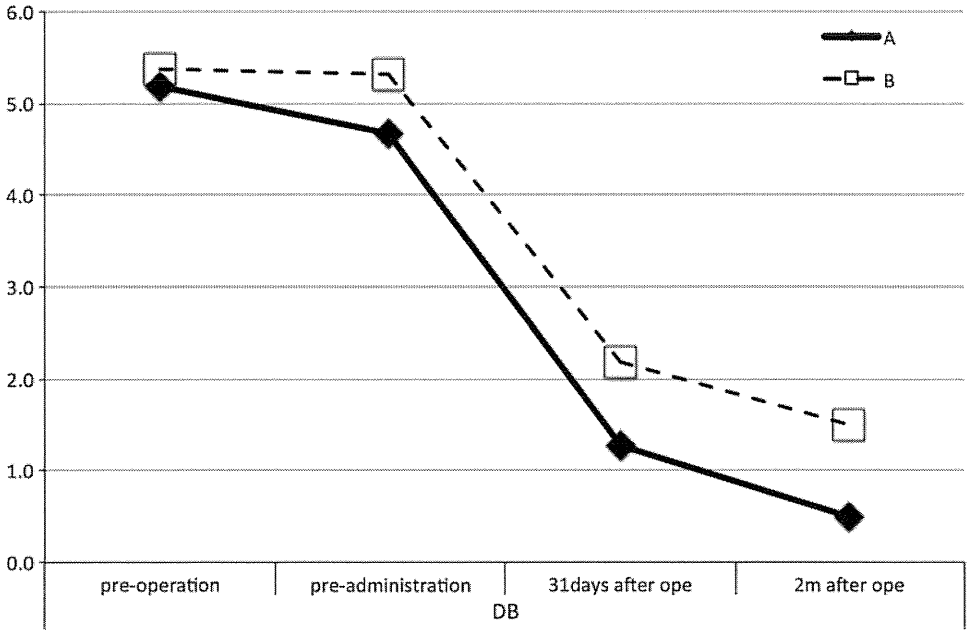


Fig. 3 Postoperative mean direct bilirubin levels in subgroup A ($n = 15$, 4 mg/kg/day) versus subgroup B ($n = 23$, 2 mg/kg/day. The level were 1.3 versus 2.2 mg/dL at 1 month, $p = 0.039$ and 0.5 versus 1.5 at 2 months, $p = 0.0157$

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.

Acknowledgments This work was supported by a Grant for Child Health and Development 17C-4 from Ministry of Health, Labour and Welfare. We are grateful to the member institutions of JBAS listed below and the many surgeons who extended their efforts to contribute to this prospective study and also each parent who gave consent for participation in this randomized trial. Institutions: Dr. kenji Iio, Aichi Prefectural Colony Central Hospital; Dr. Toshihiro Muraji, Ibaraki Children's Hospital; Dr. Yutaka Hayashi, Miyagi Children's Hospital; Dr. Shinji Uemoto, Kyoto University Hospital; Dr. Tomoaki Taguchi, Kyushu University Hospital; Dr. Kohji Oono, Saitama Medical University Hospital; Dr. Hiroo Uchida, Saitama Children's Medical Center; Dr. Yasushi Iinuma, Niigata City General Hospital; Dr. Masayuki Kubota, Niigata University Hospital; Dr. Youkatsu Ooha-ma, Kanagawa Children's Medical Center; Dr. Yasuyuki Higashimoto, Chiba Children's Hospital; Dr. Noritoshi Handa, Oita Prefectural Hospital; Dr. Kohji Masumoto, Tsukuba University Hospital; Dr. Tadashi Iwanaka, Tokyo University Hospital; Dr. Masaki Nio, Tohoku University Hospital; Dr. Tatsuya Suzuki, Fujita Health University Hospital; Dr. Akiko Yokoi, Hyogo Prefectural Kobe Children's Hospital.

References

1. Kasai M, Suzuki H, Ohashi E et al (1978) Technique and results of operative management of biliary atresia. World J Surg 2:571–580
2. Karrer F, Lilly J (1985) Corticosteroid therapy in biliary atresia. J Pediatr Surg 20:693–695
3. Muraji T, Higashimoto Y (1997) The improved outlook for biliary atresia with corticosteroid therapy. J Pediatr Surg 32:1103–1107
4. Dillon PW, Owings E, Cilley R et al (2001) Immunosuppression as adjuvant therapy for biliary atresia. J Pediatr Surg 36:80–85
5. Meyers RL, Book LS et al (2003) High-dose, ursodeoxycholic acid, and chronic intravenous antibiotics improve bile flow after Kasai procedure in infants with biliary atresia. J Pediatr Surg 38:406–411
6. Escobar MA, Jay CL, Brooks RM et al (2006) Effect of corticosteroid therapy on outcomes in biliary atresia after Kasai portoenterostomy. J Pediatr Surg 41:99–103
7. Stringer MD, Davison SM, Rajwal SR et al (2007) Kasai portoenterostomy: 12-year experience with a novel adjuvant therapy regimen. J Pediatr Surg 42:1324–1328
8. Sarkhy A, Schreiber RA, Milner R et al (2011) Does adjuvant steroid therapy post-Kasai portoenterostomy improve the outcome of biliary atresia? Systematic review and meta-analysis. Can J Gastroenterol 25:440–444
9. Muraji T, Nio M, Ohhama Y et al (2004) Postoperative corticosteroid therapy for bile drainage in biliary atresia—a nationwide survey. J Pediatr Surg 39:1803–1805
10. Davenport M, Stringer M, Tizzard S et al (2007) Randomized, double-blind, placebo-controlled trial of corticosteroids after Kasai portoenterostomy for biliary atresia. Hepatology 46:1821–1827
11. Vejchapipat P, Passsakonnirin R, Sookpotarom P et al (2007) High-dose steroids do not improve early outcome in biliary atresia. J Pediatr Surg 42:2102–2105
12. Petersen C, Harder D, Melter M et al (2008) Postoperative high-dose steroids do not improve mid-term survival with native liver in biliary atresia. Am J Gastroenterol 103:712–719
13. Kobayashi H, Yamataka A, Koga H et al (2005) Optimum prednisolone usage in patients with biliary atresia postportoenterostomy. J Pediatr Surg 40:327–330
14. Tatekawa Y, Muraji T, Tsugawa C (2005) Glucocorticoid receptor alpha expression in the intrahepatic biliary epithelium and adjuvant steroid therapy in infants with biliary atresia. J Pediatr Surg 40:1574–1580

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

Received 24 July 2012
Revised 19 September 2012
Accepted 23 September 2012
Published Online First 16 November 2012

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

To cite: Okamura A, Harada K, Nio M, *et al*. *J Clin Pathol* 2013;**66**:99–108.

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

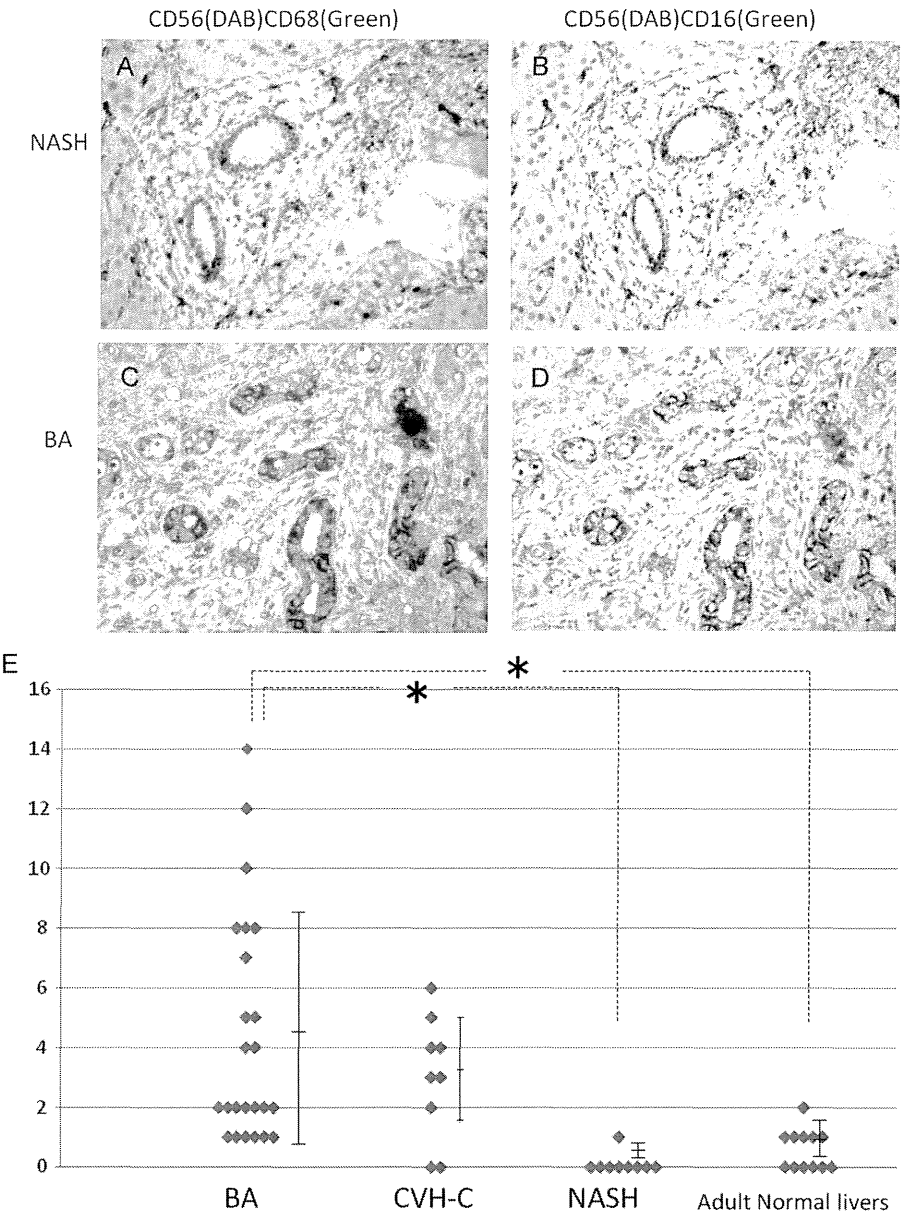


Figure 1 Density of CD56(−)CD16(+) CD68(−) natural killer (NK) cells around intrahepatic small bile ducts. (A, C) Expression of CD56 (brown) and CD68 (green). (B, D) Expression of CD56 (brown) and CD16 (green). Two photographs in the same areas of nonalcoholic steatohepatitis (NASH) (A, B) were compared. 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.

