have intact pro-oxidant/anti-oxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism [21]. When additional oxidative events occur, the pro-oxidant systems outbalance the anti-oxidant systems, potentially producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids, ultimately leading to cell death in severe oxidative stress [20]. Mild, chronic oxidative stress may alter the anti-oxidant systems by inducing or repressing proteins that participate in these systems, and by depleting cellular stores of anti-oxidant materials such as glutathione and vitamin E [22].

Oxidative stress and ROS play a crucial role in the induction and progression of various liver diseases ranging from acute hepatitis to hepatocellular carcinoma [23].

There are several reports that address the relationship between hepatic tissue injury and oxidative stress. Furthermore, the relevance of cellular redox imbalance in liver diseases is outlined by a number of studies demonstrating a correlation between liver damage and an increase in pro-oxidant cellular markers [23–27].

ROS are able to perpetuate and potentiate their own effects by acting on the transcription and activation of a large variety of cytokines and growth factors [27]. Nyhan et al. discussed the post-LTx attacks of metabolic decompensation in PPA and MMA [28].

The higher levels of serum C5a and TGF-β1 along with the higher OSI in the patients who received LTx due to an underlying IMD compared to the recipients who received LTx due to BA is probably due to the difference between a local hepatic disease like BA and a systemic diseases like IMD, especially PPA and MMA [29,30]. Furthermore, the strong negative correlation between serum antioxidants represented by the serum BAP and both C5a and TGF-β1 are in alignment with the hypothesis that the immunological stimulation and complement activity in the IMD group participated in depleting cellular stores of anti-oxidants and that these patients were prone to higher oxidative stress, as represented by their significantly higher serum OSI.

TGF-β1 is synthesized, with only a few exceptions, by virtually all cells, and TGF receptors are expressed by all cells. TGF-β1 affects nearly every physiological process in some way; its systemic and cell-specific activities are complicated. There are, however, three fundamental activities: TGF-β1 modulates cell proliferation, generally as a suppressor, and it enhances the deposition of extracellular matrix through the promotion of synthesis and the inhibition of degradation [8]. The specific action of TGF-β1 on a particular cell depends on the exact circumstances of that cell's environment.

In liver diseases, TGF-β1, which is a chief cause of liver fibrosis [6], has been proven to induce the phenotypic transition of hepatic stellate cells into proliferating myofibroblast-like cells, thus enhancing the production of extracellular components [7]. Mediators that suppressed TGF-β1 therefore caused less hepatic fibrosis in an animal model [9].

Conclusions

Our results demonstrate, for the first time, that patients who received LRLTx due to underlying IMD face more oxidative and immunological stress, most probably caused by their original disease. As a result, they face more damage to their grafted livers, and a higher risk of graft rejection. Although these findings require confirmation and amplification with a larger number of patients, the data provide insight into potential pathogenic mechanisms and may lead to protective therapies against graft rejection in pediatric patients receiving LRLTx due to underlying IMD.

Serum OSI is calculated from data acquired through a simple outpatient procedure and can serve as an index of an LRLT recipient's oxidative status. It has been proven useful in the follow-up examinations of patients who have undergone LTx due to IMD.

Accordingly, a selective form of immunosuppressive therapy targeting C5a, TGF-β1, and oxidative stress is probably advisable for pediatric patients receiving LRLTx due to underlying IMD, and the use of the serum OSI could be useful for their follow-up examinations.

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REVIEW ARTICLE

Redo surgery for biliary atresia

Masaki Nio · Hideyuki Sasaki · Hiromu Tanaka · Atsushi Okamura

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Abstract The Kasai redo surgery is important for treating biliary atresia. In the era of liver transplantation (LTx), pediatric surgeons must accurately select patients for redo surgery and ensure that potential LTx can be performed later. Although optimal timing for redo varies among cases, appropriate timing is essential. We reviewed the significance, optimal timing, operative procedures, and indications of Kasai redo surgery. Between 1989 and 2011, 2,630 patients were registered in the Japanese Biliary Atresia Registry (JBAR), and the data collected from JBAR regarding Kasai redo surgery were analyzed. Patients were divided into two groups, Group 1 (1989–1999, $n = 1{,}423$) and Group 2 (2000–2011, $n = 1{,}207$). The redo incidence significantly reduced in Group 2. Although no significant difference was found in the native liver jaundice-free survival rates between the two groups, the overall survival rate at initial registry was significantly higher in Group 2. This may be because of the limited number of patients selected for redo and increased availability of early LTx. Patients who achieved sufficient bile drainage following the initial Kasai surgery but developed sudden bile flow cessation were the best candidates for Kasai redo surgery; it should be performed only once for this subset.

Keywords Biliary atresia · Kasai portoenterostomy · Redo surgery · Liver transplantation

M. Nio (✉) · H. Sasaki · H. Tanaka · A. Okamura
Division of Pediatric Surgery, Department of Reproductive and Developmental Medicine, Tohoku University Graduate School of Medicine, Seiryo-machi 1-1, Aoba-ku, Sendai 980-8574, Japan
e-mail: mnio@ped-surg.med.tohoku.ac.jp

Introduction

The Kasai surgery for biliary atresia was developed more than 50 years ago. Since then, the surgical outcomes have markedly improved, and an increasing number of patients have survived with their native livers for very long periods. In the era of liver transplantation (LTx), the Kasai surgery is still regarded as a first-line surgical procedure for patients with biliary atresia. However, a substantial number of patients have ultimately required LTx; therefore, adequate consideration for LTx is essential at the time of the Kasai surgery and during postoperative patient care. The Kasai redo surgery has played an important role for patients who developed recurrent jaundice following the initial Kasai surgery. Because the role of the redo surgery may have changed in the era of LTx, the significance, optimal timing, operative procedures, and indications of the Kasai redo surgery were reviewed.

The significance of the Kasai redo surgery

Before the era of LTx, the Kasai surgery was the only procedure that could prolong survival for patients with biliary atresia. In these circumstances, the Kasai redo surgery was commonly performed, and patients with poor bile drainage often underwent as many as three or more surgeries.

In Japan, LTx became available in the early 1990s, a time when several arguments regarding the Kasai surgery were introduced. For instance, the Suruga II procedure, a reconstructive procedure with an external biliary conduit to prevent cholangitis that was widely performed in Japan, was abandoned because transplant surgeons criticized this procedure for impairing the LTx outcomes [1]. The role of

Kasai redo surgery was also discussed. Since the advent of LTx, an alternative procedure offering increased survival has become widely available, and the necessity and indications for the Kasai redo surgery, which may have an adverse impact on the LTx outcome, have become major topics of debate. The role of the Kasai redo surgery has apparently been changing.

Some researchers have reported that the Kasai redo surgery should not be performed because more than 70 % patients with biliary atresia have ultimately required LTx [2]. In addition, other researchers have advocated that instead of the Kasai surgery, LTx should be performed as a primary surgery [3, 4]. However, the LTx surgical outcomes have reportedly been worse in infants than in older patients, regardless of whether LTx was the primary or the rescue procedure following a failed Kasai surgery. It would be a great advantage if the Kasai redo surgery could re-establish good bile drainage or delay the need for LTx [5, 6].

Although LTx surgical outcomes have markedly improved, the possibility of surgical mortality and morbidity still exists, and long-term LTx outcomes have not yet been fully elucidated. The problems associated with LTx, such as high medical costs and shortage of donors, need to be resolved. Currently, the policy of proceeding with an appropriate combination of LTx and the Kasai surgery, including redo surgery, is preferred for selected patients [7].

Optimal timing for the Kasai redo surgery

The optimal timing for the Kasai redo surgery, which also has been a topic of debate, has not yet been determined. Lilly et al. [8] advocated prompt redo when required. One recommendation was that the redo should be performed as a quasi-emergency surgery [9] and another was that the period between the cessation of bile drainage and the redo should be less than 1 month [10]. In contrast, native liver survivors, who underwent the Kasai redo surgery several months after developing recurrent jaundice, have been reported [11]. Another author has reported that even considerably late redo surgeries have been successful, particularly in older patients in whom good bile drainage following the initial Kasai surgery was once established but suddenly stopped [12]. Recurrent jaundice because of cholangitis is likely to result in irreversible persistent jaundice leading to liver failure in infants. Granulation tissue completely blocks the biliary fistula of the porta hepatis immediately during the early postoperative course following the initial Kasai surgery; thus, prompt redo must be seriously considered in these patients. However, in older children, internal fistulae between intrahepatic bile ducts and the jejunum at the porta hepatis are well developed, and recurrent jaundice often completely recovers after medical treatment using antibiotics, ursodeoxycholic acid, and steroids. Although the optimal timing for the Kasai redo surgery may vary among cases, care should be taken to ensure appropriate timing because objective methods for assessment of the appropriate timing for each patient do not exist.

In our institution, we decided that the indication for the Kasai redo surgery is 1 month after the initial Kasai surgery, if the patient developed cessation of bile drainage immediately after the surgery. In older patients with recurrent jaundice, we try all other medical treatments before surgery. In addition, the indications for redo surgery should be considered after a complete assessment of liver pathology, hepatic functional reserve capacity using 99mTc-GSA scintigram, availability of LTx, and the patient's social background.

Procedural details of the Kasai redo surgery

Some authors have reported the efficacy of granulation tissue curettage at the porta hepatis during Kasai redo surgery or when using endoscopic techniques without reanastomosis [13, 14]. However, many pediatric surgeons have followed a procedure similar to that used during the initial Kasai surgery, which included dividing the previous hepatic anastomosis, resecting the granulation tissue, and creating a new anastomosis between the porta hepatis and jejunum in the redo surgery [7, 10].

Technically, the Kasai surgery involves initiating peritoneal adhesiolysis away from the vicinity of the porta hepatis where dense adhesions usually exist, taping the hepatoduodenal ligament during the early stage, preparing for Pringle's maneuver in case of unexpected bleeding, and dissecting in the vicinity of the hepatic hylum, taking particular care not to injure the Roux-en-Y limb to achieve secure reanastomosis. Resection of the granulation tissue and reanastomosis are similar to the maneuvers used in the initial Kasai surgery.

Indications for the Kasai redo surgery

The Kasai redo surgery may offer long-term native liver survival or increase the time until LTx is required in patients who developed cessation of bile flow following the initial Kasai surgery. In contrast, it can cause increased difficulty in LTx. Patient selection for the Kasai redo surgery is a topic of debate between pediatric surgeons and transplant surgeons.

Forty-nine patients who underwent the Kasai redo surgery in our institution between 1954 and 1990 were divided

into the following two groups, according to their postoperative courses following the initial Kasai surgery: patients who had achieved good bile drainage, Group A ($n = 29$) and patients with poor or no bile drainage, Group 2 ($n = 20$). Jaundice was resolved in 21 of 29 patients (74 %) of Group A and in no patients in Group 2. We concluded that we should perform the Kasai redo surgery only in patients with good bile drainage following the initial Kasai surgery [15]. Similar results have been reported from other institutions in other countries [8, 16].

