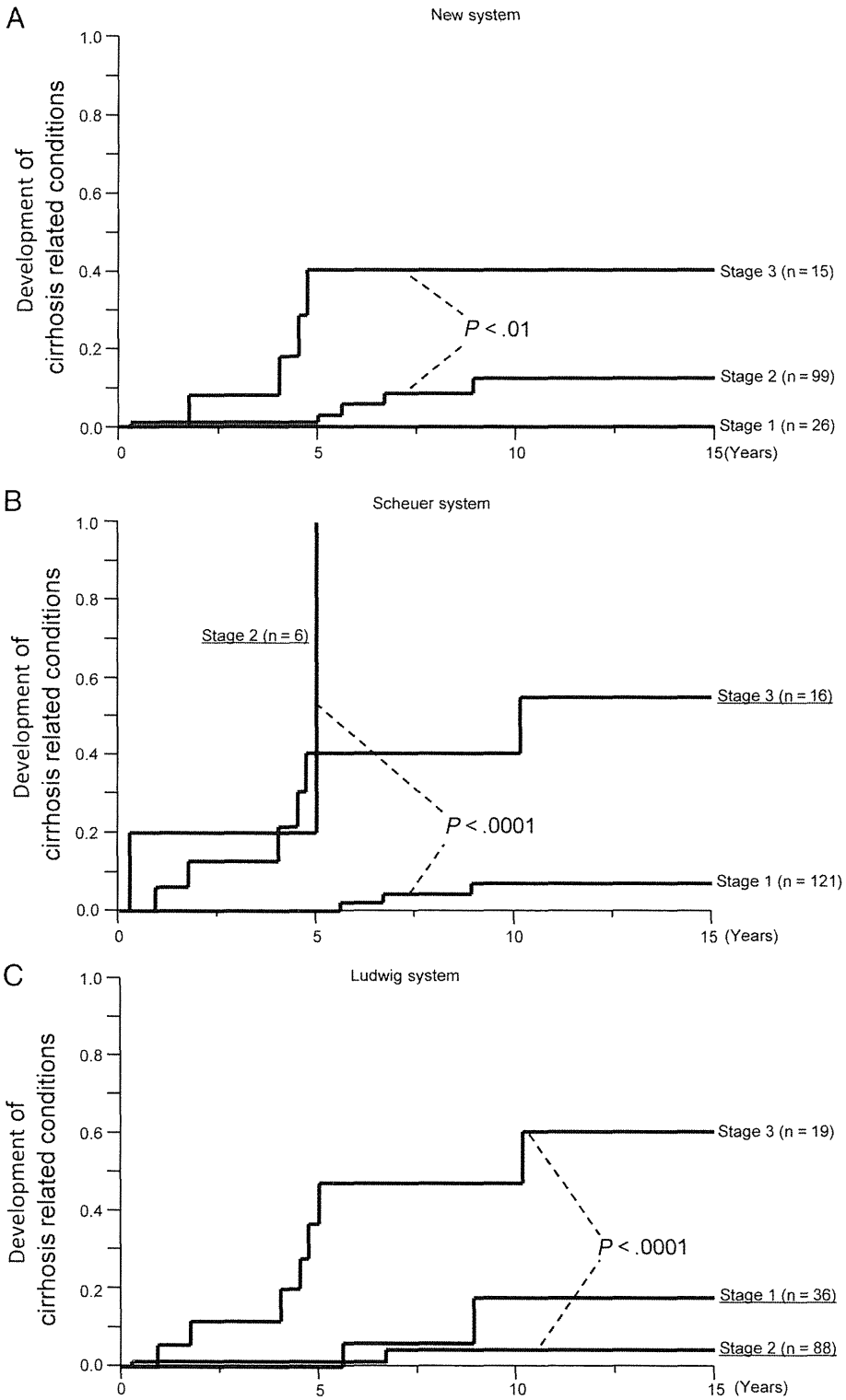

Fig. 3 Rates of development of cirrhosis-related conditions in each histologic stage. A, New staging system. B, Scheuer system. C, Ludwig system. All *P* values were calculated using the log-rank test.

stage 2 or 3 and stage 4 in the new staging, respectively, raising the possibility that an accurate evaluation of pathological progression could be performed using the new system. The new system as well as the 2 classical systems reflected liver dysfunctions before UDCA treatment. The development of cirrhosis-related conditions increased according to the stage progression of the new system on a stepwise basis. Interestingly, deposition of orcein-positive

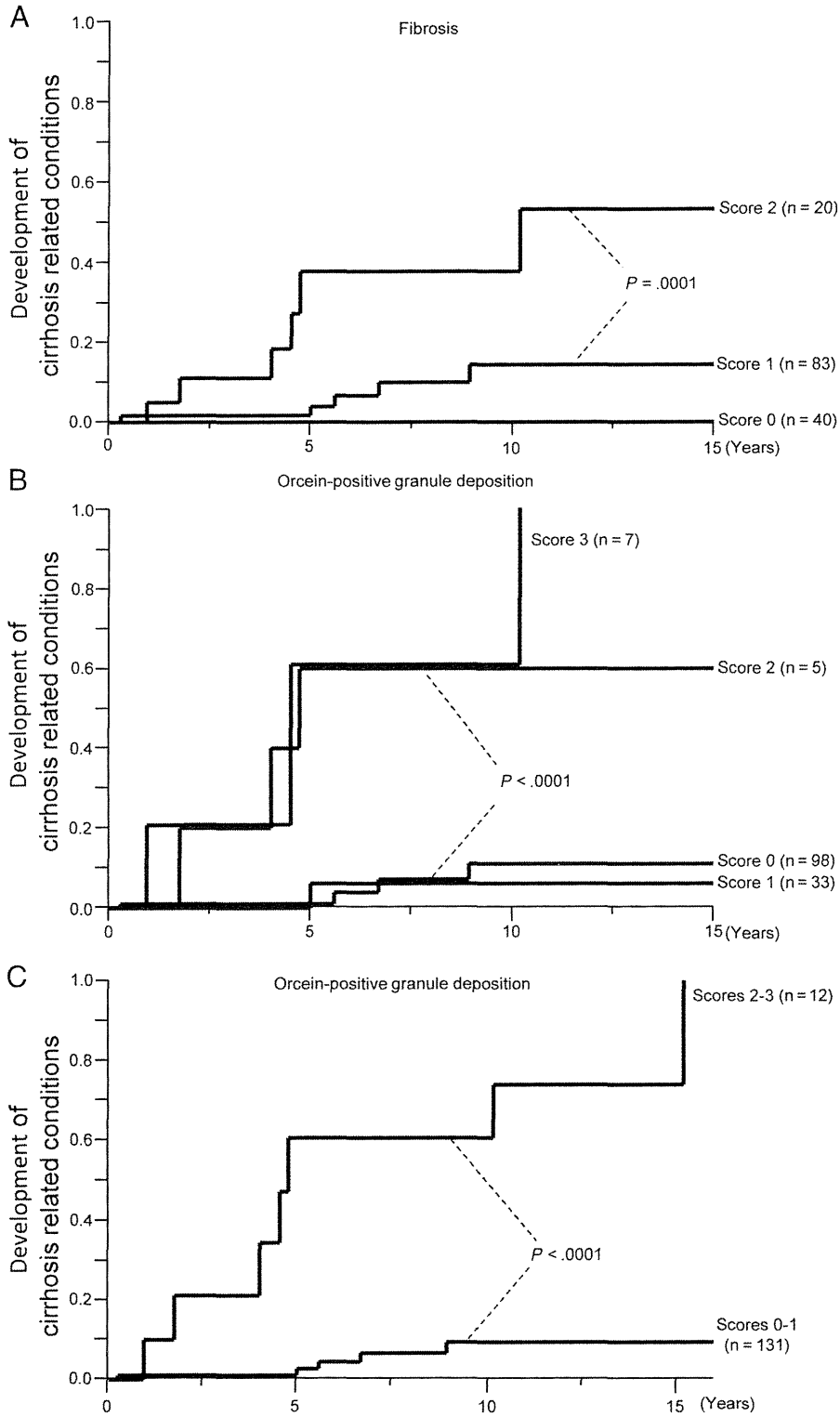

Fig. 4 Rates of development of cirrhosis-related conditions in each score of fibrosis (A) and deposition of orcein-positive granules (B and C). C, Comparison with scores 0 to 1 versus scores 2 to 3 of deposition of orcein-positive granules. All *P* values were calculated using the log-rank test.

