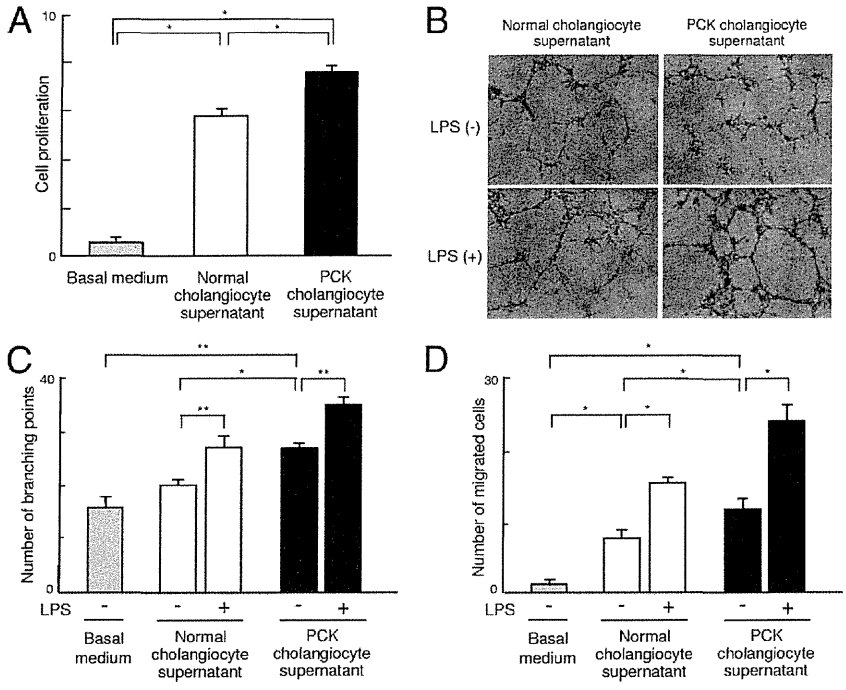


**Figure 6.** Angiogenic effects of PCK cholangiocytes. **A:** Rat aorta endothelial cells (RAOECs) were cultured in the presence of the culture supernatant of normal and PCK cholangiocytes, and cell proliferative activity was measured using the WST-1 assay. The culture supernatant of PCK cholangiocytes had more prominent effects on the induction of cell proliferative activity of RAOECs compared with that of normal rats. **B** and **C:** Tube formation assay using RAOECs demonstrated that the addition of PCK cholangiocyte culture supernatant increased the branching pattern of growth of RAOECs (**B**) (phase-contrast microscopy), and quantitative analysis of the number of branching points confirmed this tendency (**C**). **D:** Analysis using the cell migration chamber showed that the cholangiocyte culture supernatant significantly increased cell migration activity; the PCK culture supernatant had more prominent effects. The addition of LPS-treated cholangiocyte culture supernatant further increased both branching (**B** and **C**) and cell migration activity (**D**) of RAOECs. $^{*}P < 0.01$; $^{**}P < 0.05$. Original magnification, ×100.

tended to be associated with pathological progression of cholangitis. LPS-induced overexpression of VEGF in PCK cholangiocytes seemed to have a close correlation with portal neovascularization, as well as with cholangiocyte overgrowth. Increased portal neovascularization and VEGF overexpression in the biliary epithelium were also observed in Caroli's disease.

The overexpression of VEGF in cholangiocytes has been demonstrated in human PKD and in a rodent model of autosomal dominant PKD.[14,16,17] Liver cyst fluid of autosomal dominant PKD contains elevated levels of VEGF, and the cyst fluid induces vascular endothelial cell proliferation.[18,19] The contribution of signaling pathways involving ERK1/2 and mammalian target of rapamycin has been implicated in liver cyst progression of autosomal dominant PKD.[16,17] Similarly, in the kidney, overexpression of VEGF in the renal cyst epithelium has been implicated in cyst pathogenesis and pericystic hypervascularity in human PKD and also in a rodent model of PKD (Han:SPRD rat).[20] Although the significance of VEGF in PKD pathogenesis is being established, the association between VEGF expression and biliary infection has not

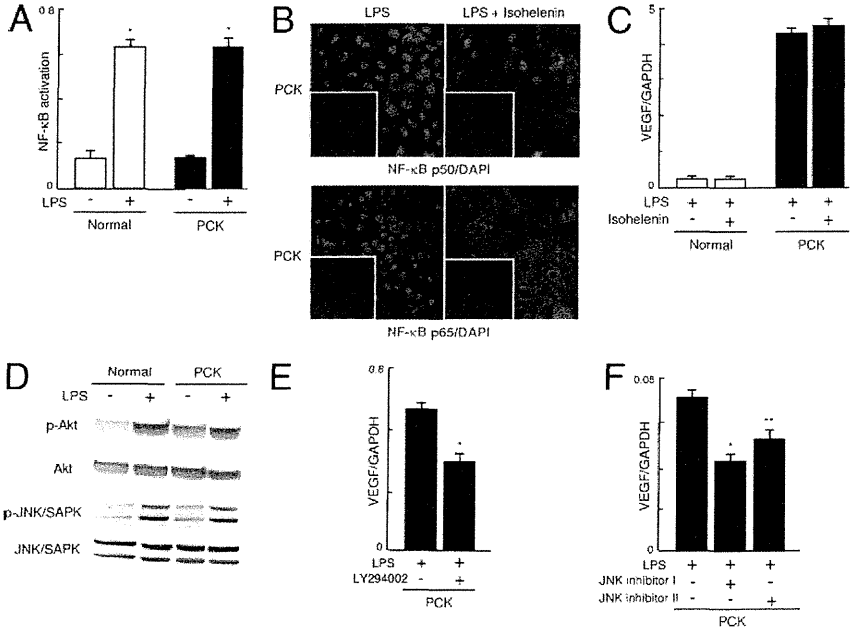


**Figure 7.** Cell signaling pathways involved in the VEGF expression of PCK cholangiocytes. **A:** After stimulation with LPS (10 μg/mL), activation of NF-κB was examined as described under *Materials and Methods*. LPS induced NF-κB activation in both normal and PCK cholangiocytes at 30 minutes after stimulation. **B:** Under immunofluorescence confocal microscopy, nuclear expression of NF-κB p50 and p65 was observed in both cell lines after LPS stimulation, and the NF-κB inhibitor isohelenin inhibited nuclear translocation of NF-κB p50 and p65; results shown are for PCK cholangiocytes. **C:** Real-time quantitative PCR showed that the expression of VEGF induced by LPS was unaffected by isohelenin, despite inactivation of NF-κB. **D:** Western blot analysis showed that LPS induced the phosphorylation of Akt and JNK/SAPK in both cell lines. **E** and **F:** The PI3K inhibitor LY294002 reduced the expression of VEGF mRNA after LPS stimulation in PCK cholangiocytes (**E**), and JNK inhibitor I and JNK inhibitor II also significantly reduced the LPS-induced VEGF mRNA expression (**F**), as determined using the real-time quantitative PCR at 3 hours after stimulation. $^{*}P < 0.01$; $^{**}P < 0.05$. Original magnification, ×1000.

been previously studied in autosomal recessive PKD or in autosomal dominant PKD.