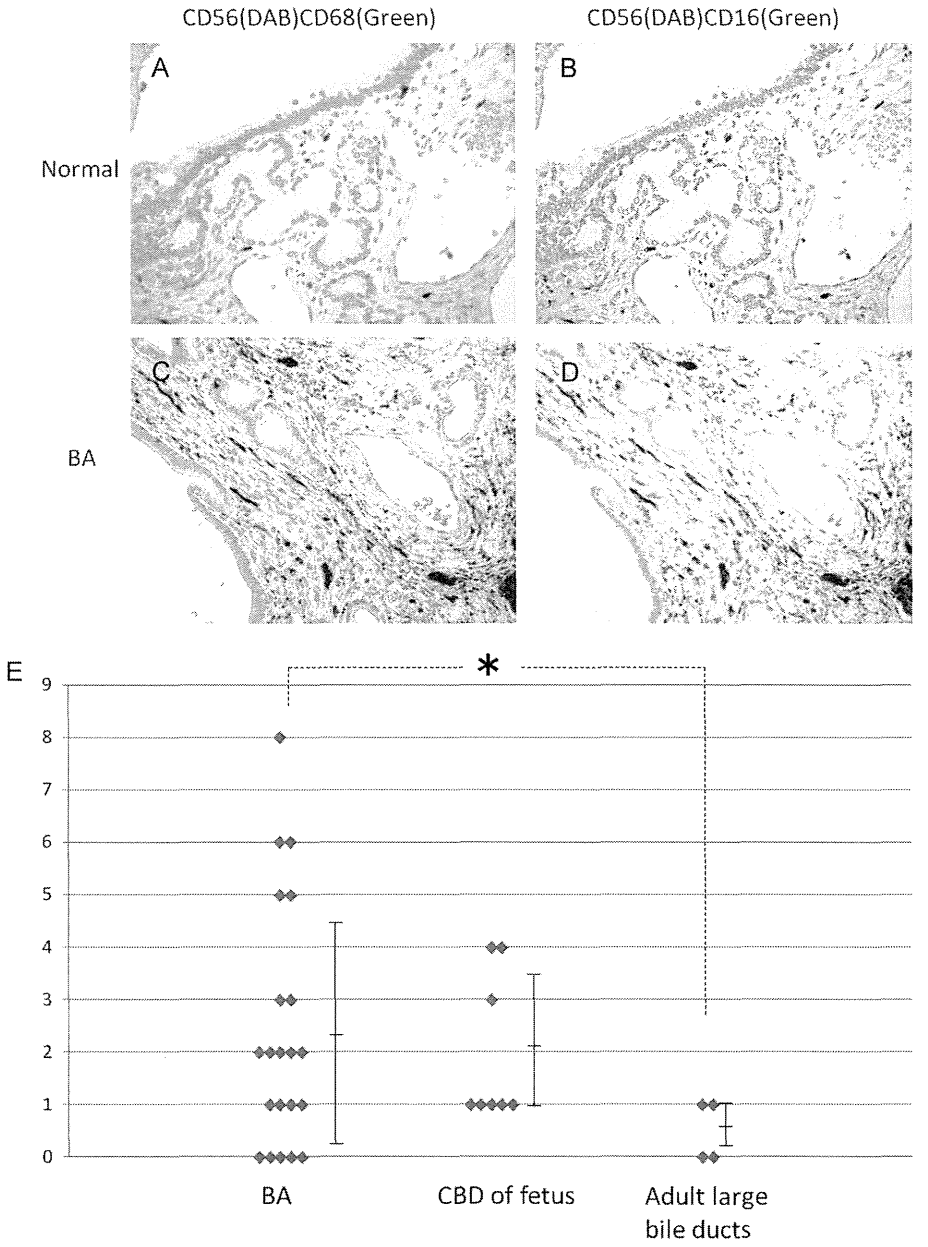


Figure 2 Density of CD56(−)CD16(+) CD68(−) natural killer (NK) cells around large bile ducts. (A, B) There were no or few CD56(−)CD16(+)CD68 (−) NK cells beneath biliary epithelia of the large bile duct in normal adult livers. (C, D) Such NK cells were identifiable in biliary atresia (BA). (E) These 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)

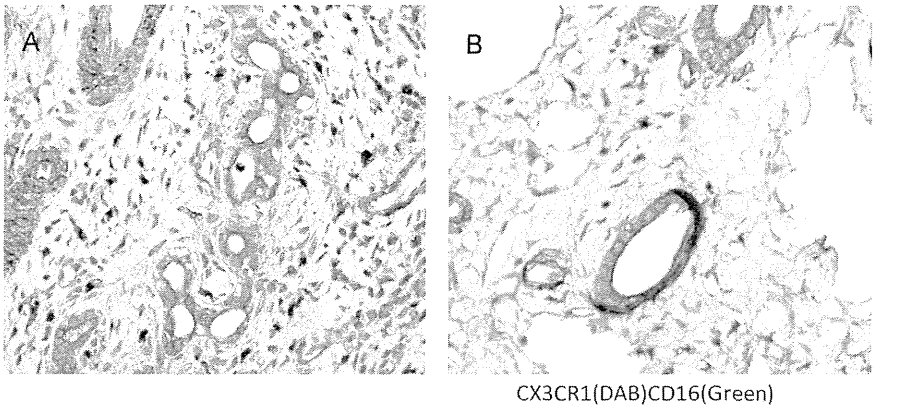
RT-PCR revealed that the amplicon of CX3CL1 mRNA could not be detected in cultured BECs without any stimulants (PBS), whereas treatment with poly(I:C) induced its expression (figure 6A). As shown in figure 6B, real-time PCR analysis revealed that treatment with poly(I:C) significantly up-regulated the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

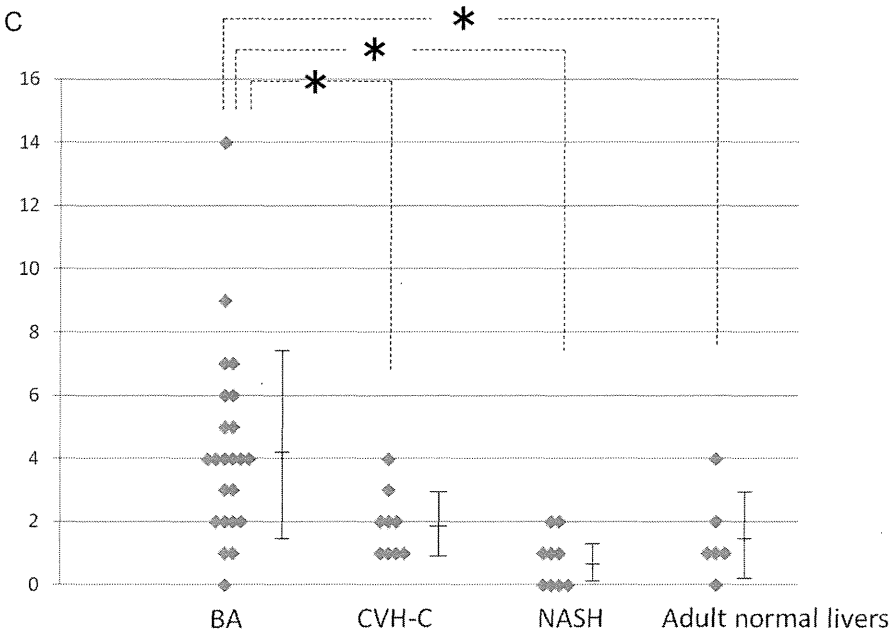
Migration of NK cells

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





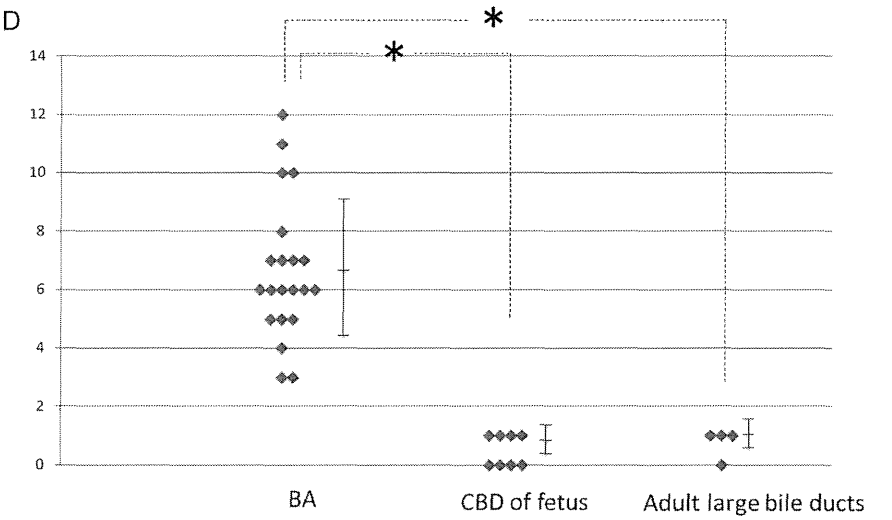


Figure 3 CX3CR1(+)CD16(+) mononuclear cells around intrahepatic bile ducts. (A) CX3CR1(+)CD16(+) mononuclear cells were frequently present in portal tracts around damaged small bile ducts in biliary atresia (BA). (B) Such cells were sparse in normal livers and other liver diseases. (C) They were rather 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