granules was a useful predictive factor for the development of cirrhosis-related conditions and was eventually a useful prognostic factor for patients with PBC. Studies with larger cohorts involving different institutions seem to be necessary to more accurately validate this new histologic grading and staging system.

Acknowledgments

The authors thank Dr Takeshi Urabe (Department of Gastroenterology, Public Central Hospital of Matto Ishikawa, Hakusan, Japan), Dr Yasutsugu Mizuno (Department of Internal Medicine, Nomi Hospital, Nomi, Japan), Dr Hisanori Oiwake (Department of Internal Medicine, Suzu General Hospital, Suzu, Japan), Dr Ryuhei Nishino (Department of Internal Medicine, Hakui Public Hospital, Hakui, Japan), and Dr Hideki Osaka (Yawata Medical Center, Komatsu, Japan) for generously donating clinical samples. They also thank Dr Yoshiaki Hitomi (Department of Environmental and Preventive Medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan) for kindly supervising the statistical analyses.

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

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Accepted 23 September 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.[7–11]

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.[12 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.[5 12 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 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 (~~CH~~-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). 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.

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.

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

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.

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 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 ~~glyceraldehyde 3-phosphate dehydrogenase (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

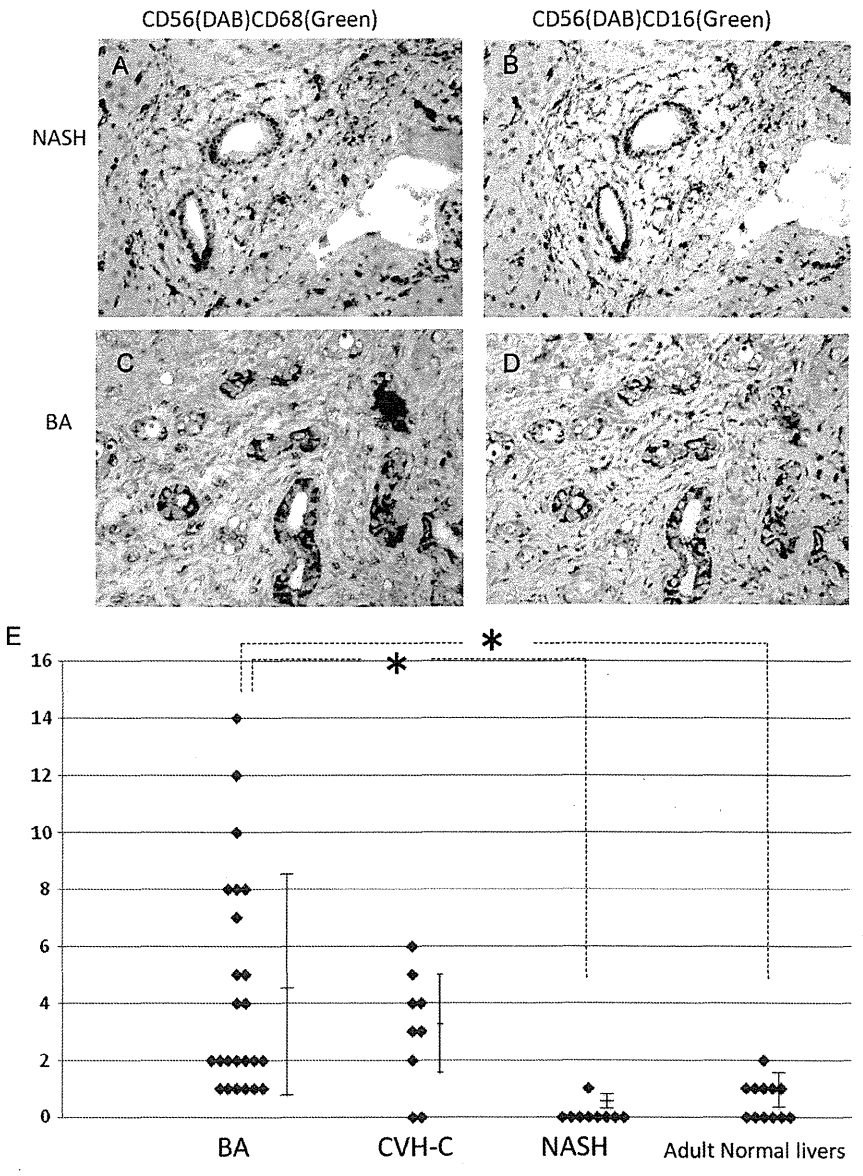

Figure 1 Density of CD56(−)CD16(+)CD68(−) natural killer (NK) cells around intrahepatic small bile ducts. (A, C) Expression of CD56 (brown) and CD68 (green). (B, D) Expression of CD56 (brown) and CD16 (green). Two photographs in the same areas of nonalcoholic steatohepatitis (NASH) (A, B) were compared.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

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 ~~PBP~~ were negative or slightly positive for CX3CL1 (figure 4A). In ~~CH~~-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 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).

