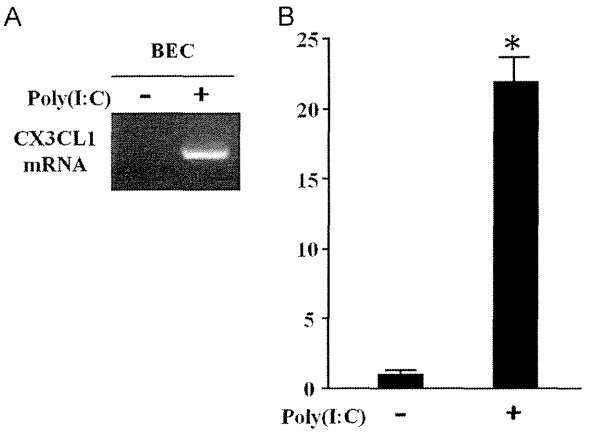

Figure 6 Expression of CX3CL1 mRNA in cultured human biliary epithelial cells (BECs). (A) Representative images of RT-PCR using cultured BECs. The amplicon of CX3CL1 mRNA could not be detected without the stimulant (–). de novo expression was found in the poly(I:C)-treated cells 3 h after treatment with 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.

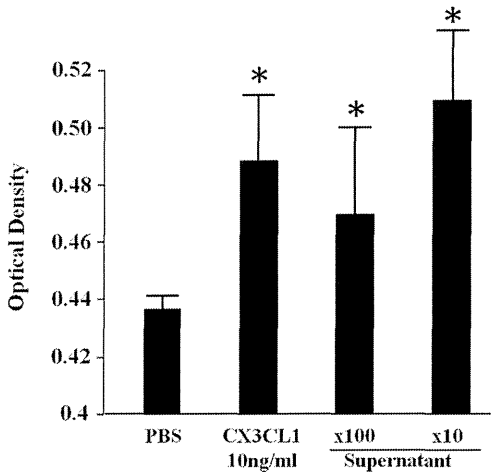

Figure 7 Migration assay of natural killer (NK) cells. Optical density (OD) reflecting the number of transmigrated NK cells was significantly increased in the lower chamber containing recombinant CX3CL1 (10 ng/ml, OD=0.49±0.02 (mean±SEM), effect size=0.66, CI −0.09 to −0.01) and supernatant of poly(I:C)-treated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

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Competing interests None.

Patient consent Obtained.

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

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

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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 ReverTra 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

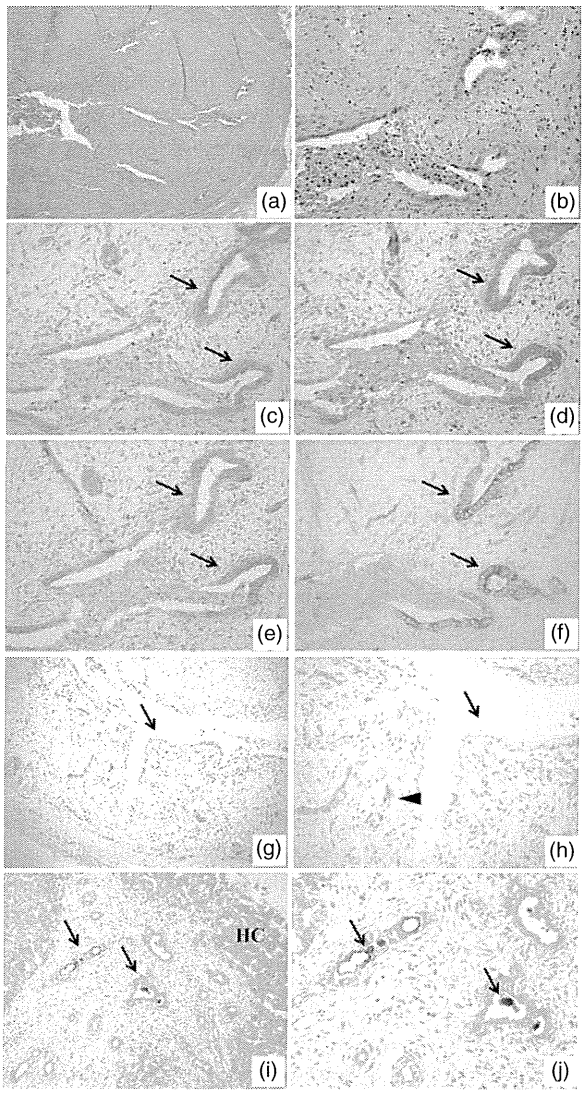

Fig. 1. Histology and immunohistochemistry for interleukin (IL)-32, Toll-like receptor (TLR)-3 and caspase 1 in biliary atresia (BA). (a,b) Transverse sections of biliary remnants. Damaged extrahepatic bile ducts line inconsistently by desquamated columnar epithelium and surrounding fibroplasia with 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.