LPS induced VEGF overexpression in PCK cholangiocytes in the present study. Because LPS induces the expression of VEGF through the TLR4-NF-κB signaling pathway in certain types of cells,[21,22] we examined the involvement of the signaling pathway in the induction of VEGF in cholangiocytes. As expected, LPS induced the activation of NF-κB in cholangiocytes. However, the NF-κB inhibitor isohelenin failed to inhibit the LPS-induced overexpression of VEGF in PCK cholangiocytes, even though it inhibited the nuclear translocation of NF-κB p50 and p65. These results suggest the involvement of the signaling pathways other than the TLR4-NF-κB pathway in the induction of VEGF expression in cholangiocytes.

Our data indicate that LPS-induced VEGF expression is mediated by PI3K-Akt and JNK. Although it is unclear whether the LPS-induced VEGF expression is a direct or an indirect effect of LPS, one possibility is that LPS induces bioactive molecules that in turn act as an inducer of VEGF in cholangiocytes. Tumor necrosis factor α (TNF-α) and cyclooxygenase-2 are two such candidate molecules that can induce VEGF via the phosphorylation of Akt.[23–25] Indeed, we have confirmed the up-regulation of TNF-α mRNA in PCK cholangiocytes after LPS stimulation (unpublished data). Hypoxia-inducible transcription factors may also be associated with the induction of VEGF in cholangiocytes.[20] Another possibility is that interferon regulatory factor 3, another downstream regulator of TLR4, may be involved in the process of VEGF induction in cholangiocytes.[26]

Several previous reports have shown that LPS stimulation can up-regulate TLR4 expression in various epithelial cells.[27] Consistent with these results, LPS induced TLR4 expression in PCK cholangiocytes *in vitro*, and immunohistochemical analysis showed that TLR4 was up-regulated in the biliary epithelium of the PCK rats. Thus, modulation of the signaling pathways through up-regulation of TLR4 may contribute to biliary pathogenesis of PCK rats.

Cholangitis was a negligible histological finding in liver of 3-week-old PCK rats. However, the microvessel density around bile ducts was significantly higher in the 3-week-old PCK rats than in normal rats. The PCK cholangiocytes initially overexpressed VEGF in the absence of LPS stimulation, and had angiogenic effects on the vascular endothelial cell growth (Figure 4). Overexpression of VEGF was accompanied by increased phosphorylation of Akt in the cells (Figure 7D). Although the mechanism of this spontaneous overexpression of VEGF in PCK cholangiocytes remains to be examined, these findings may explain the increased microvessel density around bile ducts without cholangitis in the PCK liver.

A recent study showed that VEGF stimulates proliferation of normal rat cholangiocytes via an autocrine mechanism by phosphorylating ERK1/2.[15] Although our data showed that the increase in cell proliferative activity of PCK cholangiocytes after VEGF stimulation was not mediated by phosphorylation of ERK1/2, VEGF expressed in the biliary epithelium may not only induce portal neovascularization of vascular endothelial cells via a paracrine mechanism, but may also increase cell proliferative activity of cholangiocytes via an autocrine/paracrine mechanism. Indeed, LPS induced PCK cholangiocyte proliferation in the present study, and biliary mitogens such as interleukin-6 as well as VEGF induced in cholangiocytes by LPS might be involved in the process.[28]

In summary, the present study demonstrated that biliary epithelium of PCK rats overexpresses VEGF, and LPS was identified as one of the factors leading to VEGF up-regulation in cholangiocytes. VEGF secreted from the biliary epithelium by LPS may lead to overgrowth of cholangiocytes due to hypervascularity around the bile ducts; concurrently, LPS and VEGF act as a cell proliferative factor for cholangiocytes. Thus, biliary infection is a possible exacerbating factor for biliary cystogenesis through the induction of VEGF in cholangiocytes of the PCK rats. Similar mechanisms may also exist in the pathogenesis of Caroli's disease.

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ORIGINAL ARTICLE

# Autophagy May Precede Cellular Senescence of Bile Ductular Cells in Ductular Reaction in Primary Biliary Cirrhosis

**Motoko Sasaki · Masami Miyakoshi · Yasunori Sato · Yasuni Nakanuma**

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**Abstract**
*Background and Aim* Recent studies disclosed that autophagy facilitates the process of senescence. Given that cellular senescence is involved in the pathophysiology of ductular reaction (DR) in primary biliary cirrhosis (PBC), we examined an involvement of autophagy in DRs in PBC and control livers.
*Methods* We examined immunohistochemically the expression of microtubule-associated proteins light chain 3$\beta$ (LC3) as autophagy marker, p62/sequestosome-1 (p62) as autophagy-related marker in bile ductular cells in livers taken from the patients with PBC ($n = 42$), and control livers ($n = 100$). The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also evaluated.
*Results* The expression of LC3 was seen in coarse vesicles in the cytoplasm of bile ductular cells and significantly more frequently in PBC of both early and advanced stages when compared to control livers ($p < 0.01$). The expression of p62 was seen as intracytoplasmic aggregates and significantly more frequently in PBC when compared to control livers ($p < 0.05$). The expression of LC3 and p62 significantly correlated with each other ($p < 0.01$). The expression of LC3 and p62 significantly correlated with the expression of $p16^{INK4a}$, $p21^{WAF1/Cip1}$ ($p < 0.05$).
*Conclusions* Autophagy is frequently seen in bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the advanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

**Keywords** Autophagy · Microtubule-associated proteins light chain 3$\beta$ (LC3) · p62/Sequestosome-1 · Ductular reaction · Cellular senescence · Primary biliary cirrhosis

## Introduction

Ductular reaction (DR) is a reactive lesion at the portal tract interface comprising increased bile ductules with an accompanying complex of stromal and inflammatory cells [1]. The involvement of DR has been implicated in the pathogenesis of progressive fibrosis, regeneration and hepatocarcinogenesis in chronic liver disease [1–3]. We have recently reported that bile ductular cells undergoing cellular senescence, which are characterized by the augmented expression of senescence-associated $\beta$-galactosidase (SA-$\beta$-gal), $p16^{INK4a}$ and $p21^{WAF1/Cip}$ and telomere shortening, increase in PBC along with fibrous progression, especially in PBC [4–6]. Cellular senescence is a state of stable cell arrest with active metabolism and a failsafe program against a variety of cellular insults. Cellular senescence is a delayed stress response involving multiple effector mechanisms such as the DNA damage response [7] and the senescence-associated secretion phenotype (SASP) [8–11]. Such senescent bile ductular cells may be involved in the progression of fibrosis of these diseases through the secretion of SASP [4–6, 11].