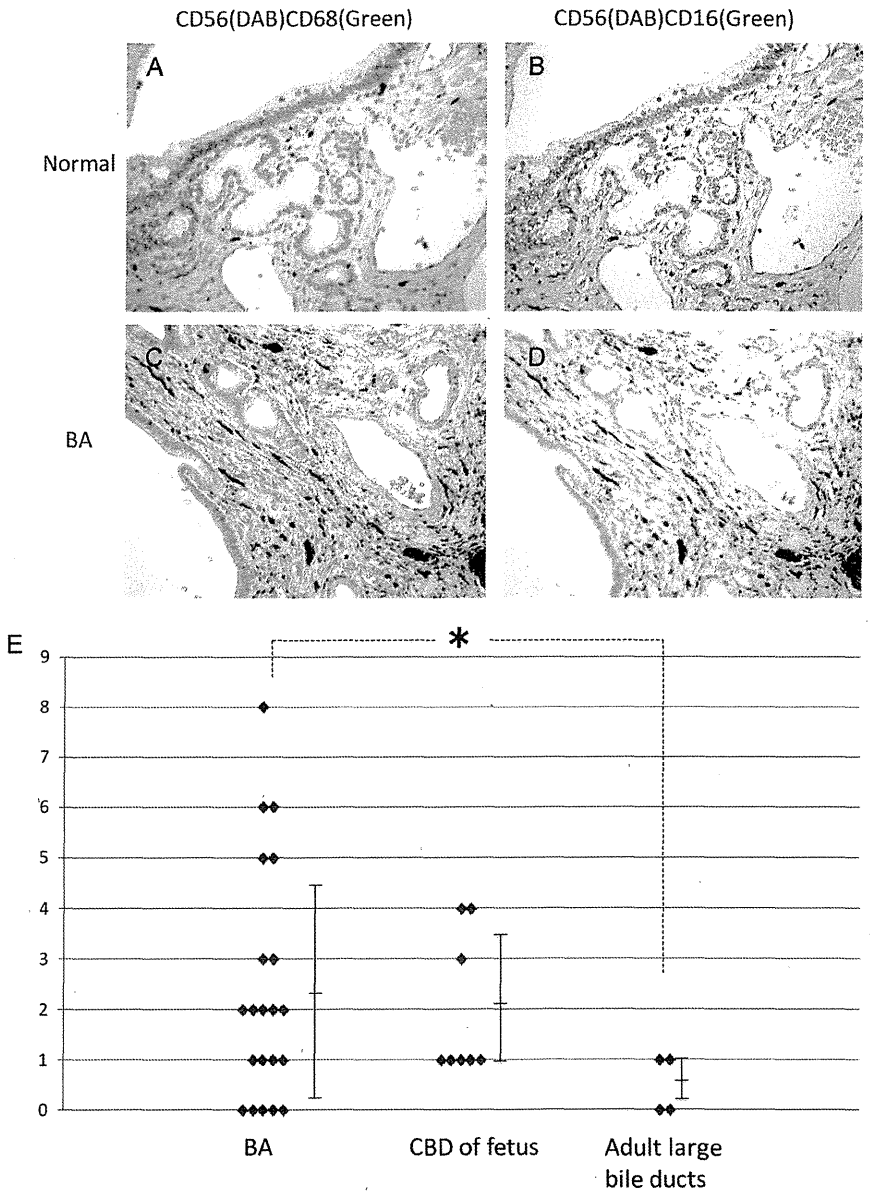

Figure 2 Density of CD56(−)CD16(+)CD68(−) natural killer (NK) cells around large bile ducts. (A, B) There were no or few CD56(−)CD16(+)CD68(−) NK cells beneath biliary epithelia of the large bile duct in normal adult livers. (C, D) Such NK cells were identifiable in biliary atresia (BA). (E) These cells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

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 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.

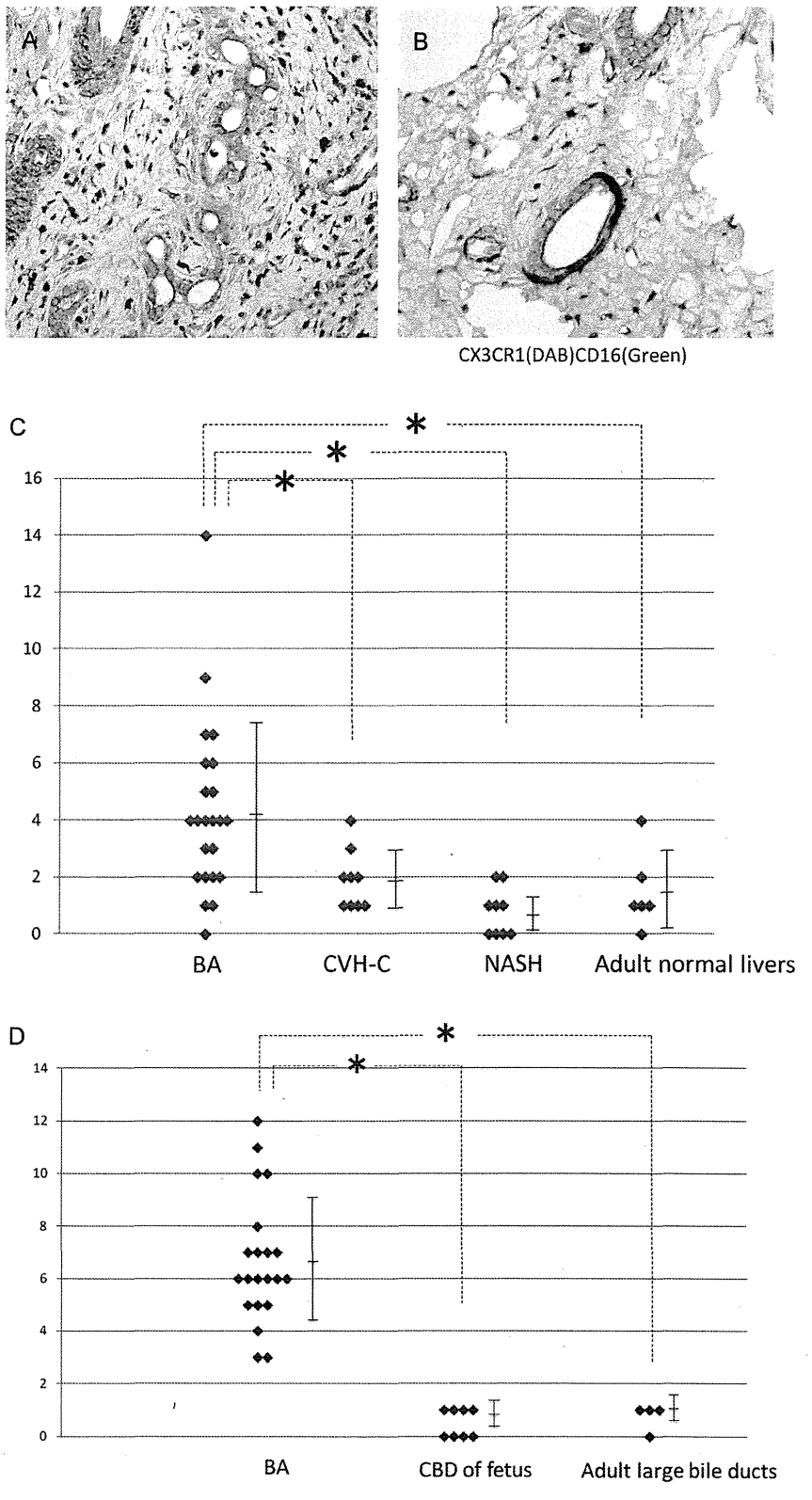

Figure 3 CX3CR1(+)CD16(+) mononuclear cells around intrahepatic bile ducts. (A) CX3CR1(+)CD16(+) mononuclear cells were frequently present in portal tracts around damaged small bile ducts in biliary atresia (BA). (B) Such cells were sparse in normal livers and other liver diseases. (C) They were rather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

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 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