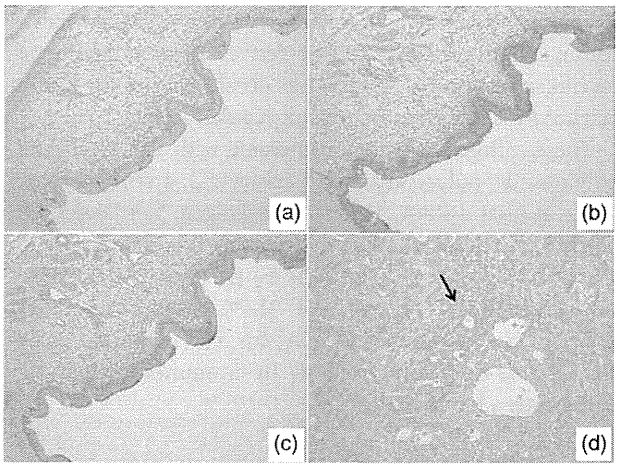

Fig. 2. Immunohistochemistry for interleukin (IL)-32 (a,d), Toll-like receptor (TLR)-3 (b) and caspase 1 (c) in age-matched controls. (a–c) Biliary epithelial cells in common bile ducts of non-hepatobiliary diseases (congenital heart anomalies) expressed TLR-3 (b) and caspase 1 (c), but lacking or faintly 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

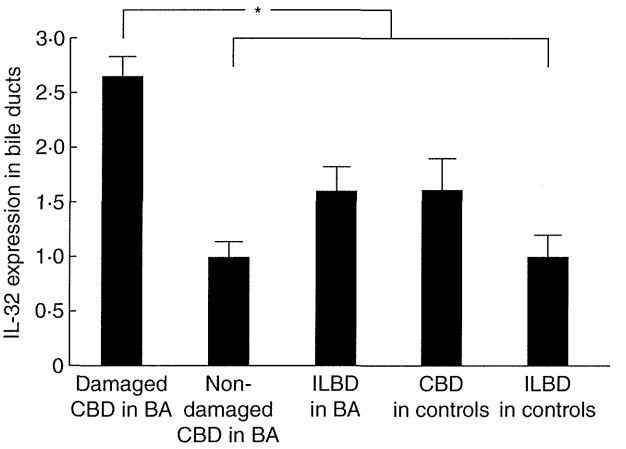

Fig. 3. Semiquantitative analysis of immunohistochemistry for interleukin (IL)-32. The expression of IL-32 in damaged common bile ducts (CBD) of biliary atresia (BA) was up-regulated significantly compared with those of non-damaged CBD and interlobular bile ducts (ILBD) in BA, and of CBD and ILBD in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

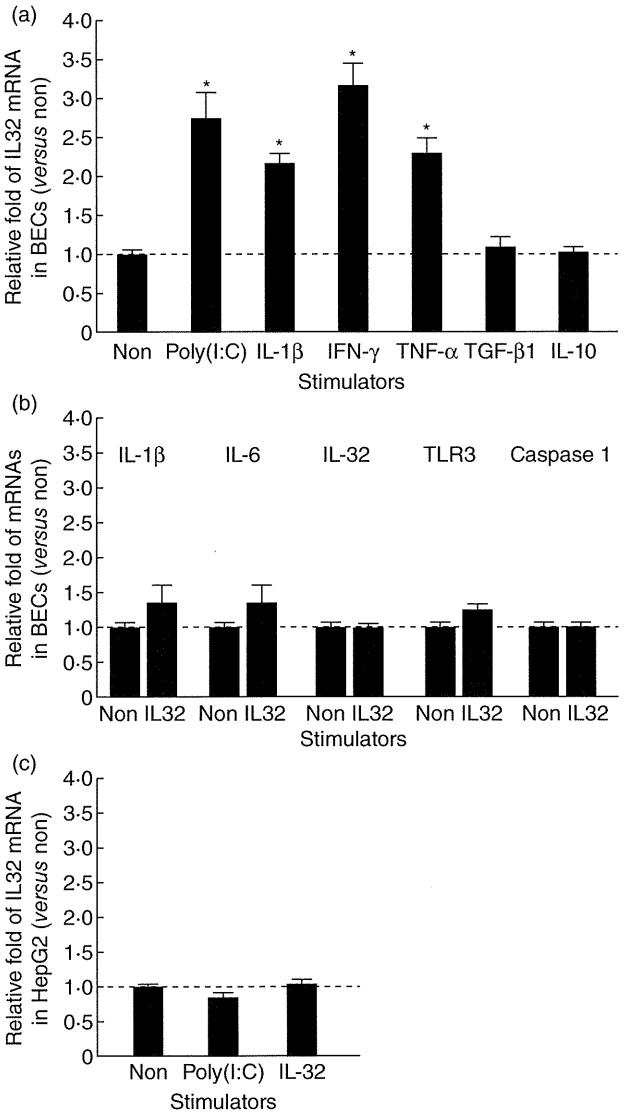

Fig. 4. (a) Induction of interleukin (IL)-32 expression by Toll-like receptor (TLR)-3 ligand (poly I:C) and cytokines in cultured biliary epithelial cells (BECs). Quantitative analysis using real-time polymerase chain reaction (PCR) revealed that a TLR-3 ligand, poly(I:C) and proinflammatory cytokines [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).