M. Sasaki (✉) · M. Miyakoshi · Y. Sato · Y. Nakanuma
Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan
e-mail: m8sasaki@med.kanazawa-u.ac.jp

M. Miyakoshi
e-mail: miyakosi@med.kanazawa-u.ac.jp

Y. Sato
e-mail: sato-ya@med.kanazawa-u.ac.jp

Y. Nakanuma
e-mail: pbcpsc@kenroku.kanazawa-u.ac.jp

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Autophagy (hereafter referred to as autophagy) in human diseases has been highlighted during the last decade [12–14] and recent data demonstrated that autophagy is significantly involved in the pathophysiology of liver diseases [15–21]. Macroautophagy is a genetically regulated program responsible for the turnover of cellular proteins and damaged organelles. This evolutionarily conserved process is characterized by the formation of double-membrane cytosolic vesicles, autophagosomes, which sequester cytoplasmic content and deliver it to lysosomes [12, 13, 22, 23]. Autophagy can enable adaptation to stress through the degradation of cellular proteins and organelles to suppress damage, maintain metabolism, and promote cellular viability and fitness [12–14, 23]. An appropriate cellular stress response is critical for maintaining tissue integrity and function and for preventing diseases. Cells respond to stress with adaptation, repair, and recovery, or are diverted into irreversible cell cycle exit (senescence) or are eliminated through programmed cell death (apoptosis) [23]. Dysfunctional autophagy appears to be associated with cellular senescence [23].

Autophagy and cellular senescence are two distinct cellular responses to stress. Recent studies disclosed that autophagy facilitates the process of senescence [22]. We have also reported that autophagy may precede biliary epithelial senescence in the damaged bile ducts in primary biliary cirrhosis (PBC) [20]. Although cellular senescence is involved in the pathophysiology of DR along with fibrous progression in primary biliary cirrhosis (PBC) [4–6], there has been no study reporting the involvement of autophagy in ductular cells in PD. In the present study, we examined an involvement of autophagy and its association with cellular senescence in DRs in PBC and control livers.

## Materials and Methods

### Classification of Intrahepatic Biliary Tree

The intrahepatic biliary tree is classified into the intrahepatic large and small bile ducts (septal and interlobular bile ducts) by their size and distributions in the portal tracts [24]. In this study, septal and interlobular bile ducts are termed small bile ducts. Bile ductules are not included in the small bile ducts. Bile ductules are characterized by tubular or glandular structures with poorly defined lumen and located at the periphery of the portal tracts and are not accompanied by parallel running hepatic arterial branches [1, 24]. Ductular cells in ductular reaction [1] including intermediate hepatobiliary cells with heterogeneous phenotype in the diseased liver were also evaluated.

### Liver Tissue Preparation

A total of 142 liver tissue specimens (all were biopsied or surgically resected) were collected from the liver disease file of our laboratory and affiliated hospitals. The liver specimens enrolled in this study were 42 PBC, 41 chronic viral hepatitis (CVH) livers, 27 nonalcoholic steatohepatitis (NASH), ten extrahepatic biliary obstruction (EBO) livers, and 22 "histologically normal" livers. All PBC were from the patients fulfilling the clinical, serological, and histological characteristics consistent with the diagnosis of PBC [25]. PBC livers were staged histologically [26] and 29 and 13 of PBC were of stages 1, 2 (early PBC) and of stages 3, 4 (advanced PBC), respectively. Twenty-seven CVH were regarded as F0-2 and 14 as F3, 4, respectively [27]. Three and 38 of CVH cases were serologically positive for hepatitis B surface antigen (HBsAg) and anti-hepatitis C viral antibody (HCVAb), respectively. The grade of activity and stage in the patients with NASH were assessed by using the criteria proposed by Brunt et al. [28], and 14 and 13 NASH were regarded as stages 1, 2 and stages 3, 4, respectively. Causes of EBO were obstruction of the bile duct at the hepatic hilum or the extrahepatic bile ducts due to carcinoma or stone, and the duration of jaundice was less than 1 month. "Histologically normal" livers were obtained from surgically resected livers for traumatic hepatic rupture or metastatic liver tumors. The liver tissues used were taken from the part sufficiently away from the trauma and tumor.

Liver tissue samples were fixed in 10% neutral buffered formalin, and embedded in paraffin. More than 20 serial sections, 4 μm thick, were cut from each block. Several were processed routinely for histopathologic study, and the remainder was processed for the following immunohistochemistry. The Committee of Ethics in Kanazawa University approved this study.

### Immunohistochemistry

We examined immunohistochemically the expression of microtubule-associated proteins light chain 3$\beta$ (LC3) as an autophagy marker, p62/sequestosome-1 (p62) [29, 30] as an autophagy-related marker in bile ductular cells. The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also examined in the same sample series. Immunostaining was performed using the antibodies shown in Table 1, as described previously [31]. In brief, after pretreatment for antigen retrieval as described in Table 1, blocking endogenous peroxidase, the sections were incubated with the primary antibody at 4°C overnight. The Envision+ solution (Dako) was then applied for 30 min at room temperature. The reaction products were visualized

**Table 1** Primary antibodies used in this study

| Primary antibody | Type (clone) | Pre-treatment | Dilution | Source |
|---|---|---|---|---|
| LC3 | Goat poly | MW–CB (95°C, 20 min) | 1:50 | Santa Cruz Biotechnology, Santa Cruz, CA |
| p62 | Rabbit poly | eARI–BA (121°C, 5 min) | 1:1,000 | MBL, Nagoya, Japan |
| $p16^{INK4a}$ | Mouse mono (JC8) | eARI–BA (121°C, 5 min) | 1:100 | Neomarkers, Freemont, CA |
| $p21^{WAF1/Cip1}$ | Mouse mono (70) | eARI–BA (121°C, 5 min) | 1:100 | BD Transduction, San Jose, CA |

*p62* p62/sequestosome-1, *LC3* microtubule-associated proteins-light chain 3*β*, *LAMP-1* lysosome-associated membrane protein-1, *MW* microwave treatment, *CB* 0.05 M citric buffer (pH 6), *eARI* electronic antigen retrieval instrument (Pascal, Dako), *BA* 0.05 M boric acid buffer (pH 8)

