased until day 3 of tBDL. Most important, both parameters were significantly higher after tBDL on day 3, the day of maximal tissue injury.

Tissue injury confers to biliary necrosis, which is a well-described phenomenon after tBDL in mice. Although also detectable after pBDL, the peak of hepatic necrosis formation was significantly lower after pBDL. The ligation of the peribiliary plexus alone does not cause hepatic necrosis, and mice only develop hepatic necrosis after a complete dearterialization of the liver. Therefore, an ischemic etiology of hepatic necrosis formation is unlikely. In contrast, the increased pressure in the biliary system after its ligation is presumably the most important mechanism of tissue injury. Consequently, cholangiocytes and hepatocytes may be more susceptible to bile acid toxicity, which may then aggravate the tissue injury.

Although the severity of cholestasis is generally attributed to the standard serum markers bilirubin, AP, and AST, these parameters were inferior to the serum bile acid concentrations and the biliary pressure in terms predicting the maximal histologic injury in our experiments on day 3. Therefore, the value of the serum bile acid concentration should be further evaluated in clinical trials as predictor of cholestatic liver injury, because the relevance of the magnitude of the bilirubin level is controversial.

In contrast with the tBDL model, biopsies from livers 14 days after pBDL failed to reveal significant histologic abnormalities, and all serum markers for cholestasis were normal. Two potential reasons for this spontaneous normalization may be considered. First, the operative manipulation might have caused a temporary complete obstruction of the bile duct, for example, owing to postinterventional local edema. Second, the slight increase in biliary

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**Fig 6.** Biliary pressure (A) and serum bile acid (B) concentrations were measured 1 and 3 days after sham-OP, pBDL (gray) and tBDL (black). The biliary pressure and the serum bile acid concentrations comparably increased on day 1 in both models. Thereafter, the biliary pressure ($P = .02$) and the serum bile acid concentrations ($P = .02$) were higher after tBDL than after pBDL on day 3.
pressure compensates the stenosis resulting in an improved flow. We have not found a prove for a temporary complete obstruction: Fig 1, D demonstrates the patency of the biliary lumen after pBDL, and Fig 1, E excludes a focal edema on day 1 after pBDL. Therefore, we favor the adaption of the biliary pressure and flow as cause of the normalization of cholestasis during the second week after pBDL as potential mechanism. Furthermore, this adaption process might also explain the biphasic course of the tBDL model. However, the exact mechanism remains unclear to date.

Consequently, experiments on acute cholestatic injury should be performed within the first 5 days of pBDL. During the second week after pBDL, this model may additionally be used to study the mechanisms of resolved acute cholestasis at various time points without the necessity of further operative procedures (eg, hepaticojunostomy). This may be particularly attractive to study the changes, for example, in transporter expression and the optimal timing of liver surgery in patients with Klatskin tumors or after complex bile duct injuries from laparoscopic cholecystectomy.

In conclusion, we have established the pBDL model as a model of pure acute cholestasis. This model seems to be highly suitable for research in acute cholestatic liver injury, because it reproducibly causes cholestasis with minimal histologic tissue injury and does not proceed to chronic cholestasis. The histologic tissue injury after BDL is presumably due to the increase in biliary pressure and consequent elevation of the serum bile acids. Moreover, the pBDL model seems to be ideal to evaluate the late effects of reversed cholestasis. The tBDL model remains the best model for research in chronic cholestasis.

REFERENCES