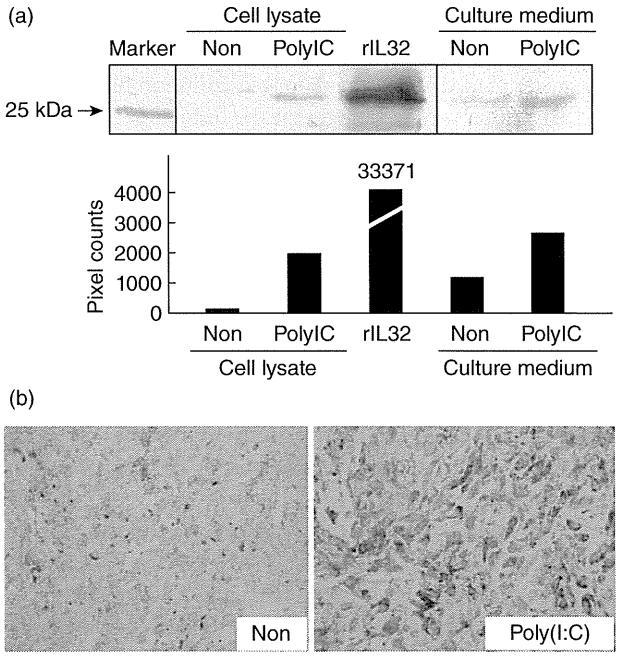

Fig. 5. Detection of intracytoplasmic and secreted interleukin (IL)-32 protein in cultured biliary epithelial cells (BECs). (a) Western blotting revealed that the culture medium as well as cell lysate of poly(I:C)-treated cultured cells contained IL-32 protein, but the level was faint in untreated cells (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.

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Disclosure

The authors declare no conflicts of interest.

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Incidentally detected cholangiocarcinoma in an explanted liver with biliary atresia after Kasai operation

Fukuda A, Sakamoto S, Kanazawa H, Shigeta T, Karaki C, Hamano I, Uchida H, Kitagawa H, Okuse C, Miyazaki O, Nosaka S, Nakazawa A, Kasahara M. Incidentally detected cholangiocarcinoma in an explanted liver with biliary atresia after Kasai operation.

Abstract: This report presents the case of a 30-yr-old woman with BA who developed incidental cholangiocarcinoma following the Kasai operation. She showed progressive liver dysfunction and cirrhosis at the age of 30 yr and underwent LDLT. A 4-cm-diameter liver tumor in the anastomotic site of portoenterostomy was incidentally found as a result of a pathological examination of the explanted native liver. The tumor was pathologically diagnosed to be intrahepatic cholangiocarcinoma. Although cholangiocarcinoma in patients with BA has been previously reported in only three cases, it should be nevertheless always considered in the differential diagnosis of hepatic tumors during a long follow-up course in patients with BA.

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Key words: biliary atresia – living donor liver transplantation – incidental carcinoma – cholangiocarcinoma

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BA causes obstructive cholangiopathy of unknown etiology that progresses to severe neonatal or infantile liver dysfunction, which leads to biliary cirrhosis of the liver. Kasai hepatic portoenterostomy (Kasai operation) is not always successful. Success depends on multiple factors, and some patients have unsuccessful Kasai operations and need to undergo liver transplantation in infancy. Kasai operation became widely accepted more than 45 yr ago, and some patients with BA who have undergone this procedure have survived beyond 20 yr of age (1). However, patients with BA can experience progression of liver dysfunction related to ongoing liver fibrosis and post-operative complications, such as cholangitis, portal hypertension, esophageal varices, and

Abbreviations: ADC, apparent diffusion coefficient; AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer; BA, biliary atresia; CA, cancer antigen; CEA, carcinoembryonic antigen; CK, cytokeratin; CT, computed tomography; DWI, diffusion-weighted magnetic resonance imaging; ICP, intrahepatic cholestasis of pregnancy; LDLT, living donor liver transplantation; MELD, model for end-stage liver disease; MRI, magnetic resonance imaging; PSC, primary sclerosing cholangitis.

hepatic failure even after initially achieving successful results with Kasai operation (2).

Patients with BA also have a risk of developing pseudo-tumors or malignant hepatic tumors. The incidence of focal nodular hyperplasia, hepatocellular carcinoma, and hepatoblastoma associated with BA is 4.8%, 0.7%, and 0.4%, respectively (3, 4). The occurrence of cholangiocarcinoma in patients with BA is extremely rare. This case report describes a patient who underwent LDLT for cirrhosis secondary to BA, and a pathological examination of the explanted liver revealed the presence of incidental cholangiocarcinoma.

Case report

Clinical presentation

A 30-yr-old woman underwent LDLT for cirrhosis secondary to BA. She was diagnosed with BA and underwent a successful Kasai operation at 105 days of age. She was followed by annual blood tests and upper gastrointestinal endoscopy every 2–3 yr. The patient remained clinically stable and exhibited normal growth, except for two episodes of cholangitis (eight months old and 23 yrs old). She has since experienced no episodes of portal hypertension, esophageal varices, and hepatic failure. She gave birth to two children at 25 and 29 yr of age, respectively. She developed progressive jaundice and cholangitis nine months after the second delivery. She was referred and assessed for liver transplantation. Her liver function tests showed total bilirubin 16.4 mg/dL, conjugated bilirubin 11.3 mg/dL, AST 68 IU/L, ALT 35 IU/L, and albumin 2.5 g/dL. Serum tumor markers showed AFP 2.2 ng/mL, CEA 6.1 ng/mL, and CA 19-9 4130 U/mL. A liver biopsy was not performed during the pretransplantation period. The patency of the portal vein and the hepatic artery was demonstrated with CT and Doppler ultrasonography.