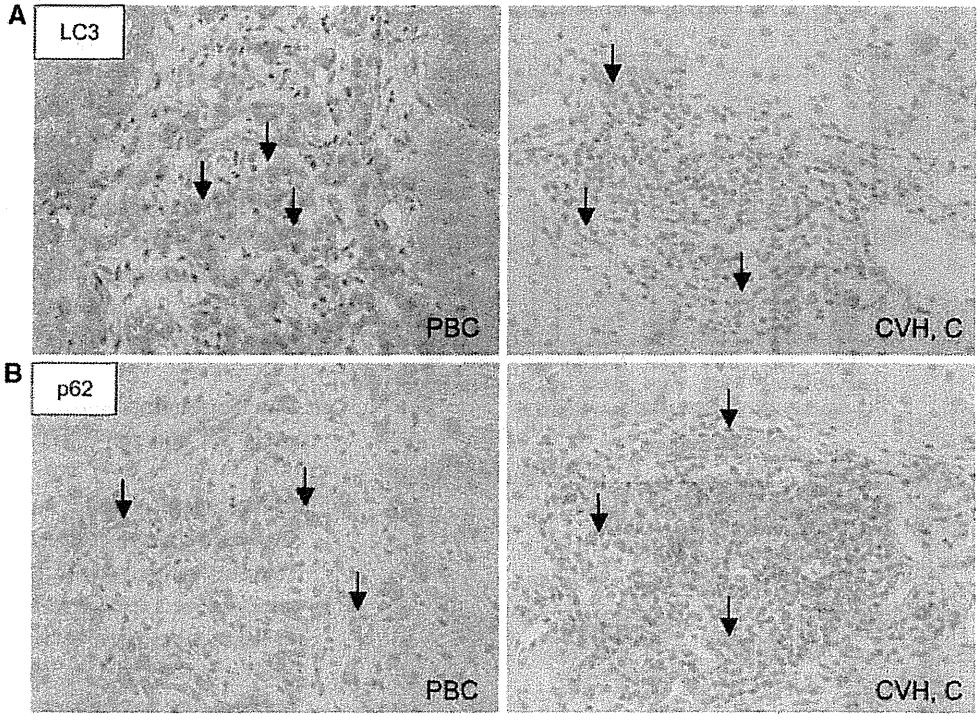


**Fig. 1** Expression of LC3 and p62 in ductular reaction (DR) in PBC and control liver. **a** Coarse vesicular expression of LC3 in DRs in PBC. Coarse vesicular expression of LC3 is seen in the cytoplasm of ductular cells in PBC (*left, arrows*), whereas no expression of LC3 is found in ductular cells in CVH, C (*right, arrows*). Immunostaining for LC3. Original magnification, ×400. **b** Coarse vesicular expression of p62 is seen in the cytoplasm of ductular cells in PBC (*left, arrows*), whereas no or faint expression of p62 is found in ductular cells in CVH, C (*right, arrows*). Immunostaining for p62. Original magnification, ×400

using 3–3′-diaminobenizidine tetra hydrochloride (Sigma Chemica, Co., St. Louis, MO) and $H_2O_2$. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of the control mouse IgG (Dako) was applied instead of the primary antibody as negative control. Positive and negative controls were routinely included.

### Assessment of Immunostaining

All fields of each liver specimen were observed under light microscope for the evaluation of immunohistochemical expression of LC3, p62, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. The extent of expression was semiquantitatively evaluated as 1+ (focal, positive cells are detected in one-third or fewer portal tracts), and 2+ (extensive, positive cells are detected in more than one-third of portal tracts).

### Statistical Analysis

Statistical analysis for the difference used the Wilcoxon rank-sum test. The correlation coefficient of two factors was evaluated using Spearman's rank correlation test. When the $p$ value was less than 0.05, the difference was regarded as significant.

## Results

### LC3

The expression of LC3 was seen in coarse vesicles in the cytoplasm of bile ductular cells, when detectable (Fig. 1a). As shown in Fig. 2a, the expression of LC3 was more frequent in ductular cells in the early stages of PBC

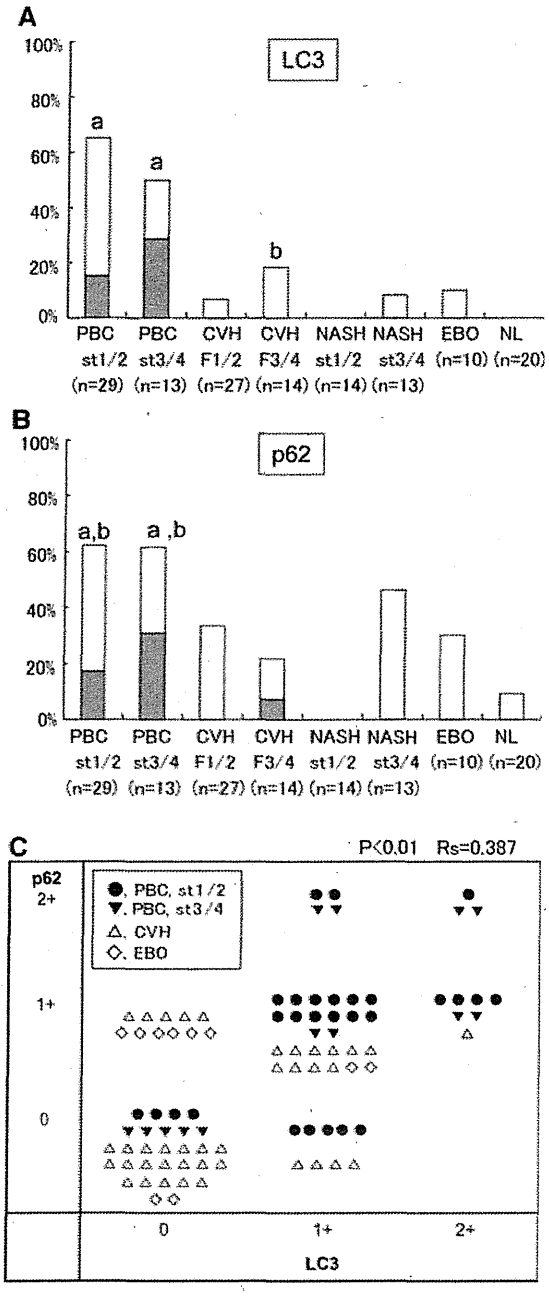


**Fig. 2** Increased expression of LC3 and p62 in ductular reaction (DR) in PBC. **a** The frequency and extent of LC3 expression. *White column*, focal expression (1+), *gray columns*, extensive expression (2+). *a* $p < 0.01$ versus other groups, *b* $p < 0.05$ versus normal liver (NL). **b** The frequency and extent of p62 expression. *White column*, focal expression (1+), *gray columns*, extensive expression (2+). *a* $p < 0.01$ versus normal liver (NL) and nonalcoholic steatohepatitis (NASH), stage (st)1 and 2, *b* $p < 0.05$ versus chronic viral hepatitis (CVH), F1 and F2 and F3 and F4. **c** Correlation between expression of LC3 and p62 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the distribution of these two molecules ($p < 0.01$, rs = 0.387)