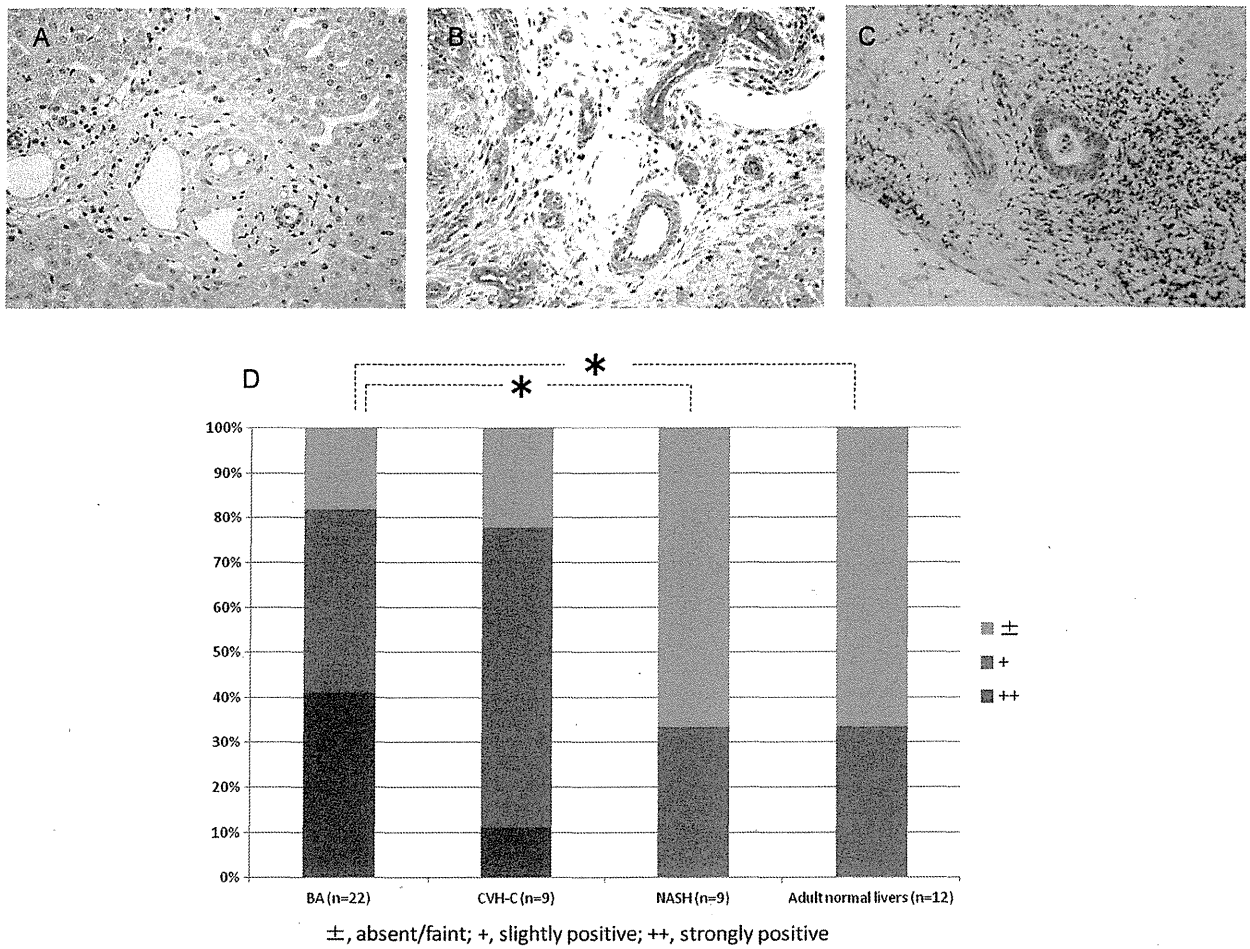

Figure 4 Expression of CX3CL1 in intrahepatic small bile duct epithelia. (A) Normal livers. Small bile ducts were generally negative or faintly positive for CX3CL1. (B) Biliary atresia (BA). Small bile ducts were strongly positive for CX3CL1. (C) The incidence of small bile ducts with mild to moderate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

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.[18] Recently, many genetic studies, moreover, have recently reported. ~~Genomic~~ study including genome-wide association study identified a susceptibility locus for BA on 10q24.2 and 2q37.3.[19 20] 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.[21–26] 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.[18 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 ~~CH~~ C patients.[5–6] These CD56(−) NK cells were functionally impaired with respect to cytokine production upon target cell recognition.[29] 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.[12] 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 possible that inadequate removal of BECs infected with cholangiotrophic virus by abundant CD56(−)CD16(+) NK

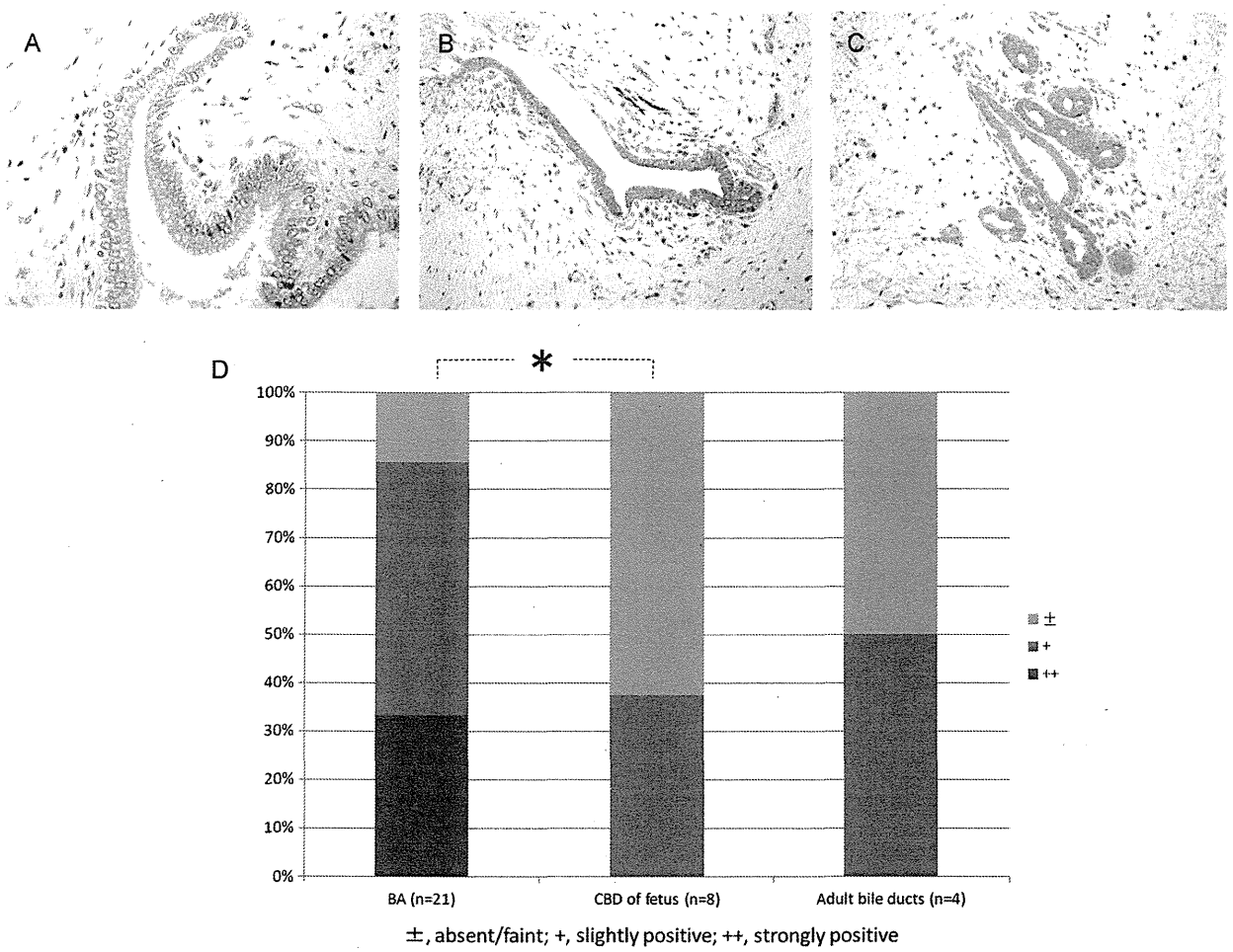

Figure 5 Expression of CX3CL1 in large bile duct epithelia. (A) CX3CL1 was not or faintly expressed in large bile ducts and peribiliary glands and ~~PBP~~ of normal livers. (B,C) It was strongly expressed in biliary epithelial cells of large bile ducts and peribiliary glands and also endothelial cells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

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.[30]

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 ~~CH 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