The patient was diagnosed to have decompensated liver cirrhosis secondary to BA (Child-Pugh score: 11 and MELD score: 23). The patient's 36-yr-old husband voluntarily donated his right hepatic lobe. There was severe adhesion around the hepatic hilum. There were no abnormal findings at the site of the portoenterostomy. An uneventful LDLT was carried out. A triple immunosuppression protocol of tacrolimus, steroids, and mycophenolate mofetil was administered post-operatively. She had an uneventful post-operative recovery and was discharged on day 39 with immunosuppressive therapy of tacrolimus, mycophenolate mofetil, and prednisolone.

The pathology report of the explanted native liver revealed a hepatic tumor measuring 4 cm diameter at the site of portoenterostomy anastomosis. The tumor was pathologically diagnosed as intrahepatic cholangiocarcinoma. CT showed recurrence of cholangiocarcinoma in the mesenteric lymph nodes, graft liver, and lung at three months after transplantation. The patient received chemotherapy with a cisplatin and gemcitabine protocol (5); however, the patient eventually died seven months after LDLT due to extensive lung metastases and lymphangiosis carcinomatosa.

Retrospective analysis of the presented case

This report presented a case with an incidentally detected cholangiocarcinoma in the explanted liver with BA after LDLT. Unfortunately, the tumor was not recognized before LDLT; however, a retrospective analysis of her clinical course and radiological examinations may have suggested its existence.

Clinical course and radiological findings

The patient had developed progressive jaundice nine months after her second delivery; however, her pregnancy and delivery assumed to be the cause of cholangitis and jaundice. CT and MRI were performed for the pre-operative evaluation. CT revealed a thickened wall at the anastomotic site of the portoenterostomy, which was suspected to have been due to severe cholangitis (Fig. 1a,b). MRI also showed the thickened wall of the anastomotic site of the portoenterostomy to be hyperintense on T1-weighted imaging and slightly hyperintense on T2-weighted imaging (Fig. 1c,d). These findings indicated that progressive jaundice could be related to a tumor obstruction at the anastomotic site of portoenterostomy. The serum level of tumor markers also indicated the presence of cholangiocarcinoma; low level of AFP 2.2 ng/mL, slightly high level of CEA 6.1 ng/mL; and an extremely high level of CA 19-9 4130 U/mL, which suggested not only a malignant tumor, but also obstructive jaundice.

Pathological findings of explanted liver

The pathological report of the explanted liver showed a complete loss of normal hepatic architecture and severe cholestasis consistent with secondary biliary cirrhosis, and adenocarcinoma (moderately differentiated cholangiocarcinoma, 4.2 × 3.4 cm in size) arising from the intrahepatic bile duct near the anastomosis of portoenterostomy, with microscopic invasion into the portal vein and regional lymph nodes (Fig. 2). It was

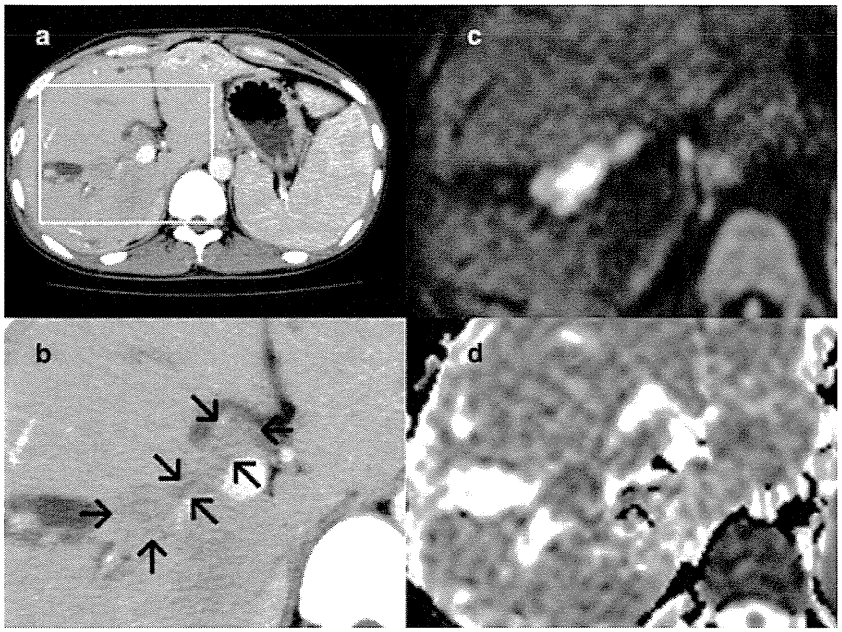

Fig. 1. Pretransplant contrast-enhanced CT and MRI showed thickened tissue on the portoenterostomy anastomosis site. (b: magnified the enclosed portion in image a; arrows indicated margin of the tumor) Diffusion-weighted image with a b-value of 1000 s/mm^2 revealed high signal intensity at thickened tissue (c) and low signal intensity on ADC map (d). ADC value of this tumor was $(0.67 \pm 0.07) \times 10^{-3}$ mm^2/s.