(1+, 50%; 2+, 15%) and the advanced stages of PBC (1+, 23%; 2+, 31%), compared to other groups; CVH, F1/2 (1+, 6.7%; 2+, 0%), CVH, F3/4 (1+, 18%; 2+, 0%), NASH stages 1 and 2 (1+, 0%; 2+, 0%), NASH stages 3 and 4 (1+, 8.3%; 2+, 0%), EBO (1+, 10%; 2+, 0%), and normal livers (1+, 0%; 2+, 0%), respectively ($p < 0.01$). The expression of LC3 in CVH, F3/4 was significantly more frequent compared to normal livers ($p < 0.05$).

p62

The expression of p62 was seen as intracytoplasmic aggregates in bile ductular cells, when detectable (Fig. 1b). As shown in Fig. 2b, the expression of p62 was more frequent in ductular cells in the early stages of PBC (1+, 45%; 2+, 17%) and the advanced stages of PBC (1+, 31%; 2+, 31v%), compared to NASH stages 1 and 2 (1+, 0%; 2+, 0%) and normal livers (1+, 9%; 2+, 0%), respectively ($p < 0.01$). The expression of p62 was more frequent in the early and advanced stages of PBC, compared to CVH, F1/2 (1+, 33%; 2+, 0%), CVH, F3/4 (1+, 14%; 2+, 7.1%), respectively ($p < 0.05$).

The Correlation Between Expressions of LC3, p62, $p16^{INK4a}$, and $p21^{WAF1/Cip1}$

The expression of LC3 and p62 ($p < 0.01$, rs = 0.38703) was significantly correlated in bile ductular cells (Fig. 2c). The expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ was significantly more frequent in bile ductular cells in PBC, stage 3, 4, when compared to control livers ($p < 0.05$), as previously reported [5, 6]. There were significant correlations between the expression of LC3 and $p16^{INK4a}$ ($p < 0.01$, rs = 0.574), the expression of LC3 and $p21^{WAF1/Cip1}$ ($p < 0.01$, rs = 0.462), the expression of p62 and $p16^{INK4a}$ ($p < 0.01$, rs = 0.336), the expression of p62 and $p21^{WAF1/Cip1}$ ($p < 0.05$, rs = 0.285) (Fig. 3a, b).

## Discussion

The data obtained in this study are summarized as follows; (1) LC3, an autophagy marker, and p62, an autophagy-related marker, were frequently expressed in ductular cells in DR in PBC compared to those in CVH, NASH, EBO, and normal livers. (2) The expression of LC3 and p62 was significantly correlated with each other in ductular cells in DR. (3) The expression of LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR.

The present study firstly disclosed that ductular cells in DR frequently show autophagy detected by immunostaining

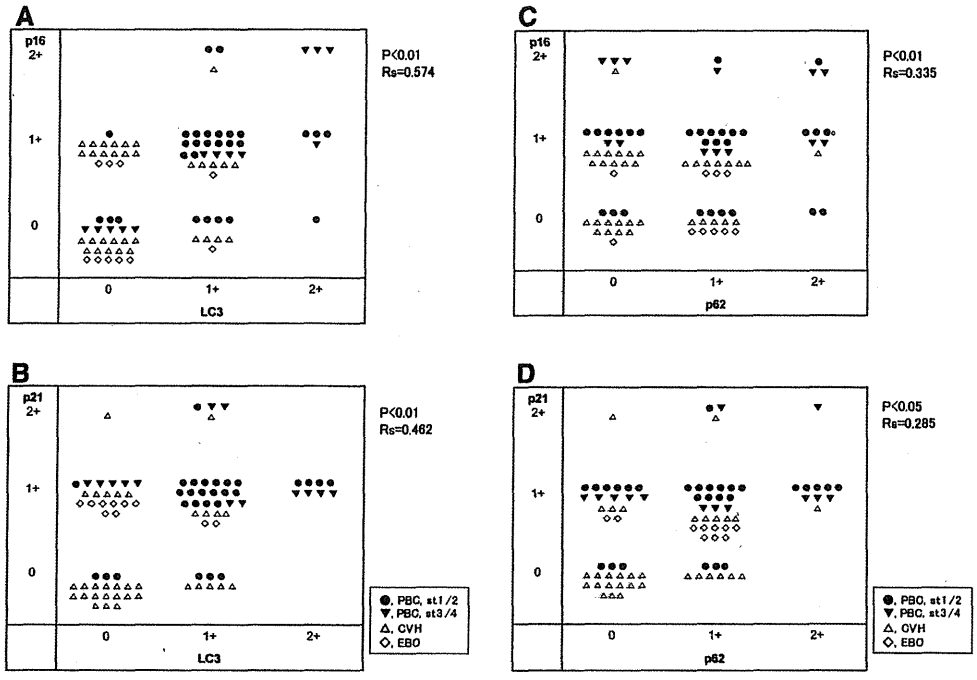


**Fig. 3** Correlation between autophagy markers and senescent markers. **a** Correlation between expression of LC3 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules is infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the distribution of these two molecules ($p < 0.01$, rs = 0.574). **b** Correlation between expression of LC3 and p21 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules is infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p < 0.01$, rs = 0.462). **c** Correlation between expression of p62 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules is infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p < 0.01$, rs = 0.335). **d** Correlation between expression of p62 and p21 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules is infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p < 0.05$, rs = 0.285)

for LC3 in PBC, whereas autophagy is infrequently detected in DR in control livers. This finding clearly indicates that autophagy is involved in the pathogenesis of DR in PBC and DR in PBC may be different from other liver disease. We have shown that autophagy is upregulated in damaged bile ducts in PBC [20]. Therefore, the autophagy appears to be a common feature of biliary epithelial cells in small bile ducts and DR in PBC.