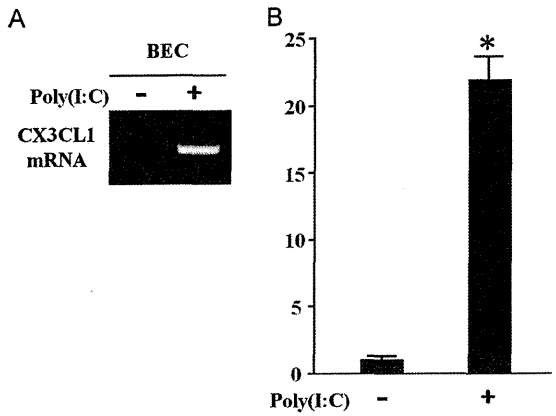

Figure 6 Expression of CX3CL1 mRNA in cultured human biliary epithelial cells (BECs). (A) Representative images of RT-PCR using cultured BECs. The amplicon of CX3CL1 mRNA could not be detected without the stimulant (−). de novo expression was found in the poly(I:C)-treated cells 3 h after treatment with 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.[18] We could confirm the increase of CD56(−)CD16(+)CD68(−) NK cells in liver specimens of ~~CH~~-C as well as BA by the immunohistochemistry, though statistical significance was not obtained in ~~CH~~-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 ~~CH~~-C, but not NASH or normal livers. 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 ~~CH~~-C and BA.

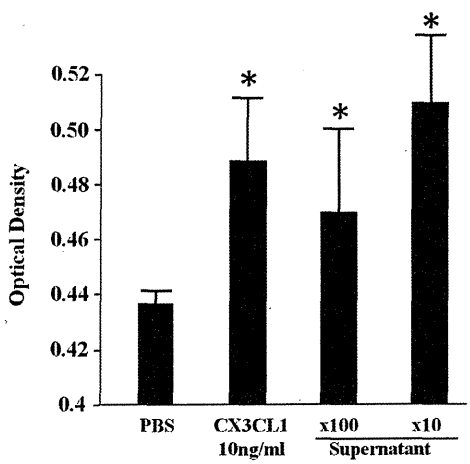

Figure 7 Migration assay of natural killer (NK) cells. Optical density (OD) reflecting the number of transmigrated NK cells was significantly increased in the lower chamber containing recombinant CX3CL1 (10 ng/ml, OD=0.49±0.02 (mean±SEM), effect size=0.66, CI −0.09 to −0.01) and supernatant of poly(I:C)-treated 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.

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.

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Med Mol Morphol (2013) 46:153–159
DOI 10.1007/s00795-013-0017-0

ORIGINAL PAPER

PPARγ ligand attenuates portal inflammation in the MRL-lpr mouse: a new strategy to restrain cholangiopathy in primary biliary cirrhosis

Yusuke Nozaki · Kenichi Harada · Takahiro Sanzen · Yasuni Nakanuma

Received: 7 May 2012 / Accepted: 13 July 2012 / Published online: 9 February 2013

Abstract Primary biliary cirrhosis (PBC) is characterized by chronic destructive cholangitis, which is associated with the reduced expression of an anti-inflammatory molecule, peroxisome proliferator-activated receptor-γ (PPARγ), in intrahepatic bile ducts. We previously demonstrated the anti-inflammatory effects of PPARγ ligands using cultured human biliary epithelial cells. In this study, we evaluated the effectiveness of PPARγ ligand against peribiliary inflammation in vivo. As an animal model of PBC, we used MRL/lpr mice in which a PBC-like cholangitis occurs naturally. Anti-inflammatory effects of the intraperitoneal administration of a PPARγ ligand, the prostaglandin D metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), were evaluated. In untreated mice, portal inflammation including cholangitis was found to some degree in the majority of portal tracts. In mice given a high-dose group, the degree of portal inflammation was significantly reduced and mice mostly lacking portal inflammation and cholangitis were also found. T cell numbers in portal tracts were markedly decreased in the high-dose group, compared with controls, whereas there was no significant difference in terms of B cells and macrophages. This study is the first to assess the therapeutic potential of a PPARγ ligand against portal inflammation including cholangitis. Anti-inflammatory effects of PPARγ ligands may prevent the progression of cholangiopathy in PBC patients.

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Keywords Peroxisome proliferator-activated receptor · Primary biliary cirrhosis · MRL/lpr mouse · Cholangiopathy · 15d-PGJ2

Introduction

Primary biliary cirrhosis (PBC) is characterized by the progressive loss of bile ducts, mainly interlobular bile ducts [1, 2]. Infectious and xenobiotics components and abnormal immune responses are implicated in the etiopathogenesis of PBC [3–8]. Our previous study showed that biliary epithelial cells (BECs) of the intrahepatic bile ducts possess an innate immune machinery consisting of bacterial recognition molecules, Toll-like receptors (TLRs), and can respond to lipopolysaccharide (LPS) followed by nuclear factor-κB (NF-κB) and inflammatory cytokines in BECs [9, 10]. Under physiological conditions, however, the intrahepatic biliary epithelium lacks any inflammatory reactions in vivo, though several pathogen-associated molecular patterns (PAMPs) including bacterial DNA and LPS exist in bile [9, 11], suggesting that BECs possess the capacity to attenuate cytokine gene expression related to the innate immune system. We have reported that peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor superfamily of ligand-activated transcription factors involved in lipid metabolism and anti-inflammatory activities, was down-regulated in the damaged bile ducts of PBC patients and speculated that increased susceptibility to biliary innate immunity is associated with the pathogenesis of cholangiopathy in PBC [12].

In addition to the biliary epithelial cells of PBC, a reduction of PPARγ expression in the colonic epithelial mucosa is reportedly associated with the pathogenesis of inflammatory bowel diseases (IBD) [13–16]. Furthermore,

the expression of PPARγ is affected by the commensal intestinal flora and ligands (agonists) for PPARγ can attenuate colitis in IBD-model mice, supporting the notion that PPARγ ligands may have salutary effects on IBD [14, 16]. As for anti-inflammatory activities, the activation of PPARγ by its ligands is shown to inhibit the expression of pro-inflammatory cytokines, the induction of which is mediated via NF-κB and mitogen activated protein kinase (MAPK) [17, 19–23]. Several PPARγ ligands have been identified, including the prostaglandin D metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) and thiazolidinedione derivatives. Our previous study demonstrated that 15d-PGJ2 treatment attenuated LPS-induced NF-κB activation and also TNF-α production via an NF-κB-dependent pathway using cultured intrahepatic cholangiocarcinoma cells and biliary epithelial cells [12].

In this study, we examined anti-inflammatory effects of PPARγ ligands in vivo using MRL/lpr mice with a naturally occurring PBC-like cholangitis to clarify possible therapeutic targets in biliary inflammation.

Materials and methods

PPARγ ligand

An endogenous ligand, 15d-PGJ2 (diluted by DMSO, Calbiochem, Darmstadt, Germany), was used as a PPARγ ligand.

Animals

MRL/lpr mice bearing the lymphoproliferative gene lpr spontaneously develop severe autoimmune diseases including a systemic lupus erythematosus (SLE) and CNSDC-like cholangitis and produce an anti-mitochondrial antibody [24]. This strain was used here as an animal model for autoimmune-mediated cholangitis similar to PBC. A total of 20 18-week-old MRL/lpr mice (male/female = 10/10) were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Animal treatments

15-deoxy-$\Delta^{12,14}$-prostaglandin J2 was administered at low (400 μg/kg/day) and high (1,000 μg/kg/day) doses (5 mice each) for 3 weeks by intraperitoneal injection. The control group ($n = 10$) received only vehicle (DMSO). After the animals were killed, major organs were obtained and 4-μm-thick sections of neutral formalin-fixed paraffin-embedded tissues were prepared for routine histologic observation and immunohistochemistry. The manipulation of these mice was done according to the Guidelines for the Care and Use of Laboratory Animals at the Takaramachi Campus of Kanazawa University (Approved Number, 050273).