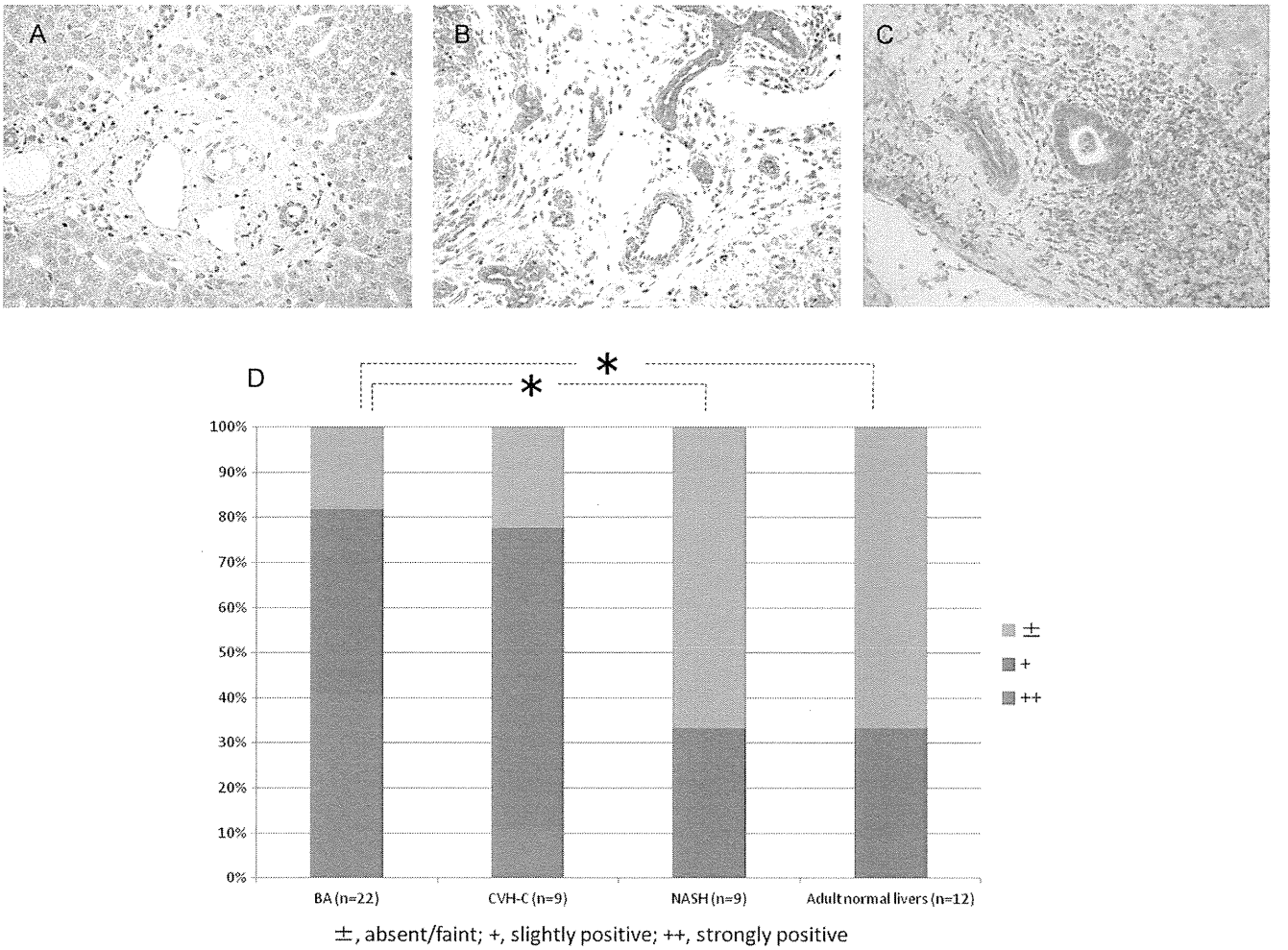


Figure 4 Expression of CX3CL1 in intrahepatic small bile duct epithelia. (A) Normal livers. Small bile ducts were generally negative or faintly positive for CX3CL1. (B) Biliary atresia (BA). Small bile ducts were strongly positive for CX3CL1. (C) The incidence of small bile ducts with mild to moderate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

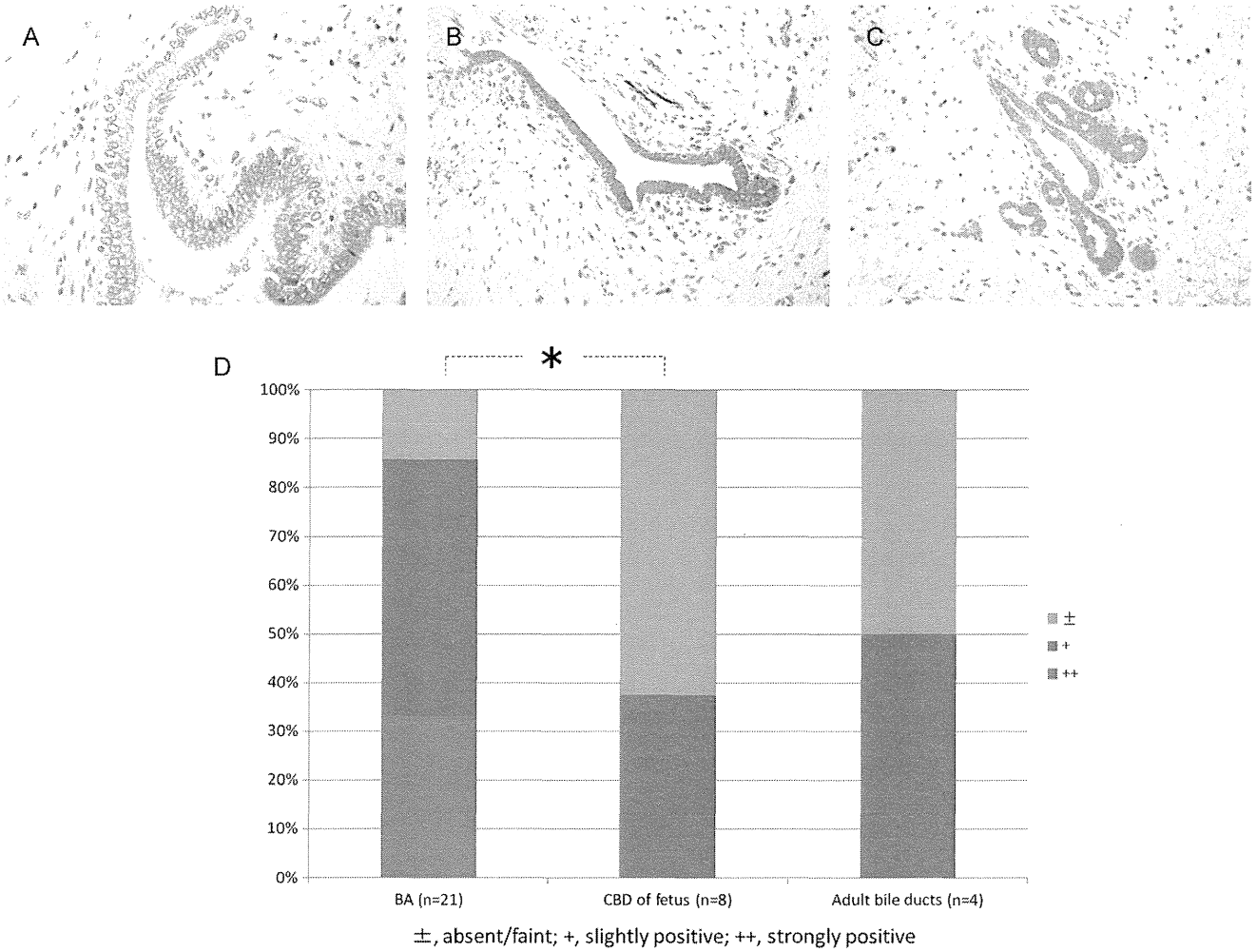


Figure 5 Expression of CX3CL1 in large bile duct epithelia. (A) CX3CL1 was not or faintly expressed in large bile ducts and peribiliary glands and peribiliary capillary plexus of normal livers. (B,C) It was strongly expressed in biliary epithelial cells of large bile ducts and peribiliary glands and 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

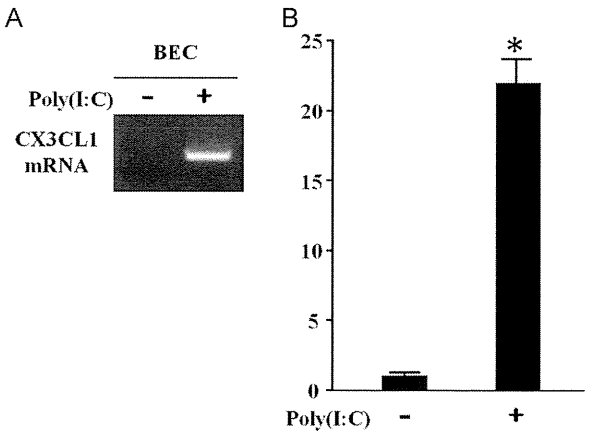


Figure 6 Expression of CX3CL1 mRNA in cultured human biliary epithelial cells (BECs). (A) Representative images of RT-PCR using cultured BECs. The amplicon of CX3CL1 mRNA could not be detected without the stimulant (–). de novo expression was found in the poly(I:C)-treated cells 3 h after treatment with poly(I:C). (B) Quantitative analysis using real-time PCR revealed the increase in the level of CX3CL1 mRNA on poly(I:C) treatment to be 21.9±2.2 (mean±SEM)-fold and statistically significant compared with that without treatment (effect size=0.97, CI −26.09 to −15.61). Results were obtained from four independent experiments. Bars indicate the mean±SEM. *<0.05.

by the chemoattraction of CX3CR1-expressing mononuclear cells including NK cells, and their adhesion to BECs.