Transplant surgeons have claimed that multiple Kasai surgeries were associated with increased operative blood loss [17], longer surgical time [7], and higher LTx morbidity [18]. Because of these reports, pediatric surgeons are hesitant to perform Kasai redo surgeries. Other researchers have reported that the Kasai surgery, including the redo surgery, did not definitively affect LTx outcomes [8, 19], and the benefit of Kasai redo surgery should be appreciated.

Currently, a consensus has been reached that patients who achieved sufficient bile drainage following the initial Kasai surgery and then developed sudden cessation of bile flow were the best candidates for Kasai redo surgery, and it should be performed only once for this selective subset of patients [7, 19].

Pediatric surgeons should strive to appropriately perform the initial Kasai surgery, properly select patients for the redo surgery, and ensure that LTx remains possible in the event that may be required later.

Analysis of data from the Japanese Biliary Atresia Registry (JBAR)

According to the data from the Japanese Liver Transplantation Society, a total of 6,195 liver transplants were performed in 65 institutions in Japan till 2010. The most frequent indication was cholestatic disease, including biliary atresia, followed by neoplastic disease. The trend in the number of patients who underwent LTx markedly increased during the 1990s in Japan (Fig. 1) [20].

The JBAR was initiated in 1989 by the Japanese Biliary Atresia Society. The aim of the JBAR is to study the epidemiology and etiology of biliary atresia and to improve surgical outcomes of the Kasai surgery. Each patient is followed up for 30 years. The initial registry data for each year were collected until August of the next year when the patient underwent the initial Kasai surgery. In total, 2,630 patients were registered in the JBAR until 2011 (Fig. 2) [21].

We used the JBAR data to analyze the incidence and outcomes of Kasai redo surgery. Patients were chronologically divided into two groups, Group 1 (1989–1999, $n = 1{,}423$) and Group 2 (2000–2011, $n = 1207$). The majority of patients in both groups underwent the Kasai surgery (Table 1). Data regarding the jaundice disappearance rate (JDR) after the initial Kasai surgery, incidence of Kasai redo surgery, JDR after redo surgery, native liver jaundice-free survival rate, and overall survival rate at the time of registration every year were compared between the two groups.

The JDR after the initial Kasai surgery was 63 % in Group 1 and 61 % in Group 2 (ns). The incidence of the redo surgery was 28 % in Group 1 and 15 % in Group 2 ($p < 0.0001$). The JDRs after redo surgery were 34 and 36 % (ns) and the native liver jaundice-free survival rates were 57 and 55 % in Groups 1 and 2, respectively (ns). The overall survival rate was 90 % in Group 1 and 95 % in Group 2 ($p < 0.0001$) (Tables 1, 2).

The incidence of Kasai redo surgery was significantly reduced in Group 2. Furthermore, no significant difference was observed in the native liver jaundice-free survival rate between the two groups, but the overall survival rate was significantly higher in Group 2. These results may be attributed to the improved availability of early LTx and limited number of patients selected for the redo surgery.

The JDRs were essentially the same between the two groups. Although no obvious improvement in the Kasai surgery outcomes was achieved for this time period, improved availability of LTx in recent years did not affect the Kasai surgery outcomes. The reduction in the incidence of Kasai redo surgery by ~50 % may be explained by

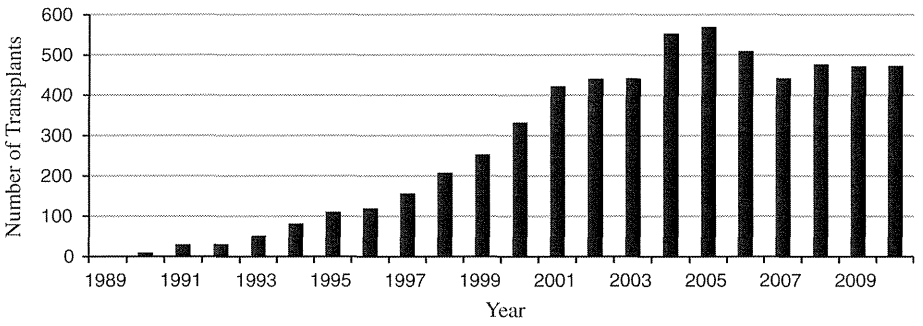

Fig. 1 Trend in the number of recipients of liver transplantation in Japan [20]. A total of 6,195 liver transplants were performed in 65 institutions in Japan until 2010. The number of recipients of liver transplantation markedly increased during the 1990s in Japan

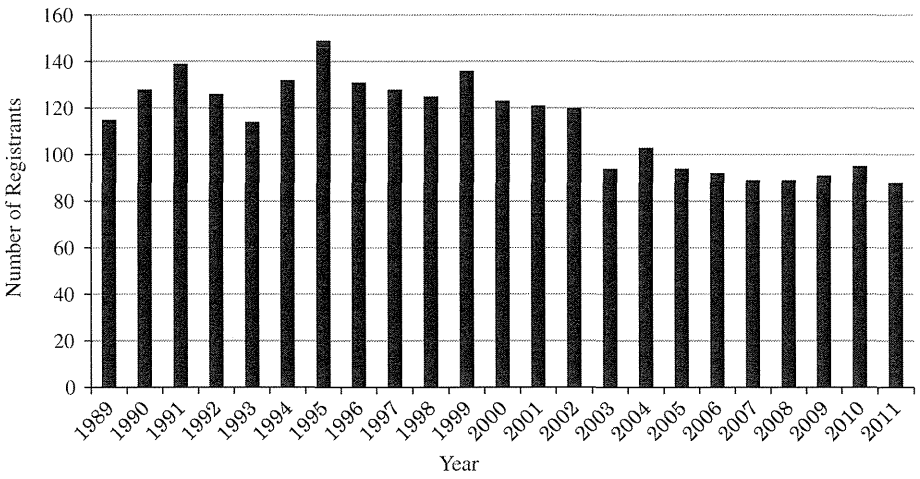

Fig. 2 Trend in the number of Japanese Biliary Atresia Registry (JBAR) registrants [21]. The number of Japanese Biliary Atresia Registry (JBAR) registrants in each year was between 110 and 150 before 2003 and approximately 90 in 2003 or later. A total of 2,630 patients were registered until 2011

Table 1 The initial surgery for registrants of Japanese Biliary Atresia Registry (JBAR) [21]

Period	1989–2011	1989–1999	2000–2011
Initial surgery			
Kasai surgery	2,598	1,415	1,183
LTx	15	2	13
Expl Lap	12	2	10
Not Performed	5	4	1

LTx liver transplantation, *Expl Lap* exploratory laparotomy

Table 2 Status of the registrants at initial registration to the Japanese Biliary Atresia Registry (JBAR) [21]

Period	1989–2011	1989–1999	2000–2011	*p*
N	2,630	1,423	1,207	
JDR % after the initial Kasai surgery	61	63	61	ns
Redo %	21	28	15	<0.0001
JDR % after redo	35	34	36	ns
NLJF survival %	56	55	57	ns
Overall survival %	93	90	95	<0.0001

JDR jaundice disappearance rate, *NLJF* native liver jaundice free

patient selection. However, if patients were appropriately selected, the JDR should have been considerably elevated. Good candidates for Kasai redo surgery may have missed their chance and directly undergone LTx. The issue of appropriate patient selection for redo surgery needs to be resolved.

In conclusion, a consensus has been obtained to some extent regarding the indications, optimal timing, and techniques of Kasai redo surgery, but further discussion is needed along with improvements in both the Kasai surgery and LTx outcomes. Currently, patients who develop sudden cessation of bile flow after achieving sufficient bile drainage following the initial Kasai surgery are the best candidates for Kasai redo surgery, and redo surgery is employed only once for this selective subset.

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ORIGINAL ARTICLE

Multicenter randomized trial of postoperative corticosteroid therapy for biliary atresia

Japanese Biliary Atresia Society · Masaki Nio · Toshihiro Muraji

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Abstract
Purpose We aimed to evaluate early response to two different corticosteroids doses after Kasai portoenterostomy for biliary atresia (BA).
Methods A prospective, randomized trial was performed in infants from the nationwide BA registry with type 3 BA. Sixty-nine infants were randomized to receive either 4 mg/kg/day (group A, $n = 35$) or 2 mg/kg/day prednisolone (group B, $n = 34$). The corticosteroids were started on postoperative day 7, and the dose was tapered toward day 30. Results of liver function tests on days 31 and 60 were compared between the groups.
Results Mean bilirubin, AST, ALT, and GGT levels did not significantly differ between the groups. However, the levels of total and direct bilirubin of infants <70 days old at surgery significantly differed between the groups. Four patients from group A and five from group B, dropped out of the study. Complications during the first month after PE were comparable between the groups.
Conclusions An initial 4 mg/kg/day dose did not significantly improve liver function, except that bilirubin levels were lower in the subgroup of infants <70 days old at surgery. There were no significant complications with either dose of corticosteroids.

Keywords Biliary atresia · Corticosteroid · Prospective randomized trial · CMV infection

Introduction

Corticosteroids have been used empirically since many years in Japan for enhancing bile flow after Kasai portoenterostomy (PE) for biliary atresia (BA). The use of steroids was found in Kasai's early publication, in which the dose of prednisolone was 20 mg/day, which equated to approximately 4 mg/kg/day [1]. Karrer and Lilly [2] subsequently reported a more aggressive use of steroids. Multiple retrospective uncontrolled studies [3–8] showed that the administration of steroids after Kasai PE for BA was beneficial. Furthermore, in 2004, the Japanese Biliary Atresia Society (JBAS) performed a nation-wide retrospective survey involving postoperative steroid use [9] and reported that only marginally significant differences in native liver survival rates between patients receiving ≥4.0 mg/kg/day steroids and a non-steroid group. Complications were uncommon when steroid administration was started 1 week after surgery.

We conducted a prospective, multi-center, randomized trial at institutions registered to JBAS to test the hypothesis that postoperative administration of 4 mg/kg/day corticosteroids compared to a 2 mg/kg/day dose starting 1 week after Kasai PE would increase bile flow, and that this dose would facilitate alleviation of jaundice in the early postoperative period in a large proportion of infants. This paper summarizes our results and details the adverse effects

Japanese Biliary Atresia Society
Departments of Pediatric Surgery, Tohoku University, Sendai, Japan

M. Nio
Division of Pediatric Surgery, Department of Reproductive and Developmental Medicine, Tohoku University Graduate School of Medicine, Seiryo-machi 1-1, Aoba-ku, Sendai 980-8574, Japan
e-mail: mnio@ped-surg.med.tohoku.ac.jp

T. Muraji (✉)
Department of Pediatric Surgery, Ibaraki Children's Hospital, 3-3-1 Futabadai, Mito, Ibaraki 311-4145, Japan
e-mail: t-muraji@ibaraki-kodomo.com

encountered during the study, which was conducted from July 2006 through August 2011.