Histological examination

To evaluate the anti-inflammatory effect of PPARγ, major organs were examined histologically. For the liver, ten representative portal tracts containing interlobular bile ducts were chosen and portal inflammation including cholangitis in each was semiquantitatively evaluated as no or mild (score 0), moderate (score 1), or severe (score 2).

Immunohistochemistry

The deparaffinized and rehydrated sections used for studying CD3, and monocytes/macrophages and CD8 were treated with proteinase K and trypsin, respectively, while those used for CD79α were microwaved 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 antibodies against CD3 (rat IgG, 10 μg/ml, Millipore Headquarters, Billerica, MA), CD8 (rat IgG1, 2.5 μg/ml, Chemicon, Tokyo), CD79α (rabbit IgG, 1.0 μg/ml, Abcam Japan, Tokyo), and macrophage/monocyte (rat IgG, 2.5 μg/ml, BMA Biomedicals, Augst, Switzerland) and then at room temperature for 1 h with a Simple Staining Kit (Nichirei, Tokyo). After a benzidine reaction, sections were lightly counterstained with hematoxylin. As a negative control, isotype-matched immunoglobulin was used as the primary antibody.

Statistical analysis

Data were analyzed using Welch's t test; $p < 0.05$ was considered statistically significant.

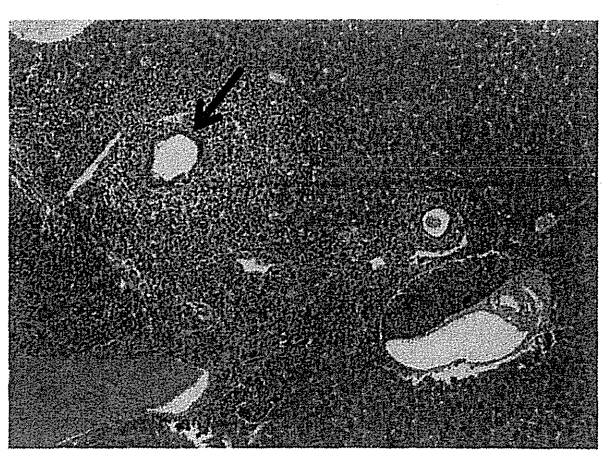

Fig. 1 The MRL/lpr mouse at 21 weeks. Marked portal inflammation involving lymphoid cells and non-suppurative destructive cholangitis-like lesions with irregular biliary epithelial polarity (*arrow*) are found

Results

Histology of MRL/lpr mice

Variation in the incidence and degree of systemic lymphadenopathy, sialoadenitis, nephritis, pneumonia, arthritis, and hepatitis including cholangitis was found in MRL/lpr mice. In particular, sialoadenitis always occurred and the inflammation was extensive and destructive in most mice. In the liver, portal inflammation involving mononuclear cells was detected and portal tracts were cellularly enlarged. Moreover, chronic cholangitis resembling CNSDC was also found at varying degrees (Fig. 1).

Effect of 15d-PGJ2

In the group injected intraperitoneally with a low dose of 15d-PGJ2, portal inflammation including cholangitis in interlobular bile ducts remained, the incidence and degree being similar to those in the controls (vehicle) (Fig. 2c, d). However, in the high-dose group, the portal inflammation and cholangitis were attenuated and their incidence and degree were significantly reduced (Fig. 2a). Almost no portal inflammation or cholangitis was found in one of the five mice in the high-dose group (Fig. 2b). Semiquantitative evaluation demonstrated that inflammation was improved in the high-dose group (Fig. 3), but not low-dose group. For sialoadenitis and pancreatitis, although some inflammatory damage remained, it was significantly less severe in the low-dose as well as high-dose group. For glomerulonephritis, there was no significant anti-inflammatory effect in 15d-PGJ2-injected mice.

Analysis of inflammatory cells in portal tracts

To analyze the population of inflammatory cells in portal tracts of MRL/lpr mice and changes in the high-dose group, T cells, B cells, and macrophages were examined by

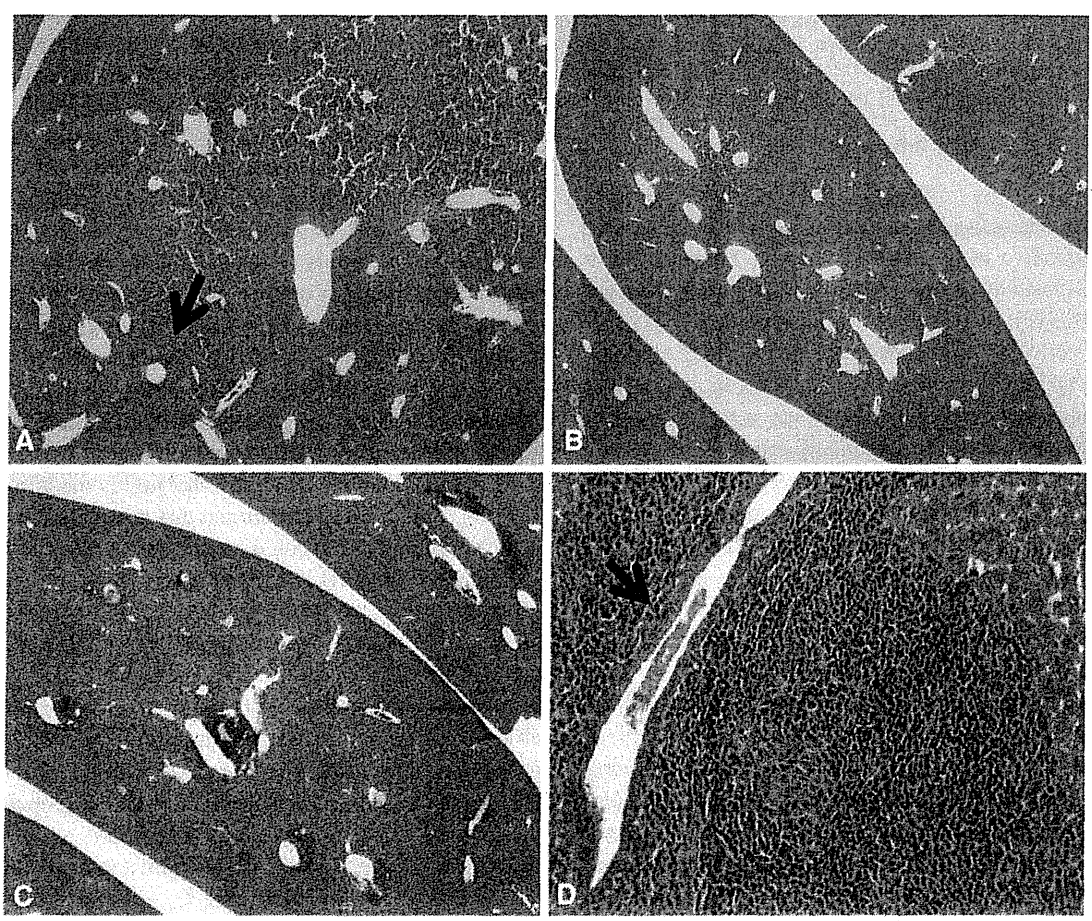

Fig. 2 MRL/lpr mice 21 weeks after the administration of high-dose 15d-PGJ2 (1,000 μg/kg/day) for 3 weeks (**a** and **b**) and controls (DMSO, **c** and **d**). **a** and **b** show the high-dose group in which portal inflammation remains (*arrow*) and has completely disappeared, respectively. **c** and **d** are controls. Most portal tracts exhibit significant inflammation (**c**) and mild bile duct damage (*arrow*, **d**) with infiltration by lymphocytes and macrophages