The elevation of CD56(–)CD16(+) NK subset was reported in the peripheral blood mononuclear cell of HCV- and HIV-infected patients.[11–13] We could confirm the increase of CD56(–)CD16(+)CD68(–) NK cells in liver specimens of CVH-C as well as BA by the immunohistochemistry, though statistical significance was not obtained in CVH-C, compared with NASH and normal liver. Therefore, impaired NK function caused by an increased CD56(–)CD16(+) NK subset in liver tissue is presumable in BA and CVH-C, but not NASH or normal livers.

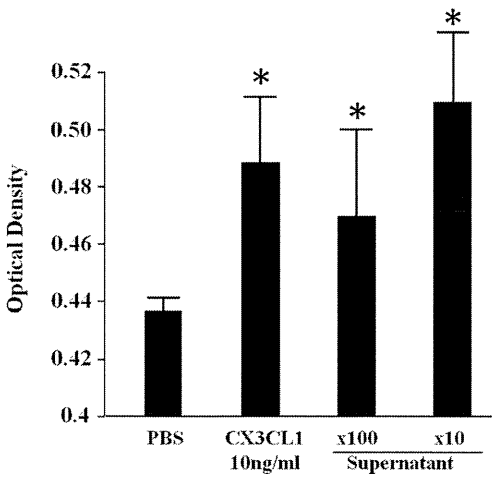


Figure 7 Migration assay of natural killer (NK) cells. Optical density (OD) reflecting the number of transmigrated NK cells was significantly increased in the lower chamber containing recombinant CX3CL1 (10 ng/ml, OD=0.49±0.02 (mean±SEM), effect size=0.66, CI −0.09 to −0.01) and supernatant of poly(I:C)-treated BEC diluted 1:100 (OD=0.47±0.02, effect size=0.51, CI −0.08 to 0.01) and 1:10 (OD=0.51±0.02, effect size=0.73, CI −0.12 to −0.02), compared with that containing the negative control medium (PBS, OD=0.44±0.008). Results were obtained from eight independent experiments. Bars indicate the mean±SEM. *<0.05.

Moreover, it is speculated that these NK cells were attracted by CXCL1 produced in BECs via an innate immunity against virus. This scenario might be common in several virus-related diseases including CVH-C and BA.

Take home messages

- CD56(–)CD16(+) NK cells with reduced NK activities accumulated around damaged small and large bile ducts may be involved in the development of BA.
- By the biliary innate immunity for dsRNA, BECs expressed CX3CL1, which may attract CD16(+) NK cells around the damaged bile ducts.
- These findings may be followed by acquired immunity against the infected bile ducts.

Contributors AO and KH contributed equally in this study, and YN and MN were mainly involved in the concept of this study and preparation of the manuscript.

Funding This work was supported by grant No.23590393 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (K.H.) and by Health and Labour Sciences Research Grants for the Research on Measures for Intractable Diseases.

Competing interests None.

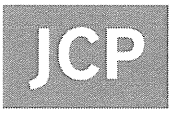
Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Sokol RJ, Mack C. Etiopathogenesis of biliary atresia. *Semin Liver Dis* 2001;21:517–24.
2 Al-Masri AN, Flemming P, Rodeck B, *et al*. Expression of the interferon-induced Mx proteins in biliary atresia. *J Pediatr Surg* 2006;41:1139–43.
3 Sharland A, Gorrell MD. Cooperation of innate and adaptive immunity in the pathogenesis of biliary atresia: there's a killer on the run. *Hepatology* 2009;50:2037–40.
4 Mack CL. The pathogenesis of biliary atresia: evidence for a virus-induced autoimmune disease. *Semin Liver Dis* 2007;27:233–42.
5 Shivakumar P, Sabla G, Mohanty S, *et al*. Effector role of neonatal hepatic CD8+ lymphocytes in epithelial injury and autoimmunity in experimental biliary atresia. *Gastroenterology* 2007;133:268–77.
6 Mack CL, Tucker RM, Lu BR, *et al*. Cellular and humoral autoimmunity directed at bile duct epithelia in murine biliary atresia. *Hepatology* 2006;44:1231–9.
7 Harada K, Sato Y, Itatsu K, *et al*. Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. *Hepatology* 2007;46:1146–54.
8 Nakanuma Y, Harada K, Sato Y, *et al*. Recent progress in the etiopathogenesis of pediatric biliary disease, particularly Caroli's disease with congenital hepatic fibrosis and biliary atresia. *Histol Histopathol* 2010;25:223–5.
9 Harada K, Nakanuma Y. Biliary innate immunity in the pathogenesis of biliary diseases. *Inflamm Allergy Drug Targets* 2010;9:83–90.
10 Harada K, Nakanuma Y. Biliary innate immunity: function and modulation. *Mediators Inflamm* 2010;2010:1–9.
11 Hong HS, Eberhard JM, Keudel P, *et al*. Phenotypically and functionally distinct subsets contribute to the expansion of CD56-/CD16+ natural killer cells in HIV infection. *AIDS* 2010;24:1823–34.
12 Hong HS, Eberhard JM, Keudel P, *et al*. HIV infection is associated with a preferential decline in less-differentiated CD56dim CD16+ NK cells. *J Virol* 2010;84:1183–8.
13 Fauci AS, Mavilio D, Kottilil S. NK cells in HIV infection: paradigm for protection or targets for ambush. *Nat Rev Immunol* 2005;5:835–43.
14 Shivakumar P, Sabla GE, Whitington P, *et al*. Neonatal NK cells target the mouse duct epithelium via Nkg2d and drive tissue-specific injury in experimental biliary atresia. *J Clin Invest* 2009;119:2281–90.
15 Isse K, Harada K, Zen Y, *et al*. Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. *Hepatology* 2005;41:506–16.
16 Nakanuma Y, Hoso M, Sanzen T, *et al*. Microstructure and development of the normal and pathologic biliary tract in humans, including blood supply. *Microsc Res Tech* 1997;38:552–70.

17 Harada K, Ohba K, Ozaki S, *et al*. Peptide antibiotic human beta-defensin-1 and -2 contribute to antimicrobial defense of the intrahepatic biliary tree. *Hepatology* 2004;40:925–32.
18 Garcia-Barceló MM, Yeung MY, Miao XP, *et al*. Genome-wide association study identifies a susceptibility locus for biliary atresia on 10q24.2. *Hum Mol Genet* 2010;19:2917–25.
19 Leyva-Vega M, Gerfen J, Thiel BD, *et al*. Genomic alterations in biliary atresia suggest region of potential disease susceptibility in 2q37.3. *Am J Med Genet A* 2010;152A:886–95.
20 Lee HC, Chang TY, Yeung CY, *et al*. Genetic variability of interleukin4 gene in Taiwanese children with biliary atresia. *Cytokine* 2012;57:402–5.
21 Lee HC, Chang TY, Yeung CY, *et al*. Association of polymorphisms in the Interleukin-18 gene with susceptibility to biliary atresia. *J Pediatr Gastroenterol Nutr* 2011;52:607–11.
22 Lee HC, Chang TY, Yeung CY, *et al*. Association of interferon-gamma gene polymorphisms in Taiwanese children with biliary atresia. *J Clin Immunol* 2010;30:68–73.
23 Lee HC, Chang TY, Yeung CY, *et al*. The VEGF +936 C/T polymorphism and particularly the C allele are associated with BA, possibly conferring increased susceptibility to the disease. *J Clin Gastroenterol* 2010;44:135–9.
24 Dong R, Zhao R, Zheng S, *et al*. Abnormal DNA methylation of ITGAL (CD11a) in CD4+ T cells from infants with biliary atresia. *Biochem Biophys Res Commun.* 2012;417:986–90.
25 Dong R, Zhao R, Zheng S. Changes in epigenetic regulation of CD4+ T lymphocytesin biliary atresia. *Pediatr Res* 2011;70:555–9.
26 Hertel PM, Estes MK. Rotavirus and biliary atresia: can causation be proven? *Curr Opin Gastroenterol* 2012;28:10–17.
27 Guo C, Zhu J, Pu CL, *et al*. Combinatory effects of hepatic CD8+ and NK lymphocytes in bile duct injury from biliary atresia. *Pediatr Res* 2012; 71:638–44.
28 Mavilio D, Lombardo G, Benjamin J, *et al*. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A* 2005; 102:2886–91.



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

Atsushi Okamura, Kenichi Harada, Masaki Nio, et al.