Patients and methods

We included patients with type 3 (obstruction at the porta hepatis) BA. Patients with intrahepatic bile ducts visualized on operative cholangiography, so-called "correctable BA", were excluded because in the correctable cases, good bile drainage is often achieved without exogenous steroid administration. Postoperative prophylactic antibiotics were standardized: intravenous cephems and aminoglycosides for the first 2 weeks, followed by oral trimethoprim/sulfamethoxazole for the next 16 days. Ursodeoxycholic acid (20 mg/kg/day) was given from day 7. No other choleretics such as phenobarbital were administered.

CMV infection was a concern among some of Japanese pediatric surgeons. We have circulated guidelines for the diagnosis and recommended management of CMV infection during the steroid challenge. Briefly, the management guideline for CMV infection is as follows: (1) preoperative determination of CMV-IgM; (2) if negative, the study proceeds. Baseline determination of CMV in urine or quantification of CMV-DNA in blood before POD 7 is recommended for future reference; (3) if positive, initiation of ganciclovir is strongly recommended at the time of seroconversion with deterioration of liver function during the steroid challenge.

The parents of eligible patients were approached to participate in this study, either preoperatively or within a few days postoperatively. After informed consent was received from patients' parents, the patients were randomized by the JBAS office into two groups (Fig. 1): group A, receiving prednisolone at 4 mg/kg/day (divided in 2 doses) orally starting on the seventh postoperative day (POD) or intravenously if oral intake was not started, with dose tapered by 1 mg/kg/day every 5 days until POD 26 or group B, receiving prednisolone at 2 mg/kg/day, one dose orally starting on POD 7 or intravenously if oral intake was not started. This dose was tapered by 1 mg/kg/day every 7 days until POD 26. In both groups, the dose was 0.5 mg/kg/day from POD 27 to 30. The total dose was 52 mg/kg for group A and 29 mg/kg for group B. A subgroup analysis was also performed in those infants who were less than 70 days old at surgery ($n = 38$) between the same two different regimens as group A and B.

The primary outcomes were laboratory values for liver function tests at 1 and 2 months after surgery, including levels of serum total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl-transpeptidase (GGTP). Ethical approval for the study was obtained at individual institutional review board in each institution and written informed consent was obtained from parents or each patient's guardian before administration of steroids.

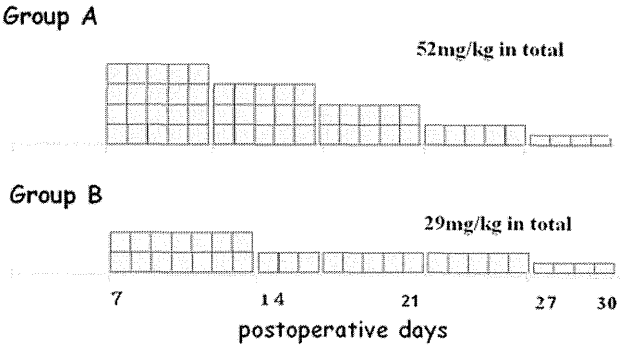

Fig. 1 Corticosteroid regimens in two groups. Corticosteroids were started from the seventh POD in both groups. Group A (*above*): 4 mg/kg/day for 5 days as an initial dose which was tapered every 5 days over the subsequent 3 weeks. A total dose is 52 mg/kg. Group B (*below*): 2 mg/kg/day for 7 days as an initial dose and a half dose was maintained over the subsequent 13 days. *One box* indicates 1 mg/kg/day. A total dose is 29 mg/kg

Before the study, a sample size was set based on the results of data analysis using sample cases from institutions wherein steroid regimen was identical to that of either group A or group B. Among the patients in the sample cases with preoperative serum bilirubin levels >7.0 mg/dL, the highest postoperative bilirubin level was 3.95 and the lowest level was 2.96, with a standard deviation of 2.15, at 1 month after surgery. Statistical analysis was performed using the Student t test with an α level of 0.05 and $1 - \beta = 0.8$. This suggested that 71 infants should be recruited into each arm of the trial. Categorical data were compared with Fisher exact test and nonparametric comparisons of liver function tests were performed with a Mann–Whitney test to assess the significance of differences. A difference was regarded as significant at $p < 0.05$.

Results

In total, 69 postoperative patients were randomized to receive an initial prednisolone dose of either 4 (group A) or 2 mg/kg/day (group B). Group A comprised 35 patients (male patients 14, female patients 21), with age at Kasai PE ranging from 21 to 153 days (median 63 days). Group B comprised 34 patients (male patients, 12; female patients, 22), with the age at Kasai PE ranging from 20 to 111 days (median 60 days). No differences were observed in the preoperative demographics between groups A and B (Table 1).

No differences were observed in the postoperative liver function tests between groups A and B. However, the mean levels of total bilirubin and direct bilirubin were

Table 1 Preoperative liver Function tests and age at Kasai portoenterostomy

	Group A ($n = 35$)	Group B ($n = 34$)	p
Age at Kasai operation (days)	63 (21–153)	60 (20–111)	NS
BASM	1	1	NS
Age at kasai operation <70 days	46 (21–63)	48 (20–67)	NS
t-/d- bilirubin (mg/dl)	9.0/5.2	8.3/5.4	NS
AST (IU/L)	118	163	NS
ALT (IU/L)	74	128	NS
GGT (IU/L)	480	452	NS

NS not significant, *BASM* biliary atresia splenic malformation

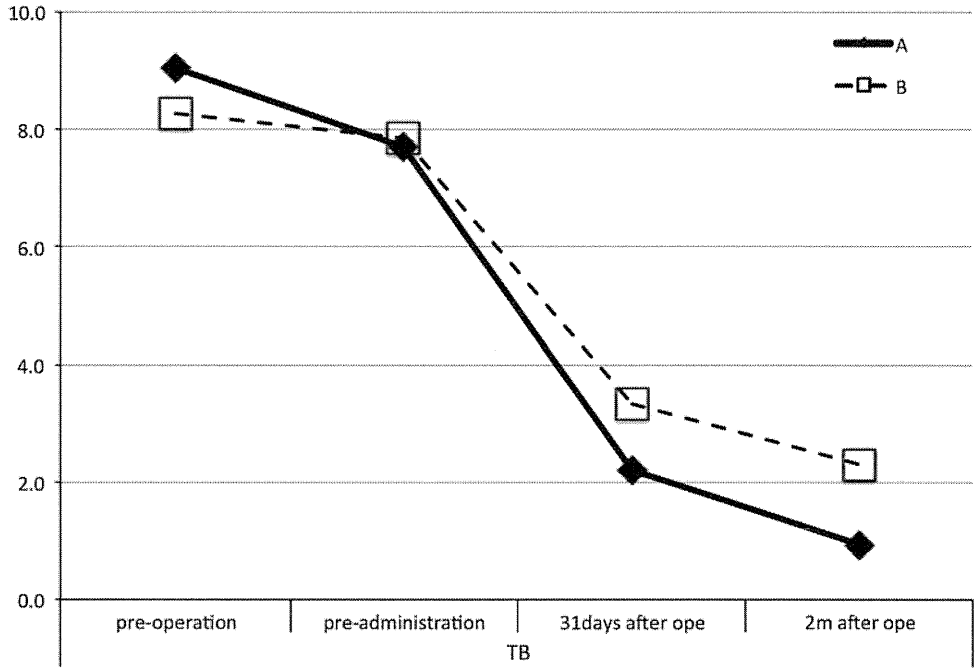

Fig. 2 Postoperative mean total bilirubin levels in subgroup A ($n = 15$, square, 4 mg/kg/day) versus subgroup B ($n = 23$, diamond, 2 mg/kg/day). The level were 2.2 versus 3.4 mg/dL at 1 month, $p = 0.04$ and 0.9 versus 2.3 at 2 months, $p = 0.013$)

significantly lower in subgroup A ($n = 15$) than in subgroup B ($n = 23$) at both 1 and 2 months, postoperatively. The total bilirubin levels were 2.2 versus 3.4 mg/dL at 1 month ($p = 0.0271$) and 0.9 versus 2.3 mg/dL at 2 months ($p = 0.0319$, Fig. 2). The direct bilirubin levels were 1.3 versus 2.2 mg/dL at 1 month ($p = 0.0275$) and 0.5 versus 1.5 mg/dL at 2 months ($p = 0.0190$, Fig. 3). No differences were observed in the other liver function test results. The mean values of the enzymes measured in postoperative liver function test did not differ significantly between the groups.

Nine patients dropped out of the study: four from group A, two because of ileus, one cholangitis, and one unknown reasons and five from group B, three because of increased dose, one cholangitis, and one gastrointestinal bleeding. Eight patients in group A and six in group B had postoperative cholangitis (Table 2).

Other complications included infections. Cytomegalovirus (CMV) infection was observed in two patients in group A on days 12 and 17, respectively, and in one patient in group B. All three cases of CMV infection were associated with liver dysfunction, but the patients were successfully treated with ganciclovir. Influenza A was observed in one patient from group A on day 41, while rotavirus infection was observed in another patient of the same group on day 13. This patient showed liver dysfunction that lasted for 7 days. One patient from group B patients developed *Candida* sepsis on day 57, which was treated with antibiotics and withdrawal of central line. One patient in group A had hypertension on day 9, with systolic pressure 120–140 mmHg that spontaneously returned to normal. Additionally, one patient from group A presented with moon face 3 weeks after surgery.