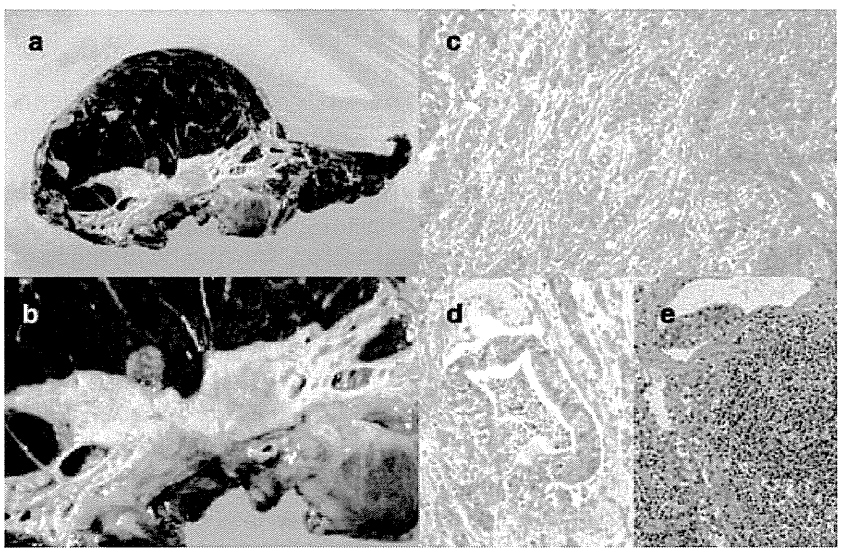

Fig. 2. Macroscopic and microscpic findings of the explanted liver. (a) Tumor located at the anastomotic site of portoenterostomy with a milky-white-colored boarder, (b) the tumor had a dumbbell shape (4.2 × 3.4 cm). (c) Hematoxylin –eosin staining showed moderately differentiated adenocarcinoma with vascular (d) and lymph node invasion (e).

classified as pT2aN1M0, stage IV-A, based on the AJCC 7th edition Pathology Staging Classification. Immunohistochemistry markers showed positivity for CK 7, CK 19, CK 20, and CA 19-9.

Discussion

The introduction of the Kasai operation has dramatically improved the survival rate of BA patients with their native livers, and liver transplantation further enhances their long-term survival. An increasing number of patients with BA have reached adulthood after the Kasai operation, and occasionally, these patients have severe late complications, such as portal hypertension, liver dysfunction, and secondary lung perfusion disorders. The prolonged lifespan of patients with BA has led to another serious issue, that is, how to manage liver function and late complications associated with pregnancy and delivery in female patients as with female patients with other liver diseases. Thirty-four deliveries from 25 female patients with BA were confirmed in Japan (6, 7). Liver dysfunction developed in eight cases and cholangitis appeared in four cases after delivery. Liver dysfunction and cholangitis after delivery are not uncommon. ICP may develop because high levels of estrogen during pregnancy may cause impaired biliary function and altered aminotransferase levels, but the impact of these changes on the maternal and fetal outcomes is

still unknown (8). Variceal bleeding most commonly occurs during the second and third trimesters when maternal blood volume is maximally expanded and the larger fetus causes increased compression of the inferior vena cava and collateral vasculature. The resultant increased intra-abdominal pressure is thought to lead to increased portal hypertension and stasis of the intestine. Digestive juice could reflux into the Roux-en-Y limb and could cause ascending cholangitis. Pregnancy may exacerbate these symptoms due to hormonal effects, compression by the gravid uterus, and the increase in intra-abdominal pressure during pregnancy and postpartum. The current patient was diagnosed with liver dysfunction and repeated cholangitis because of her second pregnancy and delivery.

There are increasing reports of BA with malignant hepatic tumors (9–11). Tatekawa reported three LDLT cases for BA with hepatic tumors (two hepatocellular carcinoma and one hepatoblastoma) in 275 LDLT for BA cases (4). The association between PSC or bile duct cysts and the development of cholangiocarcinoma is well known; however, the incidence of cholangiocarcinoma in patients with BA is extremely rare. There is only one published case of cholangiocarcinoma in patients with BA who underwent deceased donor liver transplantation: Two cases had a confirmed diagnosis of cholangiocarcinoma by autopsy (12, 13); the other case underwent liver transplantation (14). Although the association between the mechanism underlying carcinogenesis and pregnancy is not completely understood, it could be postulated that the reflux of digestive enzymes, bile stasis, and an increased concentration of intraductal bile acids contribute to the formation of malignant cells (15, 16). Recent studies suggest that intrahepatic cholangiocarcinoma expresses estrogen receptor-α and β, insulin-like growth factor 1 and that estrogens cooperate with insulin-like growth factor 1 in modulating the growth of cholangiocarcinoma (17, 18). Estrogen overexpression during pregnancy might be related to the growth of cholangiocarcinoma in the current patient.

Pretransplant CT and MRI revealed thickened tissue at the site of the anastomosis of portoenterostomy. It was difficult to recognize the thickened tissue as a tumor lesion. The combination of DWI and ADC map images is effective for the diagnosis of cholangiocarcinoma (19, 20). The pretransplant DWI and ADC map image from the current patient were retrospectively analyzed. The DWI showed high signal intensity, and the ADC map image showed low signal intensity. These findings were compatible with adenocarcinoma rather than edema or inflammation.