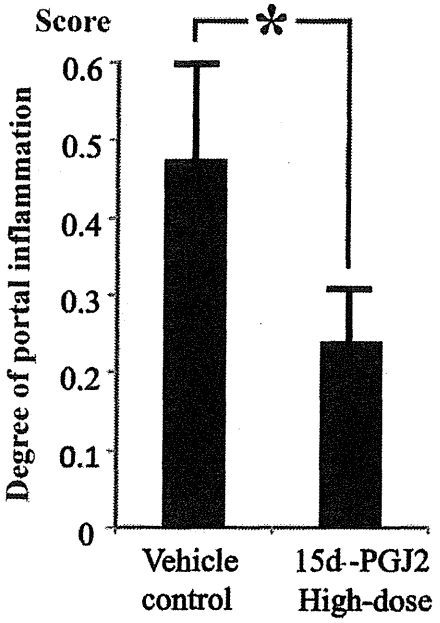

Fig. 3 Semiquantitative evaluation of portal inflammation. Ten representative portal tracts were chosen in each mouse and the inflammation in each was evaluated (score 0, 1, or 2). The average score of MRL-lpr mice treated with high-dose 15d-PGJ2 [0.48 ± 0.11 (mean ± SEM)] is significantly reduced, compared with the control (DMSO, 0.24 ± 0.07). $*p < 0.05$

immunohistochemistry. Few CD79α-positive B cells were found irrespective of 15d-PGJ2-treatment, but many scattered CD3-positive T cells and monocytes/macrophages were detected (Figs. 4, 5). In particular, the number of CD3-positive T cells was clearly decrease in 15d-PGJ2-administered mice and this reduction was significant in portal tracts without inflammation (Fig. 4). However, a few CD8-positive T cells were found irrespective of 15d-PGJ2-treatment, indicating the decrease of CD3-positive T cells is caused by that of CD4-positive T cells. A summary of the population of inflammatory cells in portal tracts is given in Table 1.

Discussion

We reported previously that PPARγ showing anti-inflammatory effects was constantly expressed in human biliary epithelial cells of intrahepatic small bile ducts including interlobular bile ducts and cultured human biliary epithelial cells [27]. Moreover, the expression of PPARγ has been reported to be down-regulated by a Th1-dominant cytokine milieu and reduced in the damaged bile ducts of PBC patients [27]. Based on these findings, we speculated that a Th1-dominant periductal cytokine milieu caused by CD4-positive T cells, following the reduction of PPARγ expression and increased susceptibility to several proinflammatory factors including innate immune responses is closely associated with the pathogenesis of cholangiopathy in PBC. Studies in vitro have shown that functional PPARγ ligands suppress inflammatory responses by limiting the production of cytokines and chemokines secreted from macrophages and epithelial cells [16, 28–30]. PPARγ ligands are divided into endogenous types and thiazolidinedione derivatives. As one of the former, the prostaglandin D metabolite 15d-PGJ2 is well known and reported to attenuate the activation of NF-κB, a master regulator of inflammation, by preventing the phosphorylation of its inhibitor protein (I-κB) [31]. Our recent study demonstrated that 15d-PGJ2 treatment attenuated LPS-induced NF-κB activation and also TNF-α production via an NF-κB-dependent pathway in cultured intrahepatic cholangiocarcinoma cells and biliary epithelial cells [9, 27].

In this study, to verify whether PPARγ ligands show anti-inflammatory effects against portal inflammation including cholangitis in vivo, we used autoimmunity-prone mice, MRL/lpr. These mice have lymphoproliferative lesions caused by a deficiency of Fas (CD95) and spontaneously develop various forms of autoimmune disease in the same individuals, including glomerulonephritis, polyarteritis, arthritis and sialoadenitis associated with excessive production of autoantibodies [25]. Therefore, MRL/lpr mice have not only been used as a model for the study of SLE, but also reported as a potentially suitable animal model of PBC [24, 26]. Although several clinical features including serum levels of total bilirubin and hepatobiliary enzymes including alanine aminotransferase (ALT), leucine aminopeptidase (LAP), and gamma-glutamyl transpeptidase (G-GTP) are incompatible with PBC, the serological and histopathological features including anti-mitochondrial antibody (AMA), cholangitis, and bile duct loss, indicate that MRL/lpr mice can be used as an experimental immune-mediated cholangitis model for PBC [24, 26]. This study demonstrated that the administration of 15d-PGJ2 (high-dose) could attenuate the degree of portal inflammation in MRL/lpr mice. Because the incidence of cholangitis was originally low and individual differences were significant, the anti-inflammatory effect was considered relatively mild as shown in Fig. 3. However, the cholangitis and portal inflammation were completely absent in the mice given a high-dose of 15d-PGJ2, suggesting an anti-inflammatory effect of PPARγ on portal inflammation. Moreover, an evaluation of the proportion of inflammatory cells in portal tracts revealed as significant reduction in T cells in PPARγ-administered mice. Moreover, although we could not directly confirm the decrease of CD4-positive T cells, because of no antibodies against mouse CD4 which are commercially available and usable for formalin-fixed, paraffin-embedded section, the decrease of CD3-positive T cells was speculated to be caused by that