Discussion

We conducted a preliminary questionnaire regarding the design of this prospective study and we learned that a vast majority of Japanese pediatric surgeons currently use corticosteroids after Kasai operation [9] and they tend to think that setting up a non-steroid group is no more ethical and

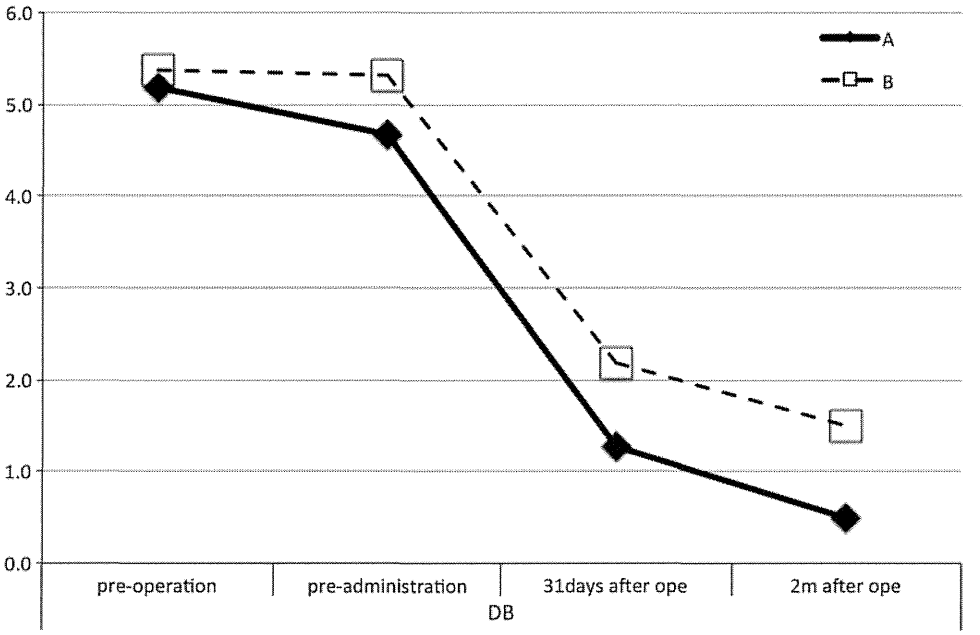

Fig. 3 Postoperative mean direct bilirubin levels in subgroup A ($n = 15$, 4 mg/kg/day) versus subgroup B ($n = 23$, 2 mg/kg/day. The level were 1.3 versus 2.2 mg/dL at 1 month, $p = 0.039$ and 0.5 versus 1.5 at 2 months, $p = 0.0157$

Table 2 Drop outs and complications in groups A and B

	Group A ($n = 35$)	Group B ($n = 34$)	Details
Drop-outs	4	5	
Infections	4 (2 CMV)	2 (1 CMV)	Ganciclovir effective in all CMV infections. Group A: influenza, rotavirus, Group B: Candida sepsis
Cholangitis	8 (1)	6 (1)	
Ileus	2 (2)	0	Day 30, non-operatively resolved
Increase of dose	0	3 (3)	Day 18, small bowel resection
GI bleeding	0	1 (1)	Stopped with H2-blocker
Others	2 (1)	1	1 unknown reason
			Others: moon face, hypertension,

they would not participate in such a study as one with non-steroid group. Thus, we tested the hypothesis that a relatively high dose of 4 mg/kg/day would enhance the clearance of jaundice more rapidly than a dose of 2 mg/kg/day in this study. The other specific purpose of our study is rather to look at the difference in efficacy of a short-term administration of corticosteroids for a short-term outcome because a long-term outcome of BA is often influenced by their subsequent eventful postoperative periods, such as viral infection or episodes of cholangitis or bile lake formation. Such a long-term outcome may not necessarily reflect the direct effectiveness of corticosteroids. Davenport [10] reported a randomized, double-blind, placebo-controlled trial in which the administration of 2 mg/kg/day corticosteroids reduced bilirubin levels the first month in infants <70 days old at surgery when compared with non-steroid group. This result is identical to our study in that postoperative corticosteroids are effective in lowering the serum bilirubin levels among a cohort of patients with earlier surgery before 70 days. Vejchapipat et al. [11] indicated that a dose of 4 mg/kg/day on alternate days, which is equal to 2 mg/kg/day every day, does not result in improvement in the patient's condition. In this study, the mean age at surgery was 89.8 days. These findings indicate that steroids may be more effective in patients who undergo early surgery. Peteresen et al. [12] reported no improvement in mid-term survival 6 months after Kasai PE when a high dose of intravenous methylprednisolone (10 mg/kg/day for 5 days) was used immediately after surgery, followed by 1 mg/kg/day until day 28. In this series, the mean age of the patients at surgery was 63 days, ranging from 20 to 151 days, but subgroup analysis according to age at surgery was not performed.

Given that corticosteroids are effective in infants undergoing early surgery, the efficacious dose of steroids may vary from patient to patient, depending on the degree of fibrosis and the reversibility of the biliary epithelial cells of an individual patient. In order to minimize the risk of

adverse effects and to determine the effective dose for each individual patient, a titrating regimen has been used [3, 13], in which a short course of an initial dose of 4 mg/kg/day prednisolone that is reduced to half every 2 days was given as needed during the first month and changes in the stool color and serum bilirubin levels were monitored. This titration process has shown that glucocorticoid receptor alfa (GcRa) correlates with the severity of liver injury, and that the required dose of the steroid was associated with the degree of GcRa expression [14]. However, this regimen is presumably difficult to employ in a multi-center prospective trial.

The complications encountered during this study provide important information. There were a few untoward but manageable adverse effects observed in both groups, and these were attributable to the effects of steroids.

A limitation of our study is the small sample size. We had expected to complete this study within 2 years considering that approximately 80 cases were annually registered to the JBAS in recent years. Contrary to our expectation, the level of participation decreased by the end of the second year and the number of registered participants decreased annually over the 4-year period; therefore, we decided to discontinue this study and to share the results at this point. In this study, no differences were observed in the enzyme levels measured during the liver function test between the groups at 1 or 2 months after surgery. The sample size is similar among other recent studies [10–12], ranging from 30 to 35 patients in one arm. Thus, the previously reported negative results may be underpowered to detect a real difference as is the case in our study.

In conclusion, our study showed that 4 mg/kg/day prednisolone only improved reduced bilirubin levels in the short-term in a subgroup of infants undergoing surgery before 70 days, but it did not improve other liver function test results. It would be preferable if future randomized trials focus on subgroups of infants divided according to age and degree of fibrosis or GcRa expression and using the dose regimen of 4 mg/kg/day for a longer duration.

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Participation of natural killer cells in the pathogenesis of bile duct lesions in biliary atresia

Atsushi Okamura,[1,2] Kenichi Harada,[1] Masaki Nio,[2] Yasuni Nakanuma[1]

[1]Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa
[2]Department of Pediatric Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan

Correspondence to
Professor Yasuni Nakanuma, Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan; nakanuma@staff.kanazawa-u.ac.jp

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ABSTRACT

Aims Immunological disturbances including innate immunity after a suspected viral infection are considered important to the pathogenesis of bile duct lesions in cases of biliary atresia (BA). In this study, we tried to evaluate whether natural killer (NK) cells and CX3CL1 (Fractalkine) and its receptor (CX3CR1) are involved in the bile duct injury.

Methods Using the section of BA (22 cases) and controls, immunohistochemistry for CD56, CD16, CD68, CX3CL1 and CX3CR1 was performed. Moreover, using cultured biliary epithelial cells (BECs) and NK cells, the production of CX3CL1 in BECs and the migration of NK cells were evaluated.

Results It was found that CD56(−)CD16(+)CD68(−) NK cells were increased around the damaged small and large bile ducts in BA and hepatitis C virus-related chronic hepatitis in comparison with other controls. CX3CL1 was strongly expressed on the damaged bile ducts in BA, while this expression was relatively weak or absent in the bile ducts of normal liver. The results suggest the CD56(−)CD16(+) NK cells to be involved in the development of bile duct injuries in BA. These CD16(+) NK cells were positive for CX3CR1, and attracted by CX3CL1 expressed on bile ducts. Further study revealed that stimulation with poly(I:C) (a synthetic analogue of viral dsRNA) increased the expression of CX3CL1 on cultured BECs followed by increased migrational activity of cultured NK cells.

Conclusions CD56(−)CD16(+) NK cells with reduced NK activity may be involved in the bile duct damage in BA, and CD16(+) NK cells expressing CX3CR1 may be attracted by and interact with bile ducts expressing CX3CL1.

INTRODUCTION

Biliary atresia (BA) is a neonatal obstructive cholangiopathy characterised by the progressive destruction of extrahepatic bile ducts. Intrahepatic large bile ducts are also involved.[1] Clinical and experimental evidence suggests that a viral infection triggers the development of bile duct lesions in BA. The infection of newborn Balb/c-mice with Reoviridae (rotavirus and reovirus, dsRNA virus) leads to bile duct obstruction and cholestasis resembling human BA.[1] In this animal model, viral infections of the biliary tree and subsequent cellular autoimmunity against the bile ducts are important for progressive cholangiopathy and loss.[2 3] Reoviridae reportedly show epitheliotropism and apoptosis in intestinal epithelial cells.[1 2 4–6] We reported that human biliary epithelial cells (BECs) possess dsRNA-related innate immune systems via a dsRNA-recognising receptor such as Toll-like receptor 3 (TLR3), suggesting that reoviridae infections directly relate to the pathogenesis of cholangiopathies in BA.[7–10]

Natural killer (NK) cells constitute an important part of the first line of defense against many microbial infections, and play a significant role in immunity and the immunopathology of hepatobiliary diseases. The majority of NK cells which are strongly cytolytic effector cells fall within the CD56(+) subset. Recently, a population of CD56(−)CD16(+) NK cells has been described in HIV and hepatitis C virus (HCV)-infected patients: these cells have impaired cytolytic functions and cytokine production.[11–13] HIV and HCV infections have been strongly associated with a loss of CD56(+) NK cells, at least partly compensated for by an expansion in the number of CD56(−)CD16(+) cells.[11–13] This replacement of CD56-expressing NK cells by functionally defective CD56(−)CD16(+) NK cells might be one of the mechanisms by which HIV and HCV impair the overall NK cell response. Shivakumar *et al* reported NK cells in the vicinity of intrahepatic bile ducts in infants with BA.[14] It remains unclear whether NK cells play an important role in the pathology of BA.

CX3CL1 (Fractalkine) plays an important role in the cell migration to target sites under physiological and pathological conditions and is expressed on vascular endothelial cells and epithelial cells in response to proinflammatory cytokines and TLR ligands. CX3CR1, a receptor of CX3CL1, is expressed on inflammatory cells including NK cells, suggesting that NK cells are attracted by CX3CL1 expressed in the liver, particularly around damaged bile ducts. Such a scenario has been shown in bile duct lesions in primary biliary cirrhosis (PBC).[15]

In this study, to clarify the participation of NK cells in the pathogenesis of cholangiopathy in BA, we first examined immunohistochemically the distribution of NK cells, particularly CD56(−)CD16(+) NK cells, in the liver tissue of BA patients. We also examined the expression of CX3CL1 on bile ducts and infiltration of mononuclear cells expressing CX3CR1, particularly around damaged bile ducts. Then, the migration of cultured NK cells was examined with respect to the expression and secretion of CX3CL1 in cultured BECs.