J Clin Pathol 2013 66: 99-108 originally published online November 16, 2012
doi: 10.1136/jclinpath-2012-201097

Clinical & Experimental Immunology
The Journal of Translational Immunology
immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12103

Interleukin-32 production associated with biliary innate immunity and proinflammatory cytokines contributes to the pathogenesis of cholangitis in biliary atresia

A. Okamura,*†‡ K. Harada,*‡ M. Nio† and Y. Nakanuma*

*Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, and †Department of Pediatric Surgery, Tohoku University, Graduate School of Medicine, Sendai, Japan

Accepted for publication 7 March 2013
Correspondence: K. Harada, Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan.
E-mail: kenichih@med.kanazawa-u.ac.jp

‡Drs Okamura and Harada contributed equally to this study.

Summary

Biliary atresia (BA) is thought to be associated with infections by viruses such as Reoviridae and is characterized histologically by fibrosclerosing cholangitis with proinflammatory cytokine-mediated inflammation. Interleukin (IL)-32 affects the continuous inflammation by increasing the production of proinflammatory cytokines. In this study, the role of IL-32 in the cholangitis of BA was examined. Immunohistochemistry for IL-32 and caspase 1 was performed using 21 samples of extrahepatic bile ducts resected from BA patients. Moreover, using cultured human biliary epithelial cells (BECs), the expression of IL-32 and its induction on stimulation with a Toll-like receptor [(TLR)-3 ligand (poly(I:C)] and proinflammatory cytokines was examined. BECs composing extrahepatic bile ducts showing cholangitis expressed IL-32 in BA, but not in controls. Caspase 1 was expressed constantly on BECs of both BA and control subjects. Furthermore, poly(I:C) and proinflammatory cytokines [(IL-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α] induced IL-32 expression strongly in cultured BECs, accompanying the constant expression of TLR-3 and caspase 1. Our results imply that the expression of IL-32 in BECs was found in the damaged bile ducts of BA and induced by biliary innate immunity via TLR-3 and proinflammatory cytokines. These findings suggest that IL-32 is involved initially in the pathogenic mechanisms of cholangitis in BA and also plays an important role in the amplification and continuance of periductal inflammatory reactions. It is therefore tempting to speculate that inhibitors of IL-32 could be useful for attenuating cholangitis in BA.

Keywords: biliary atresia, biliary epithelial cells, IL-32, innate immunity, TLR

Introduction

The obliterative lesion of biliary atresia (BA) is characterized by a progressive sclerosing cholangitis accompanying severe inflammation, fibrosis and epithelial injuries; this characteristic feature is known as fibrosclerosing cholangitis. Little is known about the aetiology and pathogenesis of BA, but infections by viruses such as Reoviridae (reovirus and rotavirus) having a double-stranded RNA (dsRNA) have been implicated, although conflicting results have also been reported [1–8]. Our recent study has demonstrated that biliary epithelial cells (BECs) possess an innate immune system consisting of Toll-like receptors (TLR), especially TLR-3, which is an innate immune-recognition receptor recognizing dsRNA, including dsRNA viruses as pathogen-associated molecular patterns (PAMPs) [9,10]. Furthermore, the biliary innate immune response to artificial dsRNA was also shown to be associated with the induction of biliary apoptosis via the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and, differing from the innate immune response to TLR-4 ligand [lipopolysaccharide (LPS)], lack of subsequent tolerance to dsRNA using cultured human biliary epithelial cells [9–11].

Interleukin (IL)-32 is a recently described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes and some epithelial cells [12,13]. Primarily, IL-32 was discovered in the synovial fluid of patients with rheumatoid arthritis and first reported as a transcript in IL-2 activated NK and T cells [14,15]. There are six isoforms (α, β, γ, δ, ε and ξ) caused by alternative mRNA splicing, resulting in

proteins with a molecular weight ranging from 14·9 to 26·7 kD. IL-32α is the most abundant transcript. IL-32 exhibits several properties typical of proinflammatory cytokines [16]. For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1α, tumour necrosis factor (TNF)-α, IL-6, IL-8 and vascular endothelial growth factor (VEGF) through the activation of nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinases (MAPKs) [15,17,18]. In contrast, the production of IL-32 is induced or enhanced by the presence of proinflammatory cytokines, including IL-1β, IFN-γ and TNF-α via the activation of caspase 1 [17,19,20]. IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, chronic obstructive pulmonary diseases, atopic dermatitis and allergic rhinitis [14,19–22].

Although human hepatocytes and hepatoma cells express IL-32 in hepatitis C virus (HCV)-associated chronic hepatitis and this expression is regulated by proinflammatory stimuli [23], the pathophysiological role of IL-32 in innate immune-related biliary diseases, including BA, remains unclear. We therefore investigated the IL-32 expression in the inflamed bile ducts of BA patients and the effect of innate immune stimulation by ligands of TLR-3 and cytokines on IL-32 expression in cultured human BECs. Our results provide evidence that biliary epithelial cells are sufficient sources of IL-32 for the biliary inflammation at sites of BA, and IL-32 may therefore play a role in the pathophysiology of BA.

Materials and methods

Patients and tissue preparations

A total of 21 patients with BA (surgical specimens; average age 1·7 months; age range 0·7–12 months; nine male/12 female) and age-matched control patients consisting of one neonatal hepatitis (giant cell hepatitis; wedge biopsy; 3 months; male) and six non-hepatobiliary diseases (congenital heart anomalies; autopsied specimens; average age 2·5 months; three male/three female) were examined. Resected common bile ducts and wedge liver biopsy specimens were obtained from patients with BA using the Kasai procedure. These specimens had been fixed in 10% neutral-buffered formalin and embedded in paraffin; 4 μm-thick sections were prepared for histological observation and immunohistochemistry.

Immunohistochemistry and immunocytochemistry

For immunocytochemistry using cultured BECs, formalin-fixed, paraffin-embedded sections of cell blocks were prepared according to the protocol reported by Mayall *et al.* [24]. The deparaffinized and rehydrated sections were heated in 10 mM citrate buffer for 20 min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at 4°C overnight with antibody against the C-terminus of IL-32 [rabbit polyclonal immunoglobulin (Ig)G, 1 μg/ml; Lifespan, Seattle, WA, USA], TLR-3 (rabbit polyclonal IgG, 1 μg/ml; Santa Cruz, Santa Cruz, CA, USA) and caspase 1 (rabbit monoclonal IgG, diluted 1:1000; Abcam, Tokyo, Japan) and then at room temperature for 1 h with anti-rabbit immunoglobulins conjugated to a peroxidase-labelled dextran polymer (Simple Staining Kit; Nichirei, Tokyo, Japan). After a benzidine reaction, sections were counterstained lightly with haematoxylin. As a negative control, normal rabbit IgG was used as the primary antibody; no staining was obtained.

For semiquantitative evaluation of the immunohistochemistry, intrahepatic bile ducts and extrahepatic common bile ducts were chosen in each section for assessment and IL-32 immunoreactivity in these bile ducts was graded semiquantitatively as follows: score 0, absence of expression; score 1, low constitutive expression; score 2, intermediate expression; and score 3, high expression.

In addition, simultaneous detection of IL-32 and cytokeratin (CK)19 was performed using double immunohistochemical staining. After IL-32 immunostaining, CK19 antibody (mouse monoclonal IgG1kappa, 0·45 μg/ml; Dako Japan, Tokyo, Japan) was applied overnight at 4°C, followed by immunoglobulins conjugated with alkaline phosphatase labelled-dextran polymer (Nichirei). Colour development of IL-32 and CK19 was achieved with diaminobenzidine (brown) and Vector blue (Vector Laboratories, Burlingame, CA, USA), respectively.

Cultured human BECs and stimulation with PAMPs and proinflammatory cytokines

A cultured cell line of human intrahepatic BECs was established from the explant liver of a 24-year-old male with BA who had already received the Kasai procedure during the newborn period, and cultured as reported previously [25]. The cultured BECs were incubated with a culture medium composed of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Tokyo, Japan), 5% newborn calf serum (Invitrogen), 0·18 mM adenine (Sigma, St Louis, MO, USA), hydrocortisone (0·4 μg/ml), cholera toxin (10 ng/ml), tri-iodo-thyronine (1·3 μg/l), ITS+ (Becton Dickinson, Franklin Lakes, NJ, USA), 25 mM sodium bicarbonate (Sigma), 1% antibiotics anti-mycotic, human epidermal growth factor (20 ng/ml) (Invitrogen) and human hepatocyte growth factor (10 ng/ml) (Invitrogen). The cells were grown as monolayers in a humidified incubator with 5% CO_2 at 37°C. More than 95% of the cells were confirmed to be biliary epithelial cells by the expression of a biliary-type cytokeratin (CK19). The cultured BECs were used between passages 4 and 9. Informed consent for human research was obtained from the patient prior to surgery. This study was approved by the Kanazawa Univer-

sity Ethics Committee. Moreover, as control cultured cells, a commercially available cell line derived from human hepatocellular carcinoma, HepG2, was obtained from the Health Science Research Resources Bank (Osaka, Japan).

These cultured cells were stimulated with a TLR-3 ligand, polyinosinic–polycytidylic acid [poly(I:C), a synthetic analogue of viral dsRNA, 25 μg/ml; Invivogen, San Diego, CA, USA] and recombinant cytokines [IL-1β, IFN-γ, TNF-α, transforming growth factor (TGF)-β1 and IL-10, l000 U/ml; PeproTech, London and IL-32, 1000 U/ml; R&D Systems, Minneapolis, MN, USA] for 3 h (molecular analysis) and 48 h (protein analysis by immunocytochemistry and Western blotting analysis).