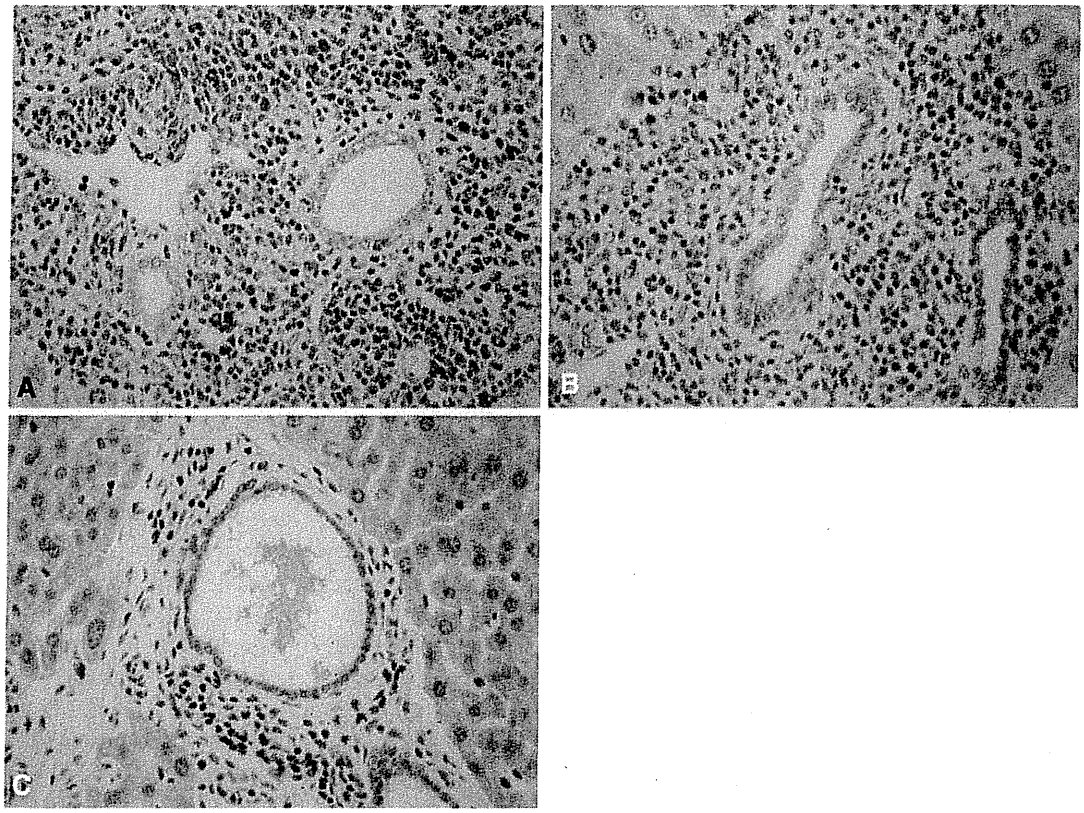

Fig. 4 Immunohistochemistry of CD3 in MRL-lpr mice treated with vehicle (DMSO, **a**) and high-dose 15d-PGJ2 (**b** and **c**). In the control groups, CD3-positive T cells are found in enlarged portal tracts (**a**). In the high-dose 15d-PGJ2 group, marked inflammation remains in portal tracts, but CD3-positive T cell numbers are significantly decreased (**b**), compared with controls (**a**). The portal tracts of the high-dose 15d-PGJ2 group contain few CD3-positive T cells (**c**)

of CD4-positive T cells from results of immunohistochemistry of CD3 and CD4. T cells, particularly autoreactive CD4-positive T cells, play an important role in the pathogenesis of cholangiopathy in cases of PBC [32]. Therefore, because the PPARγ ligand had anti-inflammatory effects caused by the attenuation of CD4-positive T lymphocytes, it is likely to show as anti-inflammatory effect on cholangiopathy in PBC patients as well as MRL/lpr mice.

In addition to cholangitis, sialoadenitis and pancreatitis in MRL/lpr mice were also improved by the low dose as well as high dose of 15d-PGJ2, suggesting that the sialoadenitis and pancreatitis are closely associated with PPARγ-dependent mechanism and show more susceptibility to PPARγ ligand, compared with cholangitis. However, as for the severity of glomerulonephritis, there was no significant anti-inflammatory effect in 15d-PGJ2-injected mice. In SLE, the deposition in kidney tissue of immune complexes and their interaction with macrophages is thought to trigger the inflammatory response leading to glomerulonephritis. Moreover, macrophage-dependent destruction in MRL/lpr mice is demonstrated in the pathogenesis of glomerulonephritis [33]. As shown in Table 1 concerning the immunohistochemistry, the inflammation of monocyte/macrophage was less attenuated by the 15d-PGJ2 treatment, compared with that of T cells, speculating that 15d-PGJ2 offers little benefit to the macrophage-dependent destruction such as glomerulonephritis in MRL/lpr mice.

Other PPARγ ligands include rosiglitazone, pioglitazone, troglitazone, and ciglitazone. Pioglitazone has been recently administered for diabetic mellitus and non-alcoholic steatohepatitis. Troglitazone had been used to treat diabetic mellitus and in one notable case, improved liver dysfunction in a patient with AMA-negative PBC [34]. It is no longer used because of its severe hepatotoxicity, but several effects of Troglitazone on inflammatory bowel diseases, carcinogenesis in the colon, diabetic mellitus, chronic pancreatitis, and sepsis in animal models have been reported [18, 35–37]. In this study, in addition to the anti-inflammatory activity of PPARγ in cultured human biliary epithelial cells, we demonstrated effected in vivo using MRL-lpr mice. This study is the first to assess the therapeutic role of a PPARγ ligand in inflammatory biliary diseases, particularly PBC. PPARγ ligands are potentially a new tool to restrain the progression of cholangiopathy in PBC patients.

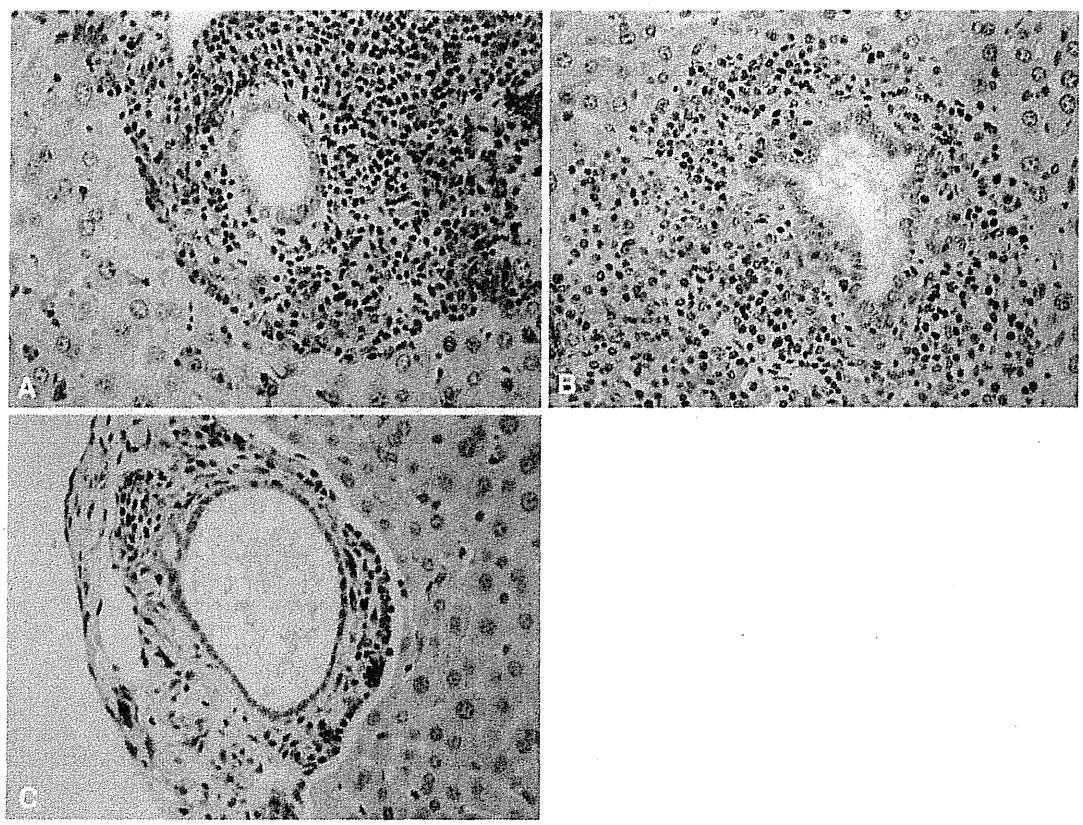

Fig. 5 Immunohistochemistry of monocytes/macrophages in MRL-lpr mice treated with vehicle control (DMSO, **a**) and high-dose 15d-PGJ2 (**b** and **c**). Many monocytes/macrophages are found in inflammed portal tracts in both the control and high-dose 15d-PGJ2 groups (**a** and **b**, respectively). The portal tracts of the high-dose 15d-PGJ2 group still contain several monocytes/macrophages (**c**)

Table 1 Summary of the population of inflammatory cells in portal tracts

MRL/lpr mice	T cells		B cells	Monocyte/ macrophage
	CD3	CD8	CD79a	
Controls (DMSO)	++	±	±	++
15d-PGJ2-treated (high-dose)	± to +	±	±	+ to ++

± none or a few, + some, ++ many

Acknowledgments 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.

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