MATERIALS AND METHODS

Tissue studies of liver and bile ducts

Anatomical classification of the biliary tree

Extrahepatic bile duct consists of the common hepatic and bile ducts, the right and left hepatic ducts, and their confluence. The branches of the right and left hepatic ducts are largely divided into the large intrahepatic bile duct and small intrahepatic bile ducts. The former roughly correspond

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Table 1 Main clinical features of cases examined

	Age (mean±SD; range)	Sex (M:F)
Cases for the study of intrahepatic small bile ducts		
Biliary atresia (n=22)	1.77±0.86 m; 0.7–12 m	10 : 12
Chronic viral hepatitis C (n=9)*	59.0±13.0 y; 27–72 y	4 : 5
Nonalcoholic steatohepatitis (n=9)†	44.4±14.4 y; 25–69 y	3 : 6
Adult normal liver (n=12)	62.1±13.1 y;47–82 y	6 : 6
Cases for study of large bile ducts		
Biliary atresia (n=21)	1.71±0.81 m; 0.7–12 m	9 : 12
Normal common bile duct (fetus)‡ (n=8)		6 : 2
Adult normal liver§ (n=4)	58.7±17.0 y; 42–76 y	2 : 2

*Staging; stage 1, 6 cases; stage 2, 0 cases; stage 3, 0 cases; stage 4, 3 cases.
†Staging; stage 1, 2 cases; stage 2, 3 cases; stage 3, 3 cases; stage 4, 1 cases.
‡Autopsy cases of fetus.
§Surgical cases.
m, months; y, years; M, male; F, female; n, number of cases.

to the first to third branches of the right and left hepatic ducts. The small bile ducts are further classified into the septal and interlobular bile ducts.[16] The peribiliary glands are present along the extrahepatic bile ducts and the large intrahepatic bile ducts, and the peribiliary vascular plexus is also identifiable around the bile ducts. In this study, the hilar bile ducts and intrahepatic large bile ducts are collectively called the large bile duct.

Case collection and preparation of liver and bile duct specimens

Case selection

The details of these cases are shown in table 1. For the examination of small intrahepatic bile ducts, 22 cases of BA, 9 cases of chronic viral hepatitis C (CVH-C), 9 cases of nonalcoholic steatohepatitis (NASH), and 12 cases of normal liver were examined (43 cases were of needle or wedge liver biopsies and the remaining 9 cases, surgically resected). For the large bile duct, 21 cases of BA, 8 autopsy cases of fetus, and 4 normal controls were examined (all cases were surgically resected). Normal livers for small intrahepatic bile ducts and large bile ducts were from non-neoplastic parts of metastatic liver carcinoma.

Tissue preparation

All of these tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. More than 20 consecutive 4-μm-thick sections were cut from each paraffin block, and some of them were stained with haematoxylin and eosin (H&E) and Azan-Mallory stain for the identification of bile duct lesions. The remaining sections were used for immunohistochemistry.

Immunohistochemistry

Immunostaining was performed using formalin-fixed, paraffin-embedded tissue sections of BA patients and controls (other diseases). The primary antibodies and their sources, optimal dilution and antigen retrieval method are shown in table 2. The small bile ducts and large bile ducts and their surrounding areas were mainly examined.

Distribution of CD56(−)CD16(+)CD68(−) NK cells

Immunostaining

After antigen retrieval (pressure with citric acid method) for 20 min, immunostaining for CD56 was performed using the CSA II System (biotin-free tyramide signal amplification system, DakoCytomation). Colour development was performed by a benzidine reaction. After microwaving with citric acid, the sections were incubated overnight at 4°C with a primary monoclonal antibody against CD68. The sections were then treated with secondary antibodies conjugated to a peroxidase-labelled polymer (EnVision system, DakoCytomation, Dako Japan, Tokyo, Japan). Colour development was performed using Histogreen. The sections were counterstained with haematoxylin. Expression of CD56 (brown) and CD68 (green) in the cytoplasm of mononuclear cells was regarded as positive. Negative controls were carried out. Cells positive for CD56 or CD68 were identified around the small bile ducts and also beneath the large bile duct epithelia. Two areas around the small bile ducts and two areas beneath the large bile duct epithelia were photographed (Photograph A) in each case. After decolourisation by microwaving with citric acid for 5 min in which green-coloured CD68 was abolished, the sections were incubated overnight at 4°C with a primary monoclonal antibody for CD16, and the sections were then treated with secondary antibodies conjugated to a peroxidase-labelled polymer (EnVision system, DakoCytomation). Colour development was performed using Histogreen. The sections were counterstained with haematoxylin. Negative controls were carried out. Cells positive for CD56 (brown) or CD16 (green) were identified around the small bile ducts and also beneath the large bile duct epithelia, and two areas in the former and two in the latter in the same areas as photographed in photo A were again photographed (Photograph B) in each case.

Semiquantitative evaluation

Photographs A and B in the same areas were compared, and CD56(−)CD16(+)CD68(−) NK cells, which were green in photograph B but not photograph A, were counted around the small bile ducts and also beneath the large bile duct epithelia. The average for the two photographs was regarded as the number of CD56(−)CD16(+)CD68(−) NK cells in each case.

Table 2 Antibodies used in this study

Primary antibody against	Type of antibody and immunised animal	Clone	Dilution	Source	Antigen retrieval method
CD16	Monoclonal (mouse)	2H7	1:200	Leica, Tokyo, Japan	Microwave
CD56	Monoclonal (mouse)	1B6	Diluted*	Nichirei, Tokyo, Japan	Pressure cooker
CD68	Monoclonal (mouse)	PG-M1	Diluted*	Nichirei, Tokyo, Japan	Microwave
CX3CL1 (Fractalkine)	Polyclonal (rabbit)		1:500	Immuno-Biological Laboratories, Fujioka, Japan	Microwave
CX3CR1	Polyclonal (rabbit)		1:1000	Immuno-Biological Laboratories, Fujioka, Japan	Microwave

*Already diluted; microwave, microwaved in 10 mM citrate buffer for 20 min in a microwave oven; pressure cooker, treated in 10 mM citrate buffer pressure cooker

Immunostaining of CX3CR1/CD16

Immunostaining

CX3CR1(+) mononuclear cells were characterised with respect to CD16 NK cells in BA. After blocking of the endogenous peroxidase and antigen retrieval for 20 min, the sections were incubated overnight at 4°C with a polyclonal rabbit anti-CX3CR1 antibody. The sections were then treated with secondary antibodies conjugated to a peroxidase-labelled polymer (EnVision system, DakoCytomation). Colour development was performed by a benzidine reaction. After microwaving with citric acid, the sections were incubated overnight at 4°C with a primary monoclonal antibody against CD16. The sections were next treated with secondary antibodies conjugated to a peroxidase-labelled polymer (EnVision system, DakoCytomation). Colour development was performed using Histogreen. The sections were counterstained with haematoxylin. Expression of CX3CR1 and CD16 in the cytoplasm of mononuclear cells was regarded as positive. Cells positive for CX3CR1 (brown) or CD16 (green) identified around the small bile ducts and also beneath the large bile duct epithelia were evaluated in individual cases. Negative controls were carried out.

Semiquantitative evaluation

Double positive cells (CX3CR1 is brown and CD16 is green) were counted around the small bile ducts (two bile ducts) and beneath the large bile ducts (two areas) in BA patients and controls, and the average of two values for each case was regarded as the number of CX3CR1(+)CD16(+) NK cells in each case.

Immunostaining of CX3CL1

Immunostaining

After blocking of the endogenous peroxidase, the sections were incubated in protein block solution (DakoCytomation). The sections were incubated overnight at 4°C with primary polyclonal antibodies against CX3CL1. The sections were then treated with secondary antibodies conjugated to a peroxidase-labelled polymer (EnVision system, DakoCytomation). After a benzidine reaction, the sections were counterstained lightly with haematoxylin. Negative controls were also done.

Semiquantitative evaluation

CX3CL1 expression in bile ducts was evaluated as either absent/faint (±), slightly positive (+), or strongly positive (++).

Culture studies

Cultures of human BECs

A line of human biliary epithelial cells (BECs) was established and cultured as previously reported.[17] BECs were established from the explant liver of a 24-year-old man with BA. More than 95% of the cultured cells were confirmed to be BECs by the expression of biliary-type cytokeratins (CK7 and CK19). Informed consent for research was obtained from the patient prior to surgery. This study was approved by the Kanazawa University Ethics Committee. Cultured BECs were stimulated with polyinosinic-polycytidylic acid (poly(I:C), TLR3 ligand, a synthetic analogue of viral dsRNA; 25 μg/ml; Invitrogen, San Diego, California, USA) and mRNA and supernatant of cells were used in the mRNA analysis and migration assay, respectively.

RT-PCR for CX3CL1

For the evaluation of the mRNA of CX3CL1 in cultured BECs, total RNA was isolated and 1 μg was reverse-transcribed with an oligo-(dT) primer and reverse transcriptase to synthesise cDNA. The cDNA was amplified by PCR using specific primers designed to specifically amplify a 262 bp portion of CX3CL1. As a positive control of the PCR, primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene mRNA were used. The PCR products were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide.

In addition, to carry out relative quantification, real-time quantitative PCR was performed for measurements of CX3CL1 mRNA according to a standard protocol using the SYBR Green PCR Master Mix and ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). Results are shown as relative mRNA expression compared with the level without any treatments (PBS). In addition, real-time quantitative PCR was performed for measurements of Notch1, Ascl1 and chromogranin A mRNAs according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan) and relative gene expression was calculated using the comparative cycle threshold method. Specific primers were as follows: CX3CL1 forward, 5′-GATGGCTCCGATATCTCTG-3′ and reverse 5′-CTGCTGCATCGCGTCCTTG-3′ and GAPDH (internal positive control), forward, 5′-GGCCTCCAAGGAGTAAGACC-3′ and reverse, 5′-AGGGGTCTACATGGCAACTG-3′.

Migration assay of NK cells with cultured BECs

Preparation of cultured NK cells

NK cells were isolated from the peripheral blood mononuclear cells of a healthy volunteer according to MACS protocols of the NK cell isolation kit (MACS, Miltenyi Biotec K.K., Tokyo, Japan). These cells were maintained on culture dishes with standard medium, lymphocyte growth medium-3 (Takara, Ohtsu, Japan) at 37°C in 95% air and 5% CO_2.

Migration assay of NK cells with cultured BECs stimulated by poly(I:C)

The chemoattractant activity of CX3CL1 secreted by cultured BECs stimulated with poly(I:C) was assessed in 96-well plates assembled with the Cultrex 96-well collagen I cell invasion assay (Treigen, Gaithersburg, Maryland, USA) according to the manufacturer's directions using isolated NK cells expressing CX3CR1 and showing efficient chemotaxis and adherence in a CX3CL1-dependent manner. Briefly, the NK cell suspension was seeded and the supernatant of BECs cultured with poly(I:C) for 3 days or the human recombinant CX3CL1 (10 ng/ml, PeproTech, Rocky Hill, New Jersey, USA) was added to lower wells at 1:100 or 1:10. After 24 h, the transferred cells were collected and their number was evaluated by optical density (OD).

Statistical analysis

Numerical data are presented as the mean±SD. Data from different groups were compared using a one-way analysis of variance and examined with the Mann-Whitney U-test. Differences in the proportions of categorical data were tested using the χ^2 test. The correlation coefficient of two factors was evaluated using Spearman's rank correlation test. For the migration assay of NK cells, Welch's t test was used. The results were considered significant if the p value was less than 0.05.