Isolation of RNA, reverse transcription–polymerase chain reaction (RT–PCR) and real-time PCR

For evaluation of mRNA of IL-32, caspase 1, TLR-3, IL-1β and IL-6 in cultured BECs, isolation of RNA from BECs and reverse transcription were performed using the RNeasy Total RNA System (Qiagen, Hilden, Germany) and Rever-Tra Ace (Toyobo, Osaka, Japan). First, to examine the presence of target molecules and the validity of the newly designed primers, conventional PCR was performed. Specific primers for IL-32, caspase 1, TLR-3 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, positive control) were designed: IL-32 forward: 5′-AGCTGGAGGACGAC TTCAAA-3′, reverse: 5′-TTGAGGATTGGGGTTCAGAG-3′ [predicted size, 258 base pairs (bp)]; TLR-3 forward: 5′-CCATTCCAGCCTCTTCGTAA-3′, reverse: 5′-GGATGT TGGTATGGGTCTCG-3′ (predicted size, 505 bp); caspase 1 forward: 5′-CCACAATGGGCTCTGTTTTT-3′, reverse: 5′-CATCTGGCTGCTCAAATGAA-3′ (predicted size, 117); IL-1β forward: 5′-CCAGGGACAGGATATGGAGCA-3′, reverse: 5′-TTCAACACGCAGGACAGGTACAG-3′ (predicted size, 129 bp); IL-6 forward: 5′-AGTGAGGAACAA GCCAGAGC-3′, reverse: 5′-AAGCTGCGCAGAATGAGAT-3′ (predicted size, 189 bp); and GAPDH forward: 5′-GG CCTCCAAGGAGTAAGACC-3′, reverse: 5′-AGGGGTCTA CATGGCAACTG-3′ (predicted size, 147 bp). The reaction profile consisted of initial denaturation at 94°C for 3 min followed by 25–40 cycles with 30 s of denaturation at 94°C, 30 s of annealing of primers at 55°C and a 60-s extension at 72°C. Next, to carry out relative quantification, real-time quantitative PCR was performed according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan). Relative gene expression was calculated using the comparative cycle threshold method and adjusted based on expression of the housekeeping gene (GAPDH). Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase and DNase free water.

Western blotting

Cell lysates of poly(I:C)-stimulated or unstimulated cultured cell lines (10 μg protein/lane) and the culture medium were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant IL-32 protein (0·1 μg; R&D) was used as a positive control. Separated proteins were transferred onto a nitrocellulose membrane; the membrane was blocked in 5% bovine serum albumin, then probed for 1 h with a primary antibody against human IL-32 (0·1 μg/ml). After washing, the membrane was incubated for 1 h with the Simple Staining Kit, and visualized with the benzidine reaction. The band density was evaluated quantitatively using NIH images.

Statistical analysis

Data were analysed using the paired *t*-test or Welch's *t*-test; $P < 0{\cdot}05$ was considered statistically significant.

Results

Expression of IL-32, caspase 1 and TLR-3 in extrahepatic bile ducts of BA

Immunohistochemistry revealed the expression of IL-32 in BECs, infiltrating inflammatory cells and endothelial cells at various intensities. In particular, damaged common bile ducts showing cholangitis in BA expressed IL-32 strongly, accompanying many IL-32-positive inflammatory cells and vessels (Fig. 1a–c). As shown in Fig. 1f, double immunohistochemistry highlighted that CK19-postive bile ducts clearly expressed IL-32. However, non-damaged biliary epithelium found at the margin of resected common bile ducts did not express IL-32 (Fig. 1g,h). In wedge liver biopsies, hepatocytes were also positive for IL-32 in addition to small bile ducts (interlobular bile ducts), but the intensity was lower than that in damaged common bile ducts (Fig. 1i,j). Moreover, congestive bile in intrahepatic bile ducts was also strongly positive for IL-32 (Fig. 1j). In contrast, BECs in common bile ducts and intrahepatic bile ducts of age-matched controls expressed only weakly or lacked IL-32 (Fig. 2a,d). The semiquantitative analysis for immunoreaction confirmed that the expression of IL-32 in damaged common bile ducts of BA was up-regulated significantly, compared with those in non-damaged/normal bile ducts of BA and age-matched controls (Fig. 3). Caspase 1 and TLR-3 were expressed constantly in BECs of extrahepatic bile ducts in both the BA and control patients (Fig. 2b,c).

Induction of IL-32 expression by PAMPs and cytokines in cultured BECs

To examine the presence of target molecules and the validity of the newly designed primers, RT–PCR at 40 cycles was

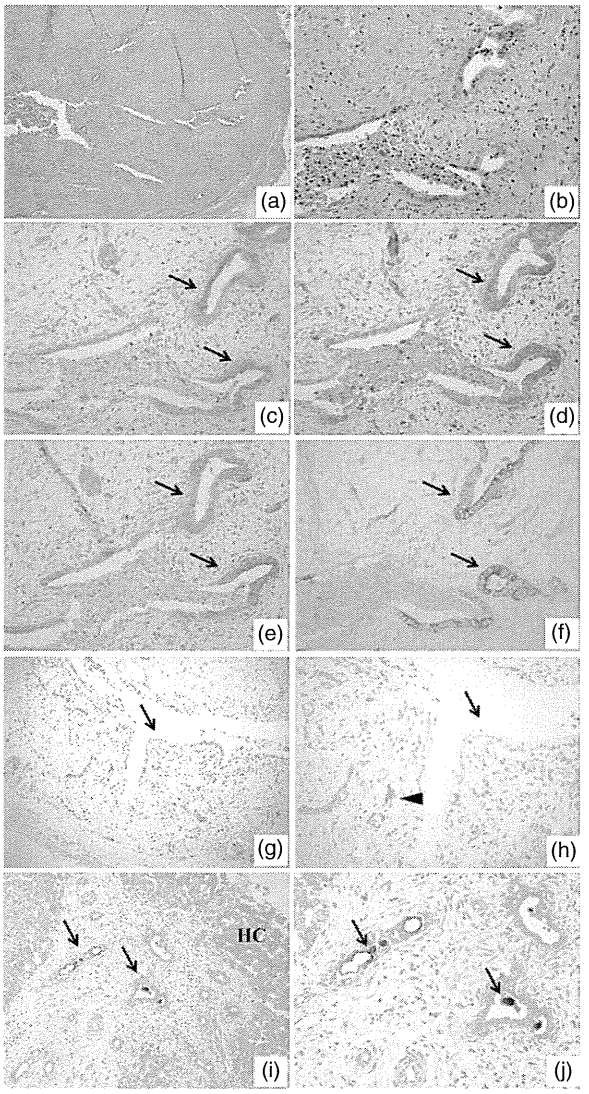


Fig. 1. Histology and immunohistochemistry for interleukin (IL)-32, Toll-like receptor (TLR)-3 and caspase 1 in biliary atresia (BA). (a,b) Transverse sections of biliary remnants. Damaged extrahepatic bile ducts line inconsistently by desquamated columnar epithelium and surrounding fibroplasia with an inflammatory cell infiltrate; (b) a higher magnification of (a). Haematoxylin and eosin (H&E) staining. Original magnification (a) ×100 and (b) ×400. Immunohistochemistry for IL-32 (c), TLR-3 (d) and caspase 1 (e). The strong expression of IL-32, TLR-3 and caspase 1 was found in biliary epithelial cells (arrows) of damage bile ducts. Original magnification ×400. (f) Double immunohistochemistry for CK19 and IL-32 highlighted the CK19-positive bile ducts (blue) clearly expressed IL-32 (brown) (arrows). Original magnification ×400. (g,h) Immunohistochemistry for IL-32. Undamaged extrahepatic bile duct located at the resected margin in BA. IL-32-positive neovascular structures (arrowhead) were found, but undamaged biliary epithelium lacked IL-32 expression (arrows); (h) is higher magnification of (g). Original magnification (g) ×200 and (h) ×400. (i,j) Immunohistochemistry for IL-32 using wedge liver specimens of BA. Interlobular bile ducts (arrows in i) and hepatocytes (HC in i) expressed IL-32. Moreover, condensed bile in dilated bile ducts was also strongly positive for IL-32 (arrows in j); (j) is a higher magnification of (i). Original magnification (e) ×200 and (f) ×400.