Furthermore, it is of interest that the accumulation of p62 is frequently seen in DR in PBC, similarly to LC3. p62 is an adaptor protein involved in the delivery of ubiquitin-bound cargo to the autophagosome and regulates the formation of protein aggregates [29, 32–34]. An accumulation of p62 is seen in autophagy-deficient condition, so, the accumulation of p62 may be a marker of dysfunctional autophagy in which the capacity of autophagy is not enough to process the damaged proteins bound to p62 [19, 21, 35]. Therefore, the accumulation of p62 may reflect dysfunctional autophagy in ductular cells in DR in PBC. In addition, the expression of LC3 and p62 was significantly correlated with each other in ductular cells in DR. Taken together, autophagy, especially dysfunctional autophagy, may be involved in the pathophysiology of ductular cells in PBC.

Recent studies have disclosed that autophagy preceded and accelerated cellular senescence [22] and we have also reported that autophagy mediates biliary epithelial senescence [20]. Our previous study shows that some of the ductular cells in DR in chronic liver diseases were at G1- arrest and undergoing cellular senescence and that such senescent cells may be involved in the progression of fibrosis of these diseases, particularly in PBC [6]. The present study revealed that the expression of autophagy markers: LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR. This finding suggests that autophagy is involved in the biliary epithelial senescence in DR. Dysfunctional autophagy may induce cellular senescence. It is of interest that autophagy is upregulated in DR in both early and advanced PBC, whereas cellular senescence is more frequent in DR in the advanced PBC in our previous study. This

may also support the hypothesis that biliary epithelial senescence may be induced via the process of autophagy. Taken together, the regulation of autophagy may be a therapeutic target to prevent the progression of fibrosis along with biliary epithelial senescence in PBC.

In conclusion, autophagy is frequently seen and correlated with cellular senescence in bile ductular cells in DRs in PBC, both in the early and advanced stages. These findings suggest that autophagy may be involved in the pathophysiology of DRs in PBC and may precede cellular senescence of bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the advanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

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**Conflict of interest** There is no conflict of interest.

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REVIEW

# Recent progress of IgG4-related hepatobiliary diseases with emphasis on pathologic aspects and differential diagnosis

Yasuni Nakanuma
Yasunori Sato
Kenichi Harada

**Abstract**
IgG4-related disease usually presents with a mass-forming or regional lesion showing considerable lymphoplasmacytic infiltration with many IgG4-positive plasma cells, sclerosis, and obliterative phlebitis. Herein, recent progress of hepatobiliary IgG4-related diseases including IgG4-related sclerosing cholangitis (IgG4-SC), hepatic inflammatory pseudotumor (HIP), and autoimmune hepatitis (AIH), are reviewed. IgG4-SC mainly affects the extrahepatic and hilar bile ducts, and is almost always associated with autoimmune pancreatitis (AIP), a prototype of IgG4-related disease. IgG4-SC has a propensity to exaggerate focally, and such IgG4-SC could be categorized as an IgG4-related HIP, mainly belonging to the lymphoplasmacytic type of HIP. IgG4-related AIH is a chronic active hepatitis with many IgG4-positive plasma cells and increased serum IgG4 level. In conclusion, several hepatobiliary diseases which had been diagnosed as other diseases, can be reclassified as IgG4-related diseases, and several local factors peculiar to the hepatobiliary system may be responsible for the development of these diseases.

**Keywords** allergy; autoimmune hepatitis; autoimmune pancreatitis; fibrosis; IgG4; inflammatory pseudotumor; phlebitis; plasma cells; regulatory T cells; sclerosing cholangitis

## Introduction

Since the 1990s, autoimmune pancreatitis (AIP) has been increasingly recognized as a special type of chronic pancreatitis.[1] Such patients show characteristically a swollen pancreas with considerable lymphoplasmacytic infiltration with many IgG4+ plasma cells (IgG4-tissue positivity) and fibrosis, and respond well to steroid therapy. This type of lesion also involves extrapancreatic tissues, including the bile duct, salivary glands, retroperitoneum and cardiovascular system.[2–4] Diseases of this type are now collectively called as IgG4-related disease,[5] are clinically characterized by elevated serum IgG4 concentrations, a high prevalence in middle to old-aged adults with a preponderance among men, steroid sensitivity, and the frequent occurrence of non-organ-specific autoantibodies such as antinuclear antibodies. Not infrequently, IgG4-related disease affects more than one organ in the same individual.[3,4] In addition, such cases are also frequently associated with lymphadenopathy showing follicular hyperplasia or Castleman-like lesions with IgG4-tissue positivity. It is becoming evident that several other diseases also show considerable infiltration of lymphoplasmacytes with IgG4-tissue positivity or elevated serum IgG4 level, making it important to distinguish IgG4-related disease as it responds well to steroid therapy.

Herein, the clinicopathological features of AIP with reference to IgG4-related disease are briefly reviewed. Then, the pathology and aetiopathogenesis of IgG4-related hepatobiliary disease are discussed in differentiation from other hepatobiliary diseases with IgG4-tissue positivity or elevated serum IgG4 level.

## Autoimmune pancreatitis and IgG4-related sclerosing disease

The concept of AIP was first reported by Yoshida et al. as a specific type of chronic pancreatitis, mainly affecting elderly men, showing diffuse or regional swelling of the pancreatic parenchyma and irregular narrowing of the main pancreatic duct.[1] Histologically, AIP is characterized by considerable lymphoplasmacytic infiltration with variable acinar atrophy and loss and also periductal infiltrates of lymphocytes and plasma cells, and is also called lymphoplasmacytic sclerosing pancreatitis (LPSP).[6] Additional characteristic features are also seen: (i) irregular or storiform fibrosis; (ii) extension of sclerosing inflammation to parapancreatic adipose tissue; and (iii) obliterative phlebitis. Eosinophilic infiltration is variably found. Hamano et al. reported that more than 90% of patients with AIP have high serum IgG levels, particularly IgG4, and many IgG4+ plasma cells are evident immunohistochemically in the affected pancreas.[7] Most importantly, steroid therapy is effective, and the size of the pancreas returns to normal.