RESULTS

Tissue studies of liver and bile ducts

Infiltration of CD56(-)CD16(+)CD68(-)NK cells

Small bile ducts

In normal livers, there were no or few CD56(−)CD16(+)CD68(−) NK cells in portal tracts. By contrast, in diseased livers including

BA, there were variable numbers of such NK cells admixed with other inflammatory cells, and these cells were rather frequent in BA (figure 1A–D). Their numbers counted around small bile ducts are plotted in figure 1E. The cells were rather dense in BA in comparison with NASH and normal livers ($p<0.01$).

Large bile ducts

There were no or few CD56(−)CD16(+)CD68(−) NK cells beneath biliary epithelia of the large bile duct in normal adult livers, while they were identifiable in BA (figure 2A–D). Their numbers are plotted in figure 2E. They were more abundant in BA than in normal livers ($p<0.01$).

Immunohistochemistry for CX3CL1

Infiltration of CX3CR1(+)CD16(+) mononuclear cells

Small bile ducts

CX3CR1(+)CD16(+) mononuclear cells admixed with other inflammatory cells were frequently present in portal tracts around damaged small bile ducts in cases of BA (figure 3A), while such cells were sparse in cases of other liver diseases and normal livers (figure 3B). Their number in the portal tracts is plotted in figure 3C. They were rather dense in BA in comparison with other liver diseases and normal livers.

Large bile ducts

CX3CR1(+)CD16(+) mononuclear cells admixed with other inflammatory cells were found around the large bile ducts in cases of BA, but were not found in normal livers. The incidence of these cells is shown in figure 3D.

Expression of CX3CL1 in bile ducts

Small bile ducts

In normal livers, small bile ducts were generally negative or faintly positive for CX3CL1, and endothelial cells of small vessels of peribiliary capillary plexus (PBP) were negative or slightly positive for CX3CL1 (figure 4A). In CVH-C and NASH livers, small bile ducts were negative or slightly positive for CX3CL1. Small bile ducts of BA patients were strongly positive for CX3CL1 (figure 4B). The incidence of small bile ducts with mild to moderate and strong expression in normal liver, BA and

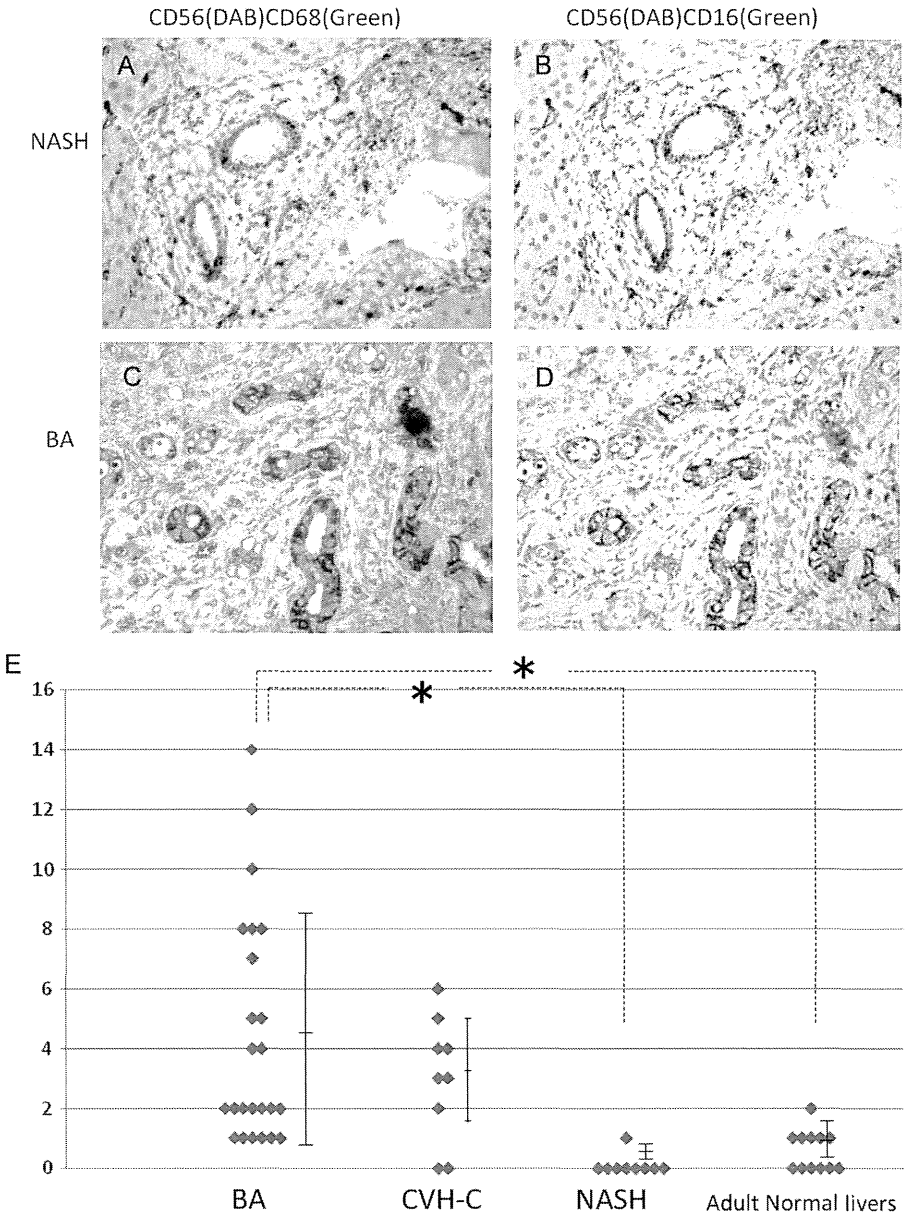

Figure 1 Density of CD56(−)CD16(+) CD68(−) natural killer (NK) cells around intrahepatic small bile ducts. (A, C) Expression of CD56 (brown) and CD68 (green). (B, D) Expression of CD56 (brown) and CD16 (green). Two photographs in the same areas of nonalcoholic steatohepatitis (NASH) (A, B) were compared. CD56(−)CD16(+) CD68(−) NK cells were green in photo B but not photo A. There were no or few CD56(−)CD16(+)CD68(−) NK cells in portal tracts. By contrast, in biliary atresia (BA) (C, D), there were variable numbers of such NK cells admixed with other infiltrated inflammatory cells. (E) The number of such NK cells around small bile ducts is rather high in BA in comparison with NASH and normal livers. Mean±SD in BA, chronic viral hepatitis C (CVH-C), NASH and adult normal livers were 4.37±3.83, 3.00±2.06, 0.11±0.33, and 0.58±0.66, respectively. Effect size and CI; BA versus CVH-C (effect size=0.18, CI −1.39 to 4.14), BA versus NASH (effect size=051, CI 1.63 to 6.90), and BA versus adult normal livers (effect size=0.50, CI 1.51 to 6.07). Bars indicate the mean±SD. *<0.01.

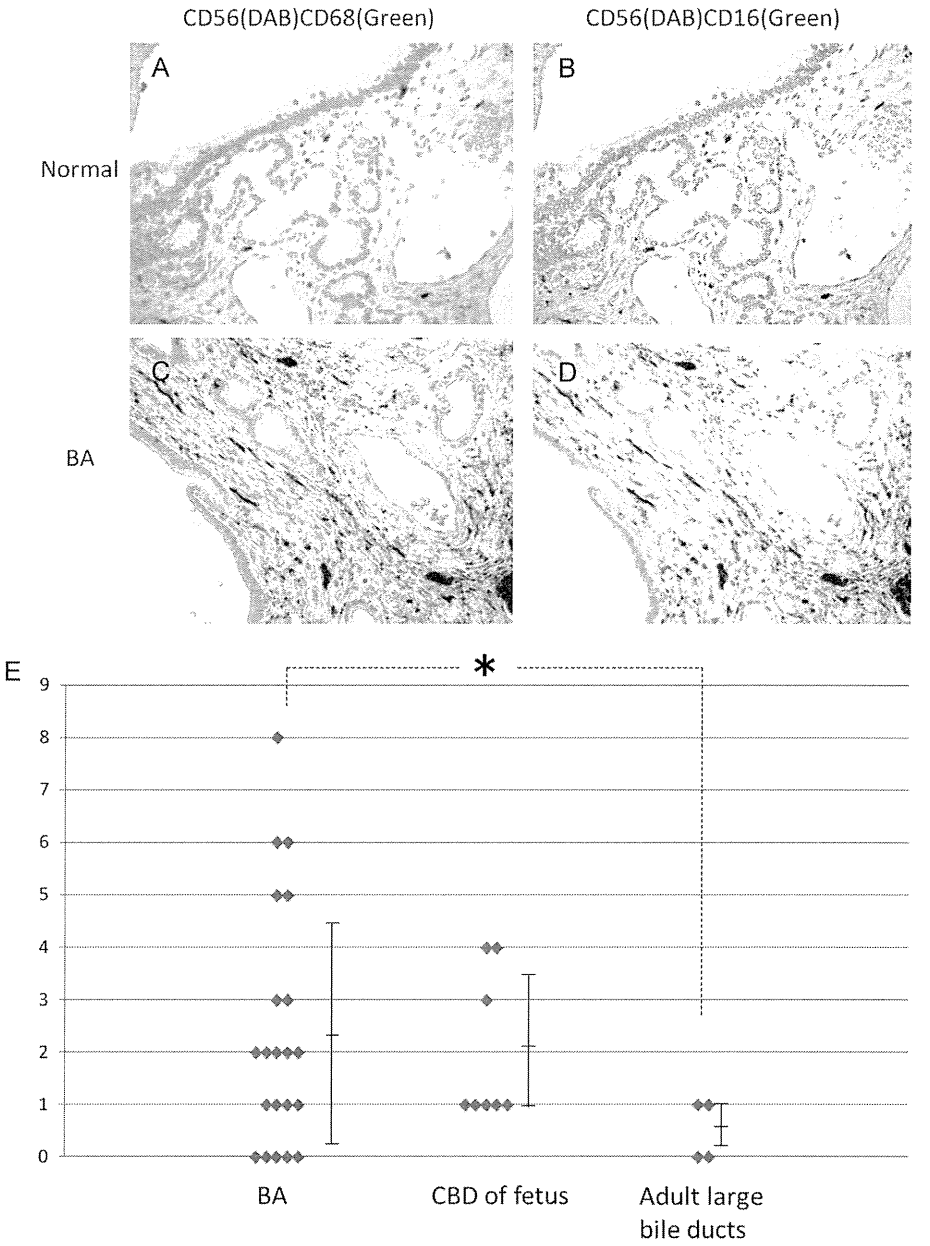

Figure 2 Density of CD56(−)CD16(+) CD68(−) natural killer (NK) cells around large bile ducts. (A, B) There were no or few CD56(−)CD16(+)CD68(−) NK cells beneath biliary epithelia of the large bile duct in normal adult livers. (C, D) Such NK cells were identifiable in biliary atresia (BA). (E) These cells were more abundant in BA than in normal livers. Mean±SD in BA, common bile duct (CBD) of fetus, and adult large bile ducts were 2.50±2.34, 2.00±1.41, and 0.33±0.57, respectively. Effect size and CI; BA versus common bile duct (CBD) of fetus (effect size=0.08, CI −1.44 to 2.20) and BA versus adult large bile ducts (effect size=0.58, CI 0.66 to 3.10). Bars indicate the mean±SD. *<0.01.

other liver diseases is shown in figure 4C. Endothelial cells around injured interlobular bile ducts of BA patients also were strongly positive for CX3CL1 and their intensity was higher in comparison with other disease controls (figure 4A,B).