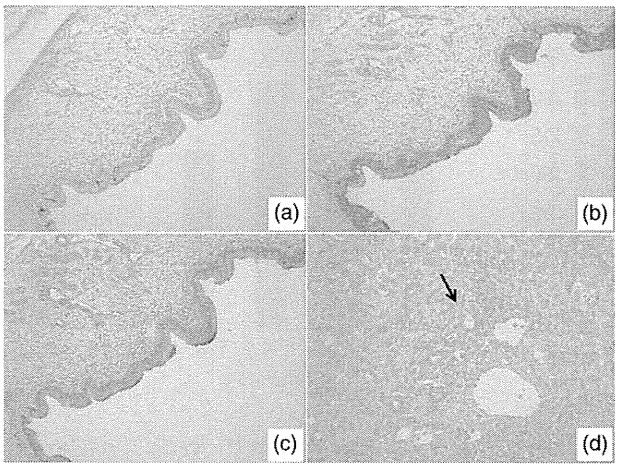


Fig. 2. Immunohistochemistry for interleukin (IL)-32 (a,d), Toll-like receptor (TLR)-3 (b) and caspase 1 (c) in age-matched controls. (a–c) Biliary epithelial cells in common bile ducts of non-hepatobiliary diseases (congenital heart anomalies) expressed TLR-3 (b) and caspase 1 (c), but lacking or faintly expressed IL-32 (a) was faint or negative. Original magnification ×200. (d) Interlobular bile duct in neonatal hepatitis was negative for IL-32 (arrow). Original magnification ×400.

performed and an amplification of all molecules could be detected as a single band from cultured BECs at the expected size. Moreover, the BECs constantly expressed the mRNA of TLR-3 and caspase 1, which is necessary for the recognition of poly(I:C) and the production of functional IL-32 protein, respectively. The real-time PCR analysis revealed that TLR-3 ligand, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), enhanced the mRNA expression of IL-32, the increases being statistically

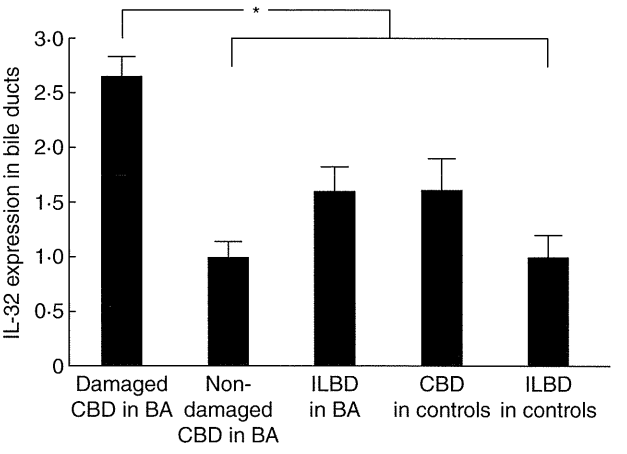


Fig. 3. Semiquantitative analysis of immunohistochemistry for interleukin (IL)-32. The expression of IL-32 in damaged common bile ducts (CBD) of biliary atresia (BA) was up-regulated significantly compared with those of non-damaged CBD and interlobular bile ducts (ILBD) in BA, and of CBD and ILBD in age-matched controls. *$P<0{\cdot}05$.

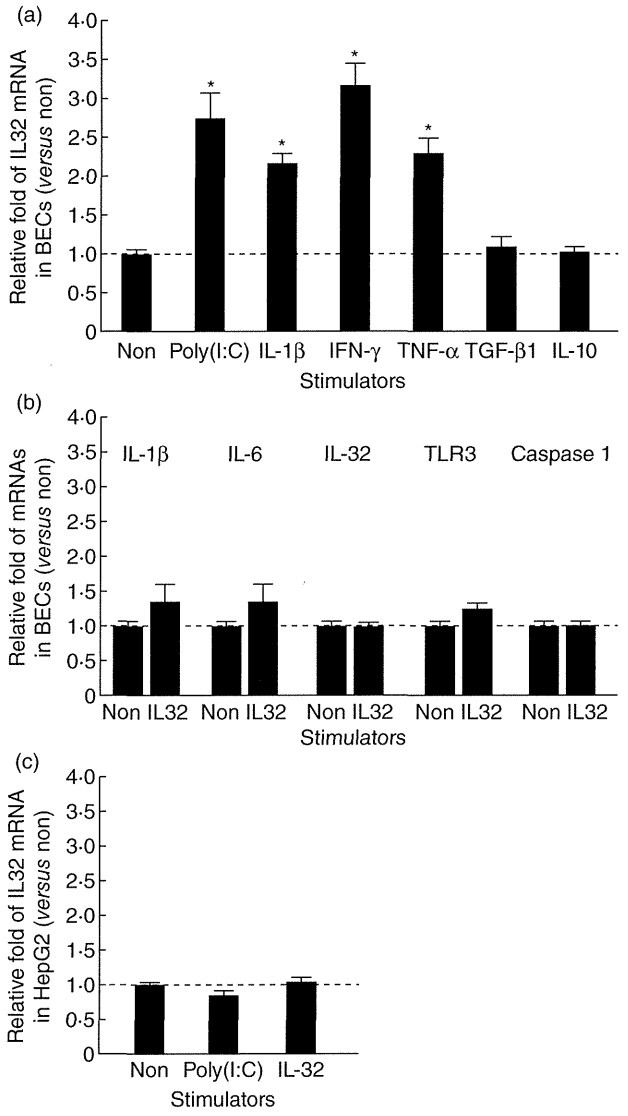


Fig. 4. (a) Induction of interleukin (IL)-32 expression by Toll-like receptor (TLR)-3 ligand (poly I:C) and cytokines in cultured biliary epithelial cells (BECs). Quantitative analysis using real-time polymerase chain reaction (PCR) revealed that a TLR-3 ligand, poly(I:C) and proinflammatory cytokines [interleukin (IL)-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α], but not regulatory cytokines [transforming growth factor (TGF)-β1 and IL-10], up-regulated significantly the mRNA expression of IL-32. (b) Detection of BEC-producing cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs. The stimulation with IL-32 did not up-regulate the expression of any cytokines, TLR-3 or caspase 1 significantly. (c) Detection of IL-32 in a control cell line, HepG2. Induction of IL-32 expression was not found by stimulation with poly(I:C) or IL-32. Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments (Non). Bars indicate the mean ± standard error of the mean. $^*P < 0{\cdot}05$.

significant (Fig. 4a). In contrast, stimulation with IL-32 did not up-regulate significantly the expression of BEC-producing cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs (Fig. 4b). Although the control cell line, HepG2, also expressed IL-32 mRNA, up-regulation of IL-32 was not significant by stimulation with poly(I:C) or IL-32 (Fig. 4c).

Detection of intracytoplasmic and secreted IL-32protein

To investigate secretion of the IL-32 protein, Western blotting was performed using the cell lysate and culture medium of BECs. IL-32 was detected in the medium as well as lysate from the poly(I:C)-stimulated BECs (Fig. 5a). Semiquantitative analysis using NIH image analysis revealed that the band density was up-regulated in cell lysate and culture medium by stimulation with poly(I:C) (Fig. 5a). Moreover, immunocytochemistry also demonstrated that IL-32 protein was expressed strongly in poly(I:C)-stimulated BECs, compared with non-stimulated BECs (Fig. 5b).

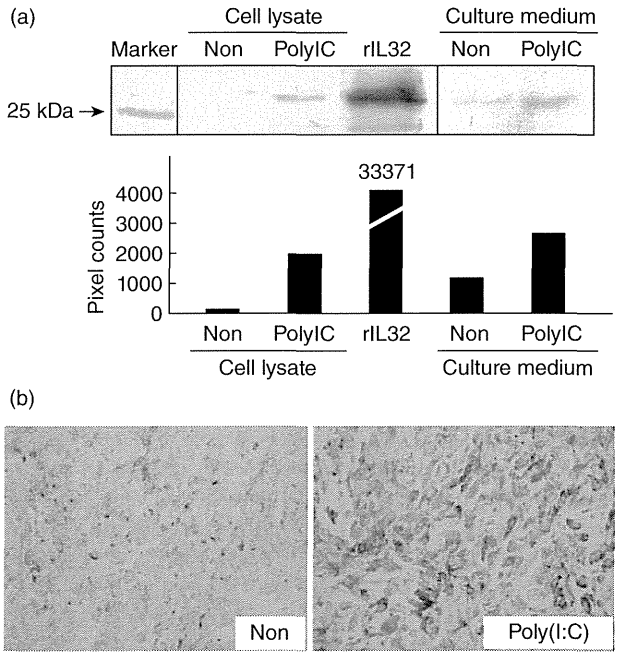


Fig. 5. Detection of intracytoplasmic and secreted interleukin (IL)-32 protein in cultured biliary epithelial cells (BECs). (a) Western blotting revealed that the culture medium as well as cell lysate of poly(I:C)-treated cultured cells contained IL-32 protein, but the level was faint in untreated cells (Non). As a positive control, recombinant IL-32 (rIL-32, 0·1 μg) was used. Semiquantitative analysis using NIH image analysis confirmed that the density of bands was up-regulated in cell lysate and culture medium by stimulation with poly(I:C). (b) Immunocytochemistry also demonstrated that IL-32 was expressed strongly in the poly(I:C)-stimulated BECs compared with unstimulated BECs (Non). Original magnification ×400.

Discussion

BA is characterized initially by periductal inflammation and fibrosis and the obstruction of common bile ducts, known as fibrosclerosing cholangitis. Recruitment of inflammatory cells results in the release of other proinflammatory cytokines and chemokines, sustaining the cholangitis associated with the biliary innate immune response and promoting chronic cholangitis associated with the subsequent acquired immune response in a later phase [26]. IL-32 is a recently described cytokine that is a strong inducer of proinflammatory cytokines whose expression is increased markedly in several inflammatory disorders, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD), and correlated with the severity of these diseases [14,19]. In the present study, human BECs were demonstrated to be the local source of IL-32. Immunohistochemical analysis showed a cytoplasmic distribution of IL-32 in BECs of the damaged common bile ducts in BA cases, although BECs of common bile ducts in age-matched controls were negative or only weakly positive for IL-32, suggesting that IL-32 is associated closely with the histogenesis of periductal inflammation in BA. However, IL-32 production in BECs is not specific to BA alone. In fact, we confirmed the expression of IL-32 in bile ducts of adult biliary diseases such as primary biliary cirrhosis, but its intensity was lower than those in the damaged common bile ducts of BA. Therefore, we speculated that the induction of IL-32 by unique factors such as viral infections in BA was stronger than those in other biliary diseases. Inflammasomes are multi-protein cytoplasmic complexes that mediate the activation of inflammatory caspase-1. For example, caspase-1 cleaves pro-IL-1β to the active form of IL-1β. In this manner, caspase-1 controls the maturation of some of the proinflammatory cytokines, and IL-32 also depends upon caspase 1 activation [17,20]. Therefore, the presence of caspase 1 is necessary for the functional expression of IL-32 in BECs. In the present study, BECs constantly expressed caspase 1 *in vitro* and *in vivo*, suggesting the expression of functional IL-32 in BECs.