Previous studies of patients with PSC who underwent liver transplantation with incidental cholangiocarcinoma found on the explanted liver (21) show the rate of recurrence of cholangiocarcinoma to be 80% at a median time of 26 months. The three-yr survival rate is only 30%. Liver transplantation for cholangiocarcinoma in conjunction with neoadjuvant chemoradiation has shown encouraging results, although the data are extremely limited for the patients within stages I to IIB of AJCC Staging system (22). The one-, two-, and three-yr Kaplan–Meier survival probabilities are 90%, 70%, and 63%, respectively, whereas the historical five-yr survival rates are 0% to 18% for intrahepatic cholangiocarcinoma when patients undergo transplantation without neoadjuvant therapy. Our patient's prognosis was very poor because of advanced-stage incidental cholangiocarcinoma without neoadjuvant therapy. It is important to diagnose cholangiocarcinoma during the early stage and to identify any oncogenic risk factors. We need to establish a follow-up system for BA patients with a long-term survival after the Kasai operation.

This report presented a case of cholangiocarcinoma occurring in a long follow-up of BA. It is an extremely rare situation, but the occurrence of such lesions should be considered as one of the causes of hepatic dysfunction and cholangitis during a long-term follow-up course in patients with BA.

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乳児期に消化管穿孔で発症し牛乳アレルギーが疑われた1例

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要　旨

症例は，在胎33週6日，切迫早産と横位のため帝王切開にて出生した男児．出生体重は2,210 gで，母乳栄養のみで発育は良好であった．52生日，突然の発熱があり，53生日に腹部レ線でfree airが認められ，当科搬送後緊急開腹術を受けた．消化管穿孔部位は不明で，腹腔内洗浄のみを施行したが，術後経過に問題なく退院．しかし，86生日に麻痺性イレウスのため再入院となった．保存的治療にて改善したが哺乳再開5日後にイレウスが再燃した．これら腹部症状発生数日前に母親の牛乳や生クリームの摂取歴があり，牛乳アレルギーが疑われ，IgE抗体検査・アレルゲン特異的リンパ球刺激試験を施行したが陰性であった．その後，乳製品制限母乳での栄養を行い，以後順調に経過している．消化管アレルギーは乳児期の消化管穿孔の原因となり，特異的検査でも陰性となることがあり，詳細な病歴聴取が診断に重要である．

索引用語：消化管穿孔，消化管アレルギー，ALST

I　はじめに

近年，新生児や乳児期における消化管アレルギーの報告が増加している．消化器症状は多彩で，軽度の嘔吐・血便から活動性低下，経口摂取不良，イレウスなどの原因となるが，消化管穿孔を来たした症例の報告は少ない．今回我々は，乳児期に原因不明の消化管穿孔と繰り返すイレウスを呈し，臨床的に牛乳アレルギーが強く疑われた症例を経験したので報告する．

II　症　　例

患児：53生日，男児．

主訴：発熱，腹部膨満．

現病歴：在胎33週6日，切迫早産・横位のため，帝王切開にて出生．2,210 gの低出生体重のため，当院NICU管理となった．母乳栄養で良好な体重増加が得られ，18生日に退院した．52生日に38度台の発熱が出現し前医を受診したが，全身状態は良好で経過観察された．翌日，発熱が持続するため，再度受診したところWBC 13,260/μl，CRP 9.98 mg/dlと炎症反応の上昇を認め，腹部レントゲン検査でfree airを認めたため，当科紹介となった．

血液・生化学検査：WBC 16,430/μl，Neut 42.3%，Eos 0.2%，RBC 317×10⁴/μl，Hb 9.4 g/dl，Ht 29.6%，Plt 44.4×10⁴/μl，CRP 10.71 mg/dl，その他は正常範囲内であった．血液ガス分析（静脈血）では，pH 7.356, HCO_3^- 21.1 mEq/l %(n　P(q/lと軽度のアシドーシスがあり，座位腹部レントゲン（図1A）で，横隔膜下にfree airを認めた．

同日，緊急開腹手術を施行したが，黄色でやや粘稠な腹水が中等量認められたが，穿孔部位は不明であり，腹腔内洗浄とドレナージのみで手術を終了した（図1B）．腹水培養では*Klebsiella pneumonia*, *Enterococcus faecalis*などの腸管内常在菌が検出された．術後，全身状態は速やかに改善し，母乳の哺乳開始後も問題なく，14病日に退院した．

退院後の体重増加も良好であったが，85生日（術後32病日）の朝から嘔吐が出現し，吐物に胆汁が混じるようになり前医を受診したが，CTで腸管全体の著明な拡張を指摘され，イレウスの診断で翌日当科に再入院した．血液生化学検査では，WBC 7,520/μl, Neut 20.7%, Eos 0.8%, CRP 0.89 mg/dlと炎症反応の上昇はほとんど認めず，RBC 289×10⁴/μl, Hb 7.9 g/dl, Ht 24.9%と貧血を認めたが，便中ヘモグロビンは陰性であった．浣腸での

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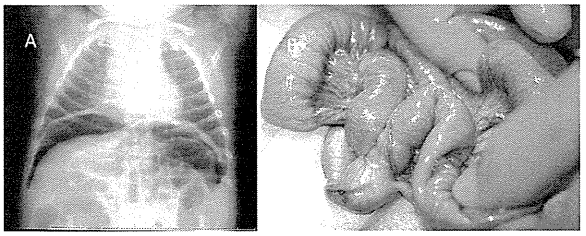

図1 初回入院時所見

A. 入院時腹部X線（座位），B. 術中消化管所見
横隔膜下にfree airを認めたが（A），術中所見では，明らかな消化管穿孔部位は同定されなかった（B）.

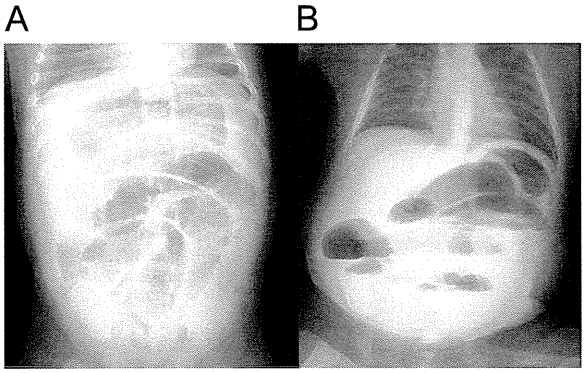

図2 2回目入院時の腹部レ線．臥位（A），座位（B）
腸管全体の拡張所見とniveauを認めた.