Large bile ducts

CX3CL1 was not expressed or only faintly expressed in large bile ducts and peribiliary glands and PBP in normal livers (figure 5A), while it was strongly expressed in biliary epithelial cells of large bile ducts and peribiliary glands in cases of BA and also endothelial cells of PBP around large bile ducts in BA (figures 5B,C), while such expression was faint or absent in normal livers. The incidence of bile ducts with mild to moderate and strong expression of CX3CL1 is shown in figure 5D.

Culture studies

Expression of CX3CL1 mRNA in cultured BECs treated with poly(I:C)

RT-PCR revealed that the amplicon of CX3CL1 mRNA could not be detected in cultured BECs without any stimulants (PBS), whereas treatment with poly(I:C) induced its expression (figure 6A). As shown in figure 6B, real-time PCR analysis revealed that treatment with poly(I:C) significantly up-regulated the expression of CX3CL1 mRNA 21.9-fold (figure 6B).

Migration of NK cells

Optical density reflecting the number of NK cells that transmigrated was significantly increased in the bottom chamber containing recombinant CX3CL1 and supernatant of poly(I:C)-treated BECs, compared with that containing the negative control medium (PBS). The effect of the supernatant was concentration (dose)-dependent (figure 7).

DISCUSSION

The findings obtained in this study can be summarised as follows: (i) CD56(−)CD16(+) NK cells were increased around the small bile ducts and beneath the biliary epithelia of large bile ducts in comparison with other diseases and normal livers, (ii) such CD16(+) cells expressed CX3CR1, a receptor of

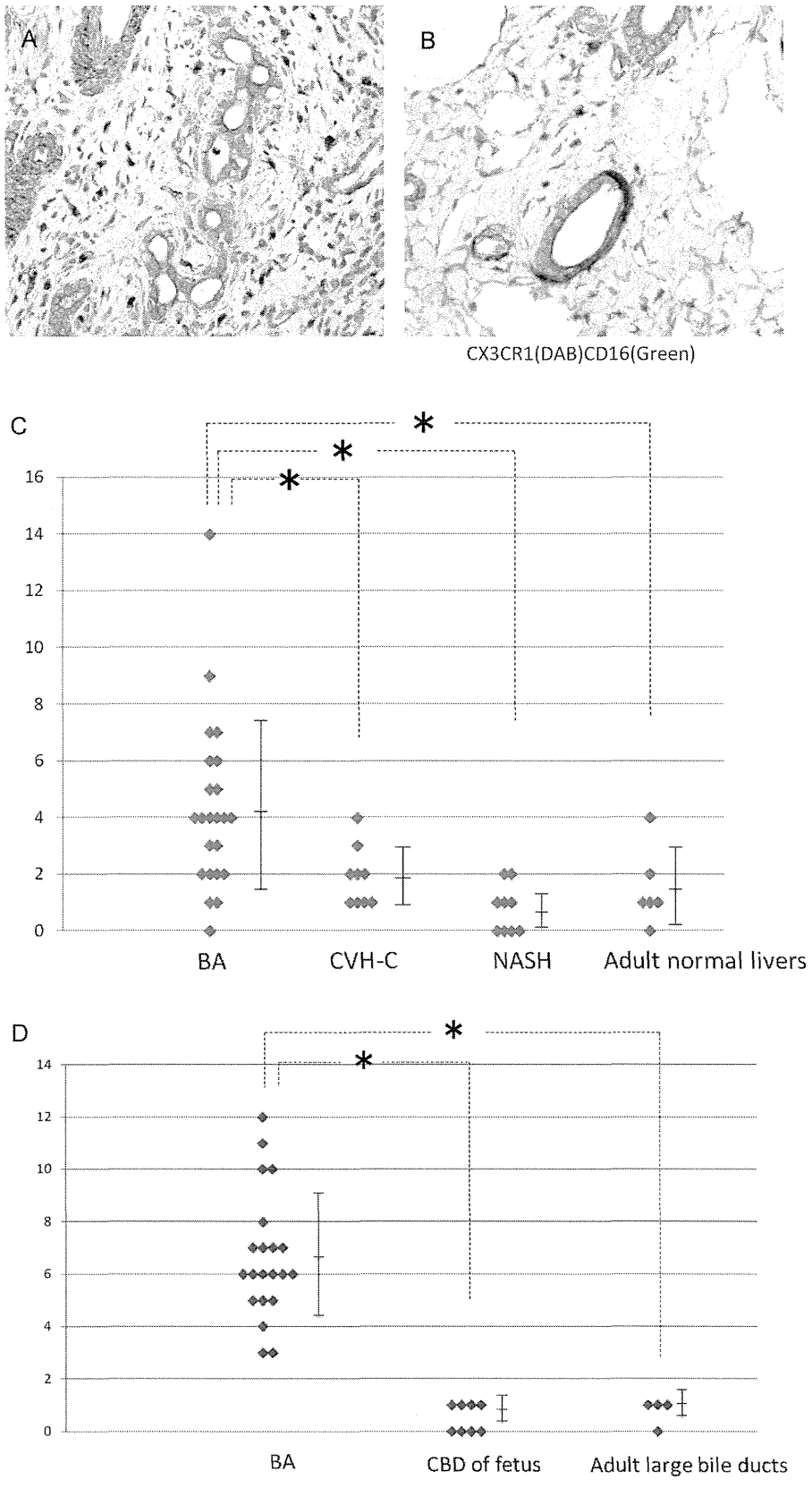

Figure 3 CX3CR1(+)CD16(+) mononuclear cells around intrahepatic bile ducts. (A) CX3CR1(+)CD16(+) mononuclear cells were frequently present in portal tracts around damaged small bile ducts in biliary atresia (BA). (B) Such cells were sparse in normal livers and other liver diseases. (C) They were rather dense in BA in comparison with other liver diseases and normal livers. Mean±SD in BA, chronic viral hepatitis C (CVH-C), nonalcoholic steatohepatitis (NASH) and adult normal livers were 4.30±3.03, 1.88±1.05, 0.77±0.83, and 1.50±1.37, respectively. Effect size and CI; BA versus CVH-C (effect size=0.39, CI 0.28 to 4.55), BA versus NASH (effect size=053, CI 1.41 to 5.64), and BA versus adult normal livers (effect size=0.39, CI 0.166 to 5.44). (D) CX3CR1(+)CD16(+) mononuclear cells were found around the large bile ducts in BA, but not in normal livers of fetuses or adults. Mean±SD in BA, common bile duct (CBD) of fetus, and adult large bile ducts were 6.66±2.41, 0.50±0.53, and 0.75±0.50, respectively. Effect size and CI; BA versus CBD of fetus (effect size=0.81, CI 4.38 to 7.95) and BA versus adult large bile ducts (effect size=0.71, CI 3.37 to 8.47). Bars indicate the mean ±SD. *<0.05.

CX3CL1, (iii) CX3CL1 was strongly expressed in BECs of small bile ducts and also of large bile ducts in BA, and (iv) stimulation with poly(I:C) (a synthetic analogue of viral dsRNA) increased the expression of CX3CL1 on cultured BECs and increased migration of cultured NK cells.

The pathogenesis of BA may be the virus-induced autoimmune-mediated injury of bile ducts.[6] In fact, Reoviridae (type 3 reovirus and type C rotavirus) and herpes virus including cytomegalovirus have all been considered possible candidates for the initiating agent.[1] Studies in the rotavirus mouse

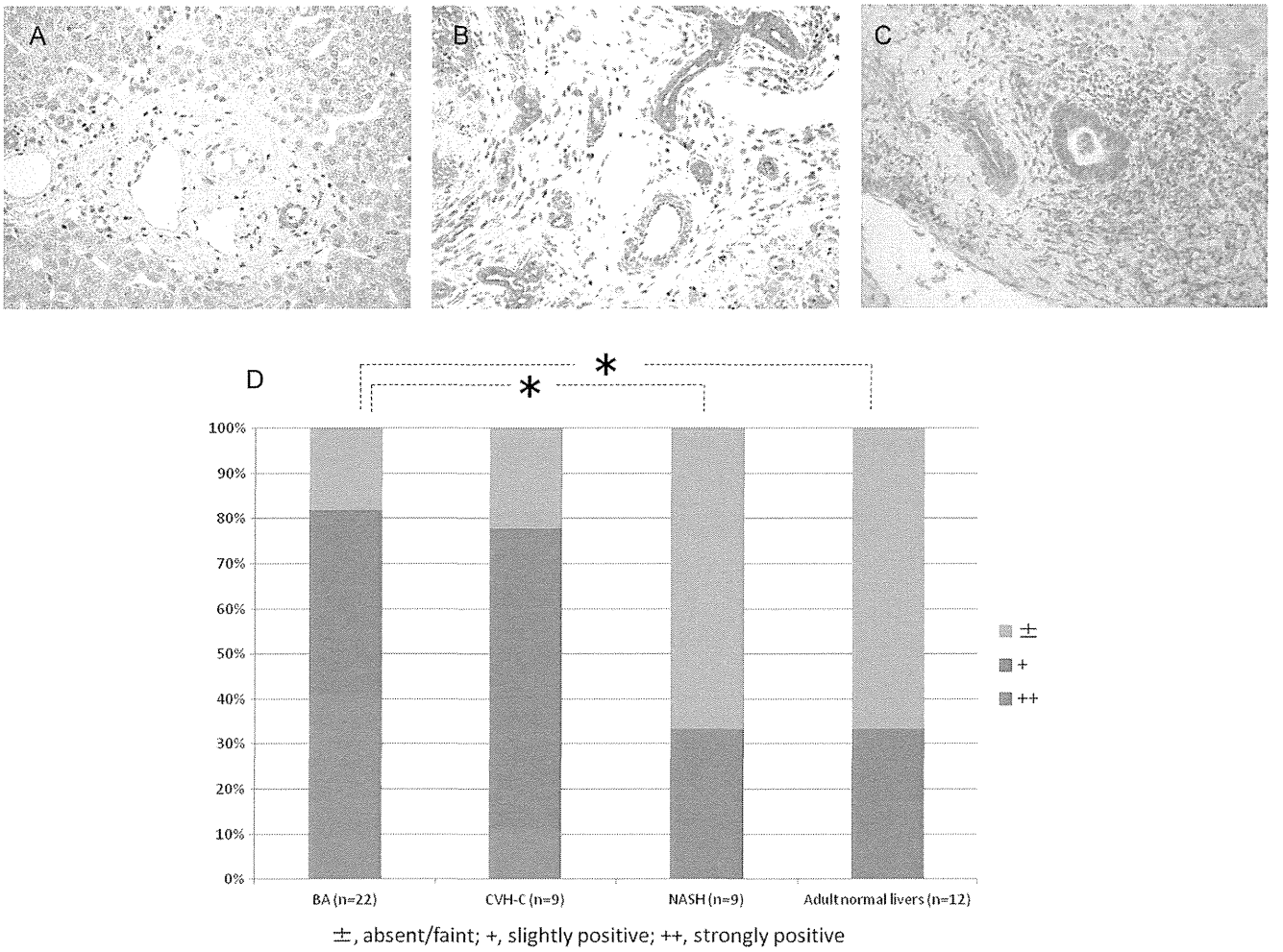

Figure 4 Expression of CX3CL1 in intrahepatic small bile duct epithelia. (A) Normal livers. Small bile ducts were generally negative or faintly positive for CX3CL1. (B) Biliary atresia (BA). Small bile ducts were strongly positive for CX3CL1. (C) The incidence of small bile ducts with mild to moderate and strong expression in normal liver, BA and other liver diseases.