As for IgG4-realted lesions in various organs, the following are representative: idiopathic retroperitoneal fibrosis, chronic sclerosing sialadenitis, Mikulicz's disease, periaortitis, and tubulointerstitial nephritis. Such diseases are now proposed to be collectively called IgG4-related diseases.[3,5] In addition, a pseudotumorous variant of IgG4-related disease diagnosed as IgG4-related inflammatory pseudotumor has been reported in several organs.[3,8] Interestingly, some of these diseases had already been reported as a disease family called multifocal fibrosclerosis proposed by Comings.[9]

At present, AIP is regarded as a pancreatic manifestation of IgG4-related disease.[4] In general, one or several organs are affected synchronously or metachronously by this disease in various combinations. Interestingly, there are some characteristic features among the affected organs,[3] suggesting the participation

***Yasuni Nakanuma*** *MD is in the Department of Human Pathology at Kanazawa University, Graduate School of Medicine, Kanazawa, Japan. Conflicts of interest: no conflict of interest.*

***Yasunori Sato*** *MD is in the Department of Human Pathology at Kanazawa University, Graduate School of Medicine, Kanazawa, Japan. Conflicts of interest: no conflict of interest.*

***Kenichi Harada*** *MD is in the Department of Human Pathology at Kanazawa University, Graduate School of Medicine, Kanazawa, Japan. Conflicts of interest: no conflict of interest.*

 

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of common immunologic mechanisms, and also of a pathologic process peculiar to individual organs.[3,5]

Regarding AIP, two types have been recently distinguished. Type 1 corresponds to a pancreatic manifestation of IgG4-related diseases as mentioned above, while type 2 AIP usually has no or very few IgG4-positive plasma cells and no elevation of serum IgG4 level. The two types share the same symptomatology and some histopathological features such as periductal lymphoplasmacytic infiltrates, though type 2 AIP shows so-called granulocytic epithelial lesion (GEL). Both types respond well to steroid treatment.

### Diagnosis of IgG4-related disease

IgG4-related disease is diagnosed based on characteristic pathologic features, such as considerable lymphoplasmacytic infiltration, fibrosis, and obliterative phlebitis. While these findings are obvious and the diagnosis is not difficult in larger specimens, a pathologic diagnosis in smaller specimens is not easy.[3] For example, obliterative phlebitis which is shown by Elastica van Gieson staining, is not usually identifiable in small specimens. Eosinophilic infiltration is not an absolute indication of this disease. As for IgG4-tissue positivity, more than 10 IgG4-bearing plasma cells/high power field (HPF), and an increased IgG4+ to IgG+ plasma cell ratio (IgG4/IgG ratio, more than 30%) are used to distinguish IgG4-related disease from other inflammatory conditions.[5] The elevated IgG4 serum level, particularly higher than 135 mg/dl, is frequently but not always observed in these patients.[7] Recently, it was shown that there are many Treg and FOXP3+ regulatory T cells (FOXP3(+) Treg) in the affected tissues,[2] and abundant infiltration by such Tregs may favour a diagnosis of IgG4-related disease.

### Hepatobiliary diseases belonging to IgG4-related disease

Some hepatobiliary diseases have been found to be IgG4-related disease.[2,8,10] They had been diagnosed as other diseases such as primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) before this entity had been recognized. Herein, the pathology of these newly reclassified diseases and their aetiopathogenesis is discussed here. In this review, the biliary tree is divided into the extrahepatic bile duct, right and left hepatic bile ducts, and intrahepatic large and small bile ducts.[11] The latter were composed of septal and interlobular bile ducts. Physiologically, peribiliary glands which are located within and outside the duct wall, are distributed along the intrahepatic large bile duct and extrahepatic bile duct.[11,12] Similar glands are also located in the gallbladder neck.

### IgG4-related sclerosing cholangitis

Sclerosing cholangitis is characterized by fibrosis and chronic inflammatory cell infiltration of the bile duct wall of the extrahepatic and intrahepatic bile ducts with luminal changes, and their distribution is segmental or diffuse. Its pathology and aetiology are heterogeneous.[10,13,14] PSC is a prototype of sclerosing cholangitis and frequently associated with inflammatory bowel disease (IBD), particularly ulcerative colitis. Sclerosing cholangitis showing abundant IgG4+ plasma cell infiltration has recently emerged and is called IgG4-related sclerosing cholangitis (IgG4-SC).[2,4,8]

IgG4-SC mainly affects elderly men, and is usually associated with AIP. Extrahepatic bile ducts, particularly intrapancreatic portions, are affected in 95% of AIP cases and the hilar or intrahepatic large bile ducts are also affected in 40% of AIP patients, though a few cases of IgG4-SC without clinical evidence of AIP are reported. However, the common hepatic duct and extrapancreatic portion of the common bile duct are not usually affected. IgG4-SC is not associated with IBD. The affected bile ducts show prominent thickening of their wall, and look whitish, medullary and fleshy, and have a border that is rather clear, but merge slightly with the surroundings. The lumens of affected bile ducts are usually stenotic, and the proximal bile ducts are usually dilated. Serological findings reported in AIP are also found in IgG4-SC, and IgG4-SC responds well to steroid therapy.

Histologically, IgG4-SC shows considerable lymphoplasmacytic infiltration with fibrosis, and lymph follicle formation and obliterative phlebitis are occasionally found (**Figure** 1a–c). It is of interest that the peribiliary glands are more severely affected and inflammatory reactions are clearly oriented towards these glands, and these glands show epithelial damage and are destroyed. Biliary lining epithelial cells of the affected large bile ducts are relatively spared in IgG4-SC as seen in the pancreatic duct in AIP. This is in sharp contrast with PSC in which biliary lining epithelial cells show degeneration, loss and ulceration. Perineural lymphoplasmacytic infiltration and extension to the periductal connective tissue are also seen. The fibrosis in IgG4-SC is not dense or hyalinized.

Immunohistochemically, there are many IgG-positive plasma cells, a majority of which are positive for IgG4, in the affected bile duct and portal tracts (**Figure** 1d). IgM, IgA, IgG1, IgG2, and IgG3-positive cells are scarce.[13] In IgG4-SC, proliferated lymphocytes are composed of T cells and also B cells, and CD4+ T cells are more common than CD8+ T cells.

**Hepatobiliary lesions secondary to or associated with IgG4-SC:** smaller portal tracts remote from the affected hilar and perihilar large bile ducts also show IgG4-tissue positivity, a useful finding for the diagnosis of IgG4-SC. In our experience, a needle liver biopsy is useful for diagnosis in about one-fourth of IgG4-SC patients by showing IgG4-positive plasma cells in portal tracts.[15] As for other hepatobiliary lesions, chronic cholestasis with frequent bile plugs, portal tract fibrosis and also biliary fibrosis due to biliary stenosis are found.[15] Periductal concentric or onion-skinning fibrosis of interlobular or septal bile ducts was also seen, though significant biliary epithelial damage, lymphoplasmacytic cholangitis, bile duct scarring and ductopenia are not usually seen in IgG4-SC. Interface hepatitis of mild degree is found in one-fourth of IgG4-SC patients.

### Involvement of gallbladder and papilla Vater in AIP or IgG4-SC

The gallbladder and duodenal papilla are also frequently affected in patients with AIP.[16] However, the clinical features or symptoms due to these tissues can be unclear, nonspecific or non-significant.