Recent studies have focused upon the role of innate immunity associated with Reoviridae (reovirus and rotavirus) in the pathogenesis of BA. Having a dsRNA genome, Reoviridae in particular are characterized by epithelial tropism [1,3,4,9,10,27,28]. The initial sensing of innate immunity is mediated by the recognition of PAMPs through TLRs. IL-32 also appears to play an important role in the host defence against invading micro-organisms [23,29,30]; that is, IL-32 is described as a proinflammatory cytokine that enhances host immunity against various microbial pathogens. The present study revealed that stimulation with poly(I:C), a mimic of Reoviridae, enhanced IL-32 expression in cultured BECs, suggesting that the biliary innate immune response directly induces the production of IL-32 in BECs. A control cell line used in this study, HepG2, also expressed IL-32 mRNA, but the up-regulation of IL-32 was not significant by stimulation with poly(I:C). It has already been reported that IL-32 expression is induced in peripheral blood mononuclear cells and monocytes by *Mycobacterium tuberculosis* [31] but, to our knowledge, this is the first description concerning the production of IL-32 in epithelial cells such as BECs via an innate immune response.

IL-1β, IFN-γ and TNF-α were reported to be inducers of IL-32 expression [16,19]. However, the regulatory mechanism of these proinflammatory cytokines remains unclear. In this study, we found that all these proinflammatory cytokines are potent stimulators of IL-32 expression in cultured BECs. In contrast, the aforementioned results suggest that the secretion of IL-32 could stimulate periductal inflammatory and/or immune cells to secrete proinflammatory cytokines and contributes to the deterioration of periductal inflammation. Because these inflammatory cytokines and an innate immunity play important roles in the immune-mediated histogenesis of BA, the inflammatory responses and innate immune response in the affected bile ducts of BA patients may be amplified by constant IL-32-induced secretion of proinflammatory cytokines from BECs and periductal inflammatory cells, suggesting that IL-32 plays a central role in the inflammatory responses involved in BA pathogenesis. However, IL-32 itself could not up-regulate the expression of inflammatory cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs, suggesting that IL-32 produced by BECs was unlikely to be involved in direct reciprocal signalling resulting in up-regulation of inflammatory cytokines and of susceptibility to virus in BECs.

In this study, we demonstrate that stimulation with poly(I:C) induced the transcription of IL-32 mRNA in BECs and also confirmed the presence of the protein in the culture medium as well as cell lysate. Moreover, immunohistochemistry also revealed that a condensed bile in intrahepatic small bile ducts was positive for IL-32. These findings suggest the secretion of IL-32 from IL-32-expressing BECs. Therefore, IL-32 is speculated to be secreted extracellularly in periductal tissue fluids and into bile in BA. As mentioned above, the secreted IL-32 induces the production of proinflammatory cytokines in inflammatory and/or immune cells, resulting in marked amplification of the inflammatory cytokine milieu, and these responses may contribute to the aggravation of BA. Moreover, it was suggested recently that IL-32 acts as a cytoplasmic protein: IL-32 was expressed at high levels in human epidermal keratinocytes after stimulation with IFN-γ and TNF-α, but was not secreted by keratinocytes [21]. Moreover, it was also shown that the up-regulation of cytoplasmic IL-32 expression induces apoptosis [21,32]. In IBD, the apoptosis of damaged colonic cells by accumulated intracellular IL-32 can be considered a host defence mechanism against invading microorganisms, by which damaged epithelial cells are

eliminated efficiently along with invading microorganisms and further invasions of microorganisms can be blocked [19,33]. In BA, our previous study found that biliary apoptosis was enhanced in the damaged common bile ducts and associated closely with bile duct loss in BA, which was caused by the production of an apoptosis-inducer, TRAIL, in BECs via the biliary innate immune response to a TLR-3 ligand, poly(I:C) [10]. However, this TRAIL-mediated biliary apoptosis is only partially involved in the poly(I:C)-induced mechanism, and other possible mechanisms could also exist [10]. Therefore, the IL-32-mediated mechanism is also likely in poly(I:C)-induced biliary apoptosis, and might be associated with enhanced biliary apoptosis in the damaged common bile ducts of BA.

In conclusion, we have demonstrated that IL-32 expression is enhanced in the damaged common bile ducts of BA patients. Expression of IL-32 in BECs was induced by the innate immune response to dsRNA [poly(I:C)] and proinflammatory cytokines (IL-1β, IFN-γ and TNF-α). This study has identified IL-32 as an important inflammatory cytokine involved in the cholangitis of BA. So far, anti-IL-32 treatment has been studied in only a few diseases, such as rheumatoid arthritis [34,35]. The regulation of IL-32 expression may form the basis of a new strategy for the treatment of BA.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan and Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure

The authors declare no conflicts of interest.

References

1 Morecki R, Glaser JH, Cho S, Balistreri WF, Horwitz MS. Biliary atresia and reovirus type 3 infection. N Engl J Med 1982; **307**:481–4.
2 Morecki R, Glaser JH, Johnson AB, Kress Y. Detection of reovirus type 3 in the porta hepatis of an infant with extrahepatic biliary atresia: ultrastructural and immunocytochemical study. Hepatology 1984; **4**:1137–42.
3 Tyler KL, Sokol RJ, Oberhaus SM *et al.* Detection of reovirus RNA in hepatobiliary tissues from patients with extrahepatic biliary atresia and choledochal cysts. Hepatology 1998; **27**:1475–82.
4 Riepenhoff-Talty M, Gouvea V, Evans MJ *et al.* Detection of group C rotavirus in infants with extrahepatic biliary atresia. J Infect Dis 1996; **174**:8–15.
5 Brown WR, Sokol RJ, Levin MJ *et al.* Lack of correlation between infection with reovirus 3 and extrahepatic biliary atresia or neonatal hepatitis. J Pediatr 1988; **113**:670–6.
6 Bobo L, Ojeh C, Chiu D, Machado A, Colombani P, Schwarz K. Lack of evidence for rotavirus by polymerase chain reaction/enzyme immunoassay of hepatobiliary samples from children with biliary atresia. Pediatr Res 1997; **41**:229–34.
7 Mack CL. The pathogenesis of biliary atresia: evidence for a virus-induced autoimmune disease. Semin Liver Dis 2007; **27**:233–42.
8 Mack CL, Tucker RM, Lu BR *et al.* Cellular and humoral autoimmunity directed at bile duct epithelia in murine biliary atresia. Hepatology 2006; **44**:1231–9.
9 Harada K, Sato Y, Isse K, Ikeda H, Nakanuma Y. Induction of innate immune response and absence of subsequent tolerance to dsRNA in biliary epithelial cells relate to the pathogenesis of biliary atresia. Liver Int 2008; **28**:614–21.
10 Harada K, Sato Y, Itatsu K *et al.* Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. Hepatology 2007; **46**:1146–54.
11 Harada K, Isse K, Sato Y, Ozaki S, Nakanuma Y. Endotoxin tolerance in human intrahepatic biliary epithelial cells is induced by upregulation of IRAK-M. Liver Int 2006; **26**:935–42.
12 Heinhuis B, Netea MG, van den Berg WB, Dinarello CA, Joosten LA. Interleukin-32: a predominantly intracellular proinflammatory mediator that controls cell activation and cell death. Cytokine 2012; **60**:321–7.
13 Dahl CA, Schall RP, He HL, Cairns JS. Identification of a novel gene expressed in activated natural killer cells and T cells. J Immunol 1992; **148**:597–603.
14 Joosten LA, Netea MG, Kim SH *et al.* IL-32, a proinflammatory cytokine in rheumatoid arthritis. Proc Natl Acad Sci USA 2006; **103**:3298–303.
15 Kim KH, Shim JH, Seo EH *et al.* Interleukin-32 monoclonal antibodies for immunohistochemistry, Western blotting, and ELISA. J Immunol Methods 2008; **333**:38–50.
16 Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: a cytokine and inducer of TNFalpha. Immunity 2005; **22**:131–42.
17 Netea MG, Azam T, Ferwerda G *et al.* IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. Proc Natl Acad Sci USA 2005; **102**:16309–14.
18 Kobayashi H, Huang J, Ye F, Shyr Y, Blackwell TS, Lin PC. Interleukin-32beta propagates vascular inflammation and exacerbates sepsis in a mouse model. PLoS ONE 2010; **5**:e9458.
19 Shioya M, Nishida A, Yagi Y *et al.* Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease. Clin Exp Immunol 2007; **149**:480–6.
20 Jeong HJ, Shin SY, Oh HA, Kim MH, Cho JS, Kim HM. IL-32 up-regulation is associated with inflammatory cytokine production in allergic rhinitis. J Pathol 2011; **224**:553–63.
21 Meyer N, Zimmermann M, Burgler S *et al.* IL-32 is expressed by human primary keratinocytes and modulates keratinocyte apoptosis in atopic dermatitis. J Allergy Clin Immunol 2010; **125**:858–65 e10.
22 Calabrese F, Baraldo S, Bazzan E *et al.* IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2008; **178**:894–901.
23 Moschen AR, Fritz T, Clouston AD *et al.* Interleukin-32: a new proinflammatory cytokine involved in hepatitis C virus-related liver inflammation and fibrosis. Hepatology 2011; **53**:1819–29.
24 Mayall F, Chang B, Darlington A. A review of 50 consecutive cytology cell block preparations in a large general hospital. J Clin Pathol 1997; **50**:985–90.