反応便は緑色粘稠で，便培養では腸管病原菌は検出されなかった．腹部レントゲンでは腸管の拡張とniveauを認めた（図2）.

保存的治療で症状は速やかに改善し，入院5日目から母乳の経口哺乳を再開した．しかし，入院10日目にイレウスが再燃したため，再度禁乳としたところ翌日には改善が得られ，入院19日目に退院となった.

入院中に施行した各種IgE抗体は陰性であったが（表1），臨床経過より消化管アレルギーが疑われたため，詳細な病歴聴取を行った．その結果，児の出生前から母は約200 ml/日の牛乳を摂取していた（85生日以降は意図せず摂取を控えていた）こと，53生日（消化管穿孔）と86生日（イレウス）の入院の1週間前に丸1日人工乳の哺乳を行っていたこと，イレウス再燃前日に母が牛乳摂取を再開していたことが判明した（図3）．なお，後にIgEが陽性となった卵（表1）に関しては，発症の前後に母が多く摂取していたということはなかった．生後4か月半時にも同様のイレウスで入院したが，入院の前に母親が生クリームを少量摂取していたというエピソードがあった．母親の乳製品摂取を禁止とし，母乳での栄養を継続した．なお，アレルゲン特異的リンパ球刺激試験（ALST）は陰性であり（表2），症状が改善した生後5か月時に施行した直腸肛門内圧検査では，直腸肛門反射は陽性であり，ヒルシュスプルング病は否定された.

その後は症状なく体重増加も良好であり，体重が7 kgを越えた生後8か月より，外来にて2週ごとに米・大豆・小麦の順に1品ずつ少量から開始していき，安全性を確認した．3品をクリアしたところで，経過中にIgEが高値となった卵のみを極力避けた食生活を行い，順調に体重増加を得ることができた．人工乳負荷試験については生後1歳5か月より入院・静脈ライン確保したう

表1 各種IgE検査結果

	生後3か月	生後8か月	生後10か月
非特異的IgE（IU/ml）	9.0	31.6	37.3
卵白	<0.34（class 0）	8.21（class 3）	9.46（class 3）
卵黄	<0.34（class 0）	0.82（class 2）	0.82（class 2）
オボムコイド	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
ミルク	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
αラクトアルブミン	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
βラクトグロブリン	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
カゼイン	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
牛肉	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
鶏肉	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
米	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
小麦	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）
大豆	<0.34（class 0）	<0.34（class 0）	<0.34（class 0）

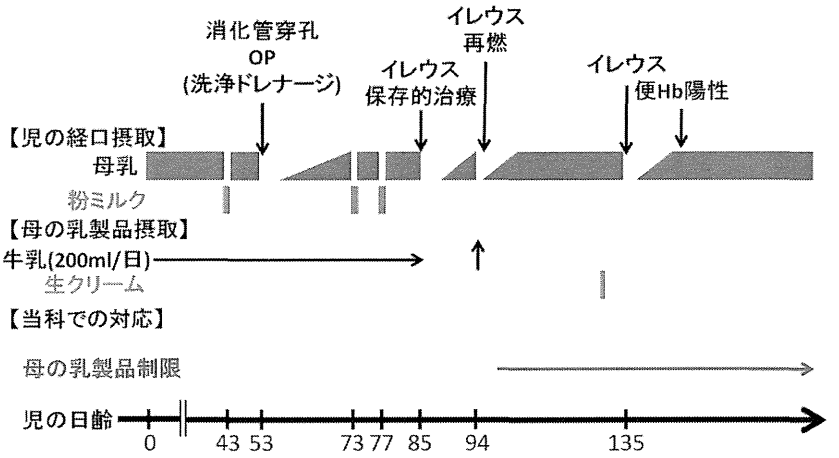

図3　臨床経過図

児の人工乳摂取と母の乳製品摂取後に消化管穿孔とイレウスを発症している.

表2　ALST 検査結果

抗原	Stimulation index	Cut off 値	判定
αラクトアルブミン	1.5	2.0	(-)
βラクトグロブリン	1.1	2.0	(-)
αカゼイン	0.4	2.0	(-)
βカゼイン	0.6	2.0	(-)
κカゼイン	0.5	2.0	(-)

えで少量から開始し，腹部症状の出現なく終了した.

III　考　　察

食物アレルギーは，IgE 依存型の即時型反応と，IgE 非依存型で細胞性免疫が関与する遅延型反応と，その中間のものとの3つのグループに分類することができる(表3). この中で IgE 非依存型の食物アレルギー（本症）は，近年報告が増加し注目されている[1].