model of BA indicate that a viral infection of the biliary epithelium is an initial event leading to biliary inflammation and obstruction and autoreactive T cells and autoantibodies specific to bile duct epithelia have been reported.[3] [18] Specific host factors related to innate and acquired immunopathological processes with respect to viral infection may also play a key role in experimental BA.[3] Recently, many genetic studies, moreover, have recently reported. For example, genomic study including genome-wide association study identified a susceptibility locus for BA on 10q24.2 and 2q37.3.[18] [19] Moreover, DNA hypermethylation at the CD11a locus in CD4+ cells, polymorphisms of vascular endothelial growth factor gene, and two microRNAs (miR-29a/29b1) may contribute significantly to BA susceptibility, but polymorphisms of IL-4, IL-18, IFN-γ genes were unlikely.[20–25] These genetic analyses revealed a link to the susceptibility to BA with respect of immunopathological processes.

Recent studies showed the roles of NK cells in addition to T cells in the destruction of extrahepatic bile ducts in BA.[26] [27] That is, the inflammatory milieu from portal tracts and/or biliary remnants showed greater numbers of T cells and NK cells, and up-regulation of CD8(+) costimulatory molecules in BA.[27] In experimental BA, activated NK cells were reportedly the most abundant cells in extrahepatic bile ducts and such NK cells were regarded as key initiators of bile duct injury.[14] However, the exact roles of NK cells and their phenotypic and functional alterations have not been studied in BA.

The CD56(−)CD16(+) NK subset is greatly expanded in HIV-viremic individuals.[28] The CD56(−) NK fraction was associated with extremely poor in vitro cytotoxic functions.[28] In addition, the secretion of certain cytokines important for initiating antiviral immune responses was markedly reduced in the CD56(−) NK cells. Elevated levels of CD56(−) NK cells are also found in many CVH-C patients.[11–13] These CD56(−) NK cells were functionally impaired with respect to cytokine production upon target cell recognition.[28] Furthermore, high levels of these cells reveal a disturbance in innate cellular immunity that is associated with an impaired ability to respond to antiviral treatment with IFN-α and ribavirin. Taken together, these findings suggest that the expansion of this highly dysfunctional CD56(−) NK cell subset in humans infected with HIV-1 and HCV largely accounts for the impaired function of the total NK cell population.[11–13] So far, such issues have not been examined in BA.

It was found in this study that CD56(−)CD16(+) NK cells were increased around the damaged small and large bile ducts in BA, and the proportion of these cells was relatively high in BA in comparison with controls, suggesting that increased CD56(−)CD16(+) NK cells with reduced NK activities were involved in the development of bile duct injuries in BA. It seems

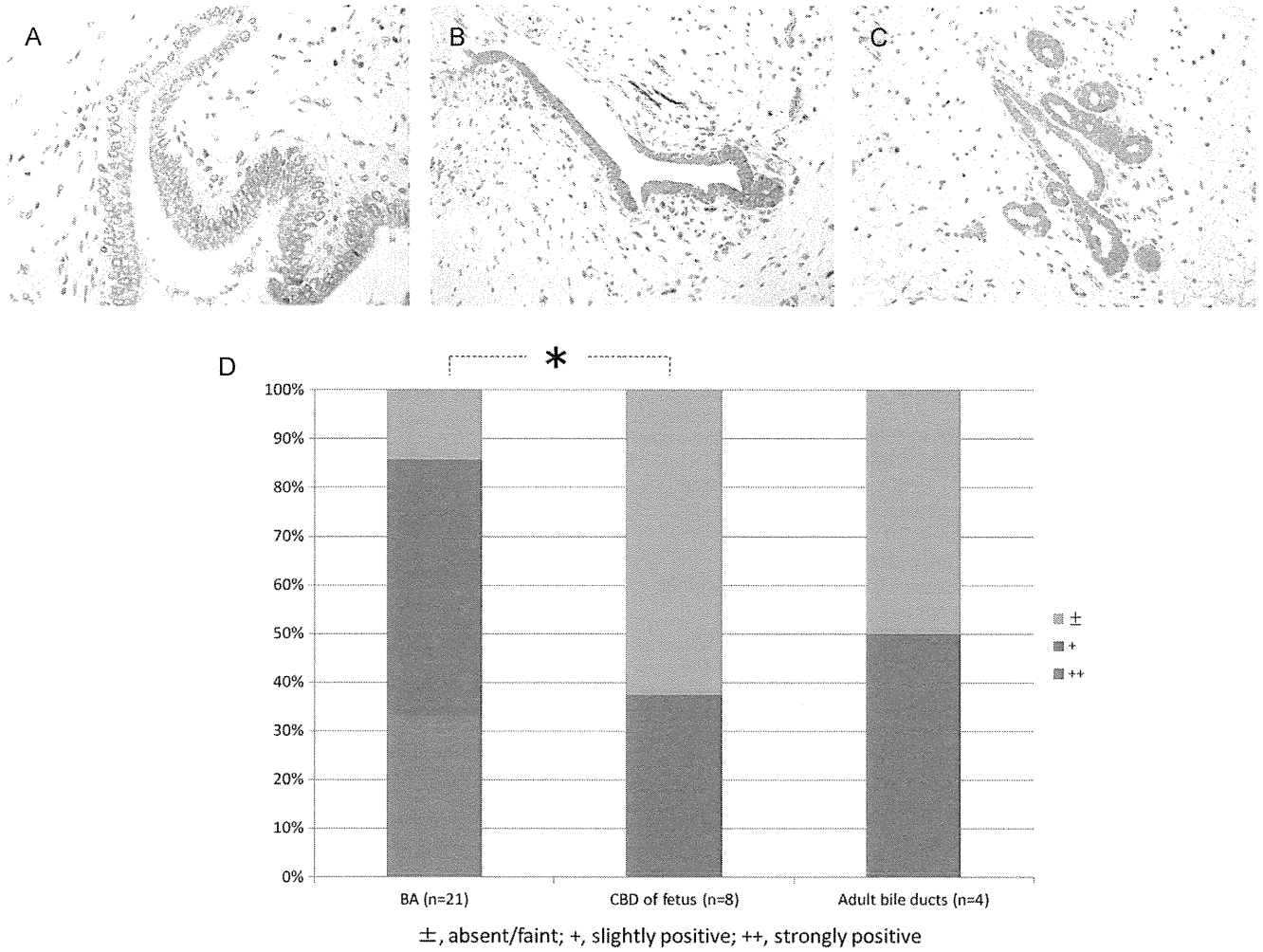

Figure 5 Expression of CX3CL1 in large bile duct epithelia. (A) CX3CL1 was not or faintly expressed in large bile ducts and peribiliary glands and peribiliary capillary plexus of normal livers. (B,C) It was strongly expressed in biliary epithelial cells of large bile ducts and peribiliary glands and also endothelial cells of PBP around large bile ducts in BA. (D) The incidence of bile ducts with mild to moderate and strong expression of CX3CL1. *<0.01.

possible that inadequate removal of BECs infected with cholangiotrophic virus by abundant CD56(−)CD16(+) NK cells with reduced antiviral activities leads to the induction of secondary immunisation against the cholangiotrophic virus as well as BECs in BA. Cross-reactivity between viral and self-antigens is also proposed to trigger secondary autoimmunity.[2 6] This may be in turn followed by extensive autoimmune-mediated destruction of the bile ducts by CD8(+) cytotoxic T cells and other effector cells. CD8(+) T cells were reportedly necessary for induction of bile duct injury and obstruction in an experimental model of BA with autoimmune features.[5]

It was also found in this study that CD16(+) NK cells were positive for CX3CR1, and CX3CL1 was strongly expressed on the damaged bile ducts in BA. While the expression of CX3CL1 was relatively weak or absent in the bile ducts of normal liver and CVH-C, CX3CL1 was also strongly expressed in the damaged bile ducts in PBC, in which the interaction of CX3CR1-expressing lymphocytes and CX3CL1-expressing bile ducts and endothelial cells of PBP is important in the bile duct destruction.[15] CX3CL1 is a chemokine with both chemoattractant and cell-adhesive functions, and in the intestine it is involved with its receptor CX3CR1 in the chemoattraction and recruitment of intraepithelial lymphocytes.[15] It seems likely that CD16(+) NK cells with expression of CX3CR1 may be chemoattracted and infiltrate around the bile ducts expressing CX3CL1 and this may be followed by the immunological interaction of NK cells and bile ducts, possibly virus infected.

Expression of CX3CL1 in human BECs in response to a TLR3 ligand, poly(I:C), was examined using a human intrahepatic BEC line. Consequently, the expression of CX3CL1 mRNA was low under normal conditions, but significantly up-regulated by the stimulation with poly(I:C). We have already reported that BECs express multiple functionally active TLRs and respond to the corresponding bacterial or viral TLR ligands including poly(I:C).[7] Moreover, we previously demonstrated the diffuse expression of TLR3 in extrahepatic and intrahepatic bile ducts of patients with biliary atresia. Therefore, BECs infected by Reoviridae (reovirus and rotavirus) having a double-strand RNA are speculated to induce the expression of CX3CL1 via biliary innate immunity in biliary atresia patients. Moreover, the chemotaxis of human NK cells expressing the CX3CL1 ligand CX3CR1, and showing efficient chemotaxis and adherence in a CX3CL1-dependent manner was assayed using a cell invasion assay kit. The human NK cells showed chemotaxis toward recombinant CX3CL1 and also the culture medium which was speculated to contain CX3CL1 secreted by poly(I:C)-stimulated BECs. Therefore, dsRNA viruses in the microenvironment of injured bile ducts resulting from BA induce the upregulation of CX3CL1 expression in BECs, followed