欧米においては，IgE 非依存型として food protein-induced enterocolitis syndrome（FPIES）という概念が確立されており，その他に food protein-induced proctocolitis syndrome，food protein-induced enteropathy syndrome，celiac disease などといった疾患概念も挙げられているが，本邦ではこれらの疾患概念はまだ確立されていない[2][3].

本症の症状は嘔吐や血便，下痢の様な軽症なものから，血圧低下やイレウスなど重症のものまで多彩であり，稀ではあるが本症例の様に消化管穿孔での開腹例も報告されている[4]-[7]. 多くの症例は小児科で診断・治療されているが血便を主訴に小児外科を受診することもある. 本症は症状が現れるまでに1～数時間以上を要すると考えられ，実際には数時間～3日以内に発症することが多いとされている[3]. 本症例において1週間後に症状が生じたことより，遅延型反応が生じていたと考えられる.

本症の診断に用いられる検査には，末梢血の好酸球数，便粘液の細胞診，腸管粘膜組織検査，特異的 IgE 抗体，ALST などがあるが，遅延型反応の場合は，診断が難しいとされ，本症例では好酸球の上昇や牛由来ミルク IgE の上昇は経過中認めなかった. 本症の 1/3 は好酸球の上昇を認めず，IgE についても初発時の陽性率は 18～30％程度であるとされており[8][9]，陰性であるからといって本症を否定することはできない. 反対に，IgE が陽性でも本症とは直接結びつくものではないが，卵による遅延型の消化管アレルギーの報告もなされている[10]. 本症例では，母への病歴聴取から患児の発症前に普段より多く卵を食べたという経過はなかったとのことで，原因物質としての可能性は低いと判断したが，注意を要する食物であると考えられる. ALST については，κ-カゼイン-ALST で感度は 90％以上とも言われており[11]，有用性は高いと考えられる. しかし，本症の確定診断は，除去試験と負荷試験によってなされるのが原則であり，

表3 食物アレルギーの分類

	Group 1	Group 2	Group 3
病態	IgE 依存性 (即時型)	IgE 依存性と 非依存性の複合型	IgE 非依存性 (遅延型)
発症時期	乳児期	乳児期	生後1日～1年 (新生児期に多い)
症候	蕁麻疹 血管性浮腫 アナフィラキシー 嘔吐・下痢 喘息症状	嘔吐・下痢 腸炎 アトピー性皮膚炎 喘息	嘔吐・下痢・血便 皮膚炎 成長障害 胃食道逆流
診断法	病歴 スキンプリックテスト陽性 IgE 検査	病歴 スキンプリックテスト陽性 消化管内視鏡検査・生検 除去試験・負荷試験	除去試験・負荷試験 ALST 陽性 便中好酸球陽性 消化管内視鏡検査・生検 スキンプリックテスト陰性

文献 5)-7) より改変

上記検査は補助的なものと位置づけられる．軽症～中等症の本症であれば負荷試験を行い確定診断に至るが，重症～最重症に該当する症例では負荷試験を行わないことも選択肢の一つとなる．

Katz らの大規模な前向き研究によると，本症の70%近くが1歳6カ月までに，85%が2歳までに，90%以上が生後900日までに寛解するとされている[8]．他の報告でも母集団は少ないが1歳までに75%が寛解し[9]，2歳までにほとんどが改善するとされている[9)12)]．本症例の様に消化管穿孔を起こした症例において乳製品の経口摂取を再開するためには，十分なインフォームドコンセントを行い，適切な時期を見て負荷試験を行うのが望ましいと思われる．また，母乳を介したアレルゲン摂取のリスクについても指摘されており[13]，本症例においても母乳を介したアレルゲン摂取後の発症を含め合計3回の負荷試験＋除去試験が行われたと考えることもでき，本症を強く疑う根拠となった．なお本症例では開腹手術の所見から器質的疾患は否定され，また直腸肛門反射は陽性でヒルシュスプルング病が否定されていることも，本症の診断に近づくものと思われる．

本症における治療乳の開始には，原因食物（乳成分）によって異なるが，母乳（乳製品除去あり・なし），加水分解乳，アミノ酸乳がある．本症例においては，人工乳の直接摂取と母乳を介した摂取に反応して症状が出現しており，後者については感作が成立したためと考えられた．そのため，乳製品を除去した母乳での治療を開始した．また，離乳食の開始にあたっても，米や大豆，小麦に反応する場合があるとされており[9)14)15)]，注意が必要である．中でも米への反応を示すことが多く，人工乳以外で反応を示した場合には他の複数の食品にも反応を示すことが多いとされ[14)15)]，より気をつけなければならない．

ただし，本症例では上述した様に，牛乳アレルギーの確定診断に至る根拠は得られていない．経口摂取から1週間後に発症するというのも非典型的であり，何より暴露抗原の正体が一貫していない．しかし，たとえ診断に至らずとも，まず本症を疑うことから始め，詳細な病歴聴取や各種検査を進めていくことは重要なことと思われる．

本症例の様に新生児・乳児期に原因不明の消化管穿孔やイレウスを呈する症例では，本症を念頭に置き対応することが必要であると思われた．

（ALST 検査のみならず，臨床面及び本稿作成につきましても御助言を頂きました，国立成育医療研究センターアレルギー科の野村伊知郎先生，同免疫アレルギー研究部の森田英明先生に深謝致します．）

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