**IgG4-related chronic sclerosing cholecystitis (IgG4-CSC):** in AIP/IgG4-SC, the gallbladder shows moderate or marked mucosal and transmural lymphoplasmacytic inflammatory infiltrates with IgG4-tissue positivity, extramural inflammatory nodules, tissue eosinophilia and obliterative phlebitis as seen in AIP or IgG4-SC. IgG4-tissue positivity could be helpful in differentiating it from

 

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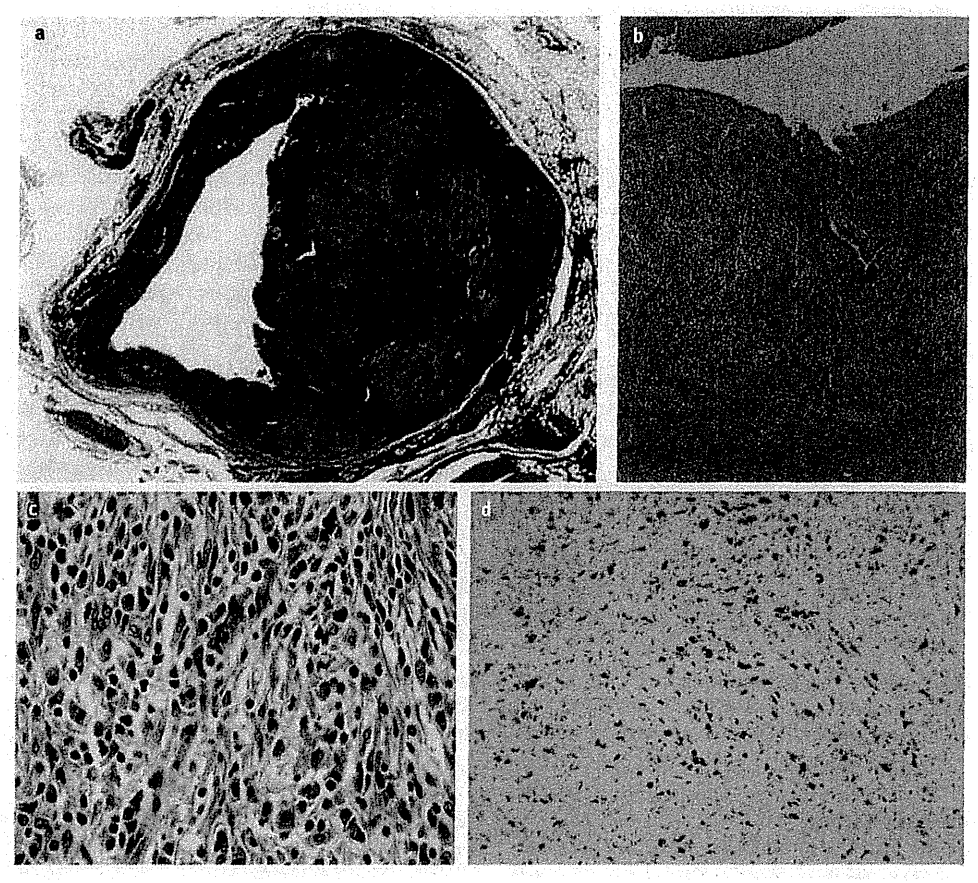


**Figure 1** (**a**) About a half circumference of the extrahepatic bile duct is markedly inflamed, while the other part is apparently normal. IgG4-related sclerosing cholangitis. H&E. (**b**) The affected bile wall shows fibrosis and lymphoplasmacytic infiltration, rather diffusely. The lining biliary epithelia are relatively preserved. IgG4-related sclerosing cholangitis. H&E. (**c**) Lymphoplasmacytic infiltration and fibroblasts are seen. IgG4-related sclerosing cholangitis. H&E. (**d**) There are many IgG4-positive plasma cells in the affected bile ducts. Immunostaining for IgG4 and haematoxylin.

other cholecystopathies. The presence of a dense extramural inflammatory infiltrate, extramural inflammatory nodules, and >10 IgG4 plasma cells per HPF in the gallbladder favours the diagnosis of IgG4-CSC.

IgG4-tissue positivity in the duodenal papilla is found in AIP, and is helpful in the diagnosis of AIP. Kawakami et al. reported that the diagnostic sensitivity of IgG4 immunostaining of both ampullary and bile duct biopsy specimens was 52% (15/29) for symptomatic patients with AIP or IgG4-SC. Two-thirds of patients had more than 10 IgG4+ plasma cells in biopsy specimens. Furthermore, the bile duct biopsy specimens not only indicated the number of IgG4+ plasma cells but also showed other characteristic histological features, including storiform fibrosis.

### Hepatic inflammatory pseudotumor

Hepatic inflammatory pseudotumor (HIP) is characterized by the irregular proliferation of fibroblasts intermixed with infiltrating inflammatory cells, mainly lymphocytes and plasma cells. It is regarded as a heterogeneous disease and subcategorized histologically into several types including a fibrohistiocytic type, lymphoplasmacytic type, and sclerosed or fibrosed type. The fibrohistiocytic type is characterized by xanthogranulomatous inflammation, multinucleated giant cells, and neutrophilic infiltration, and mostly occurs in the peripheral hepatic parenchyma as a mass-forming lesion. In contrast, the lymphoplasmacytic type shows diffuse lymphoplasmacytic infiltration with variable eosinophilic infiltration, and is usually found around the hepatic hilar and perihilar region. Obliterative phlebitis and cholangitis with periductal fibrosis are common features of the lymphoplasmacytic type (Figure 2a,b).[17] Interestingly, IgG4-positive plasma cells were significantly more numerous in the lymphoplasmacytic than fibrohistiocytic type. However, a few cases of fibrohistiocytic type had also relatively many IgG4-positive plasma cells. Taken together, a majority of lymphoplasmacytic HIPs may belong to the so-called IgG4-related diseases. In fact, such cases are known to respond well to steroid therapy. In contrast, fibrohistiocytic HIP might still be a heterogeneous group of disorders. Several cases of fibrohistiocytic type with relatively abundant IgG4-positive plasma cells could be IgG4-related disease with secondary histopathologic modifications.

HIP is not infrequently associated with AIP/IgG4-SC,[2,17] and the pathogenesis may be similar or closely related to AIP and IgG4-SC. In HIP, the bile ducts and their surrounding tissues including peribiliary glands and connective tissue are severely inflamed and the inflammation expands into the surroundings, suggesting that such HIP is an exaggerated inflammatory condition in IgG4-SC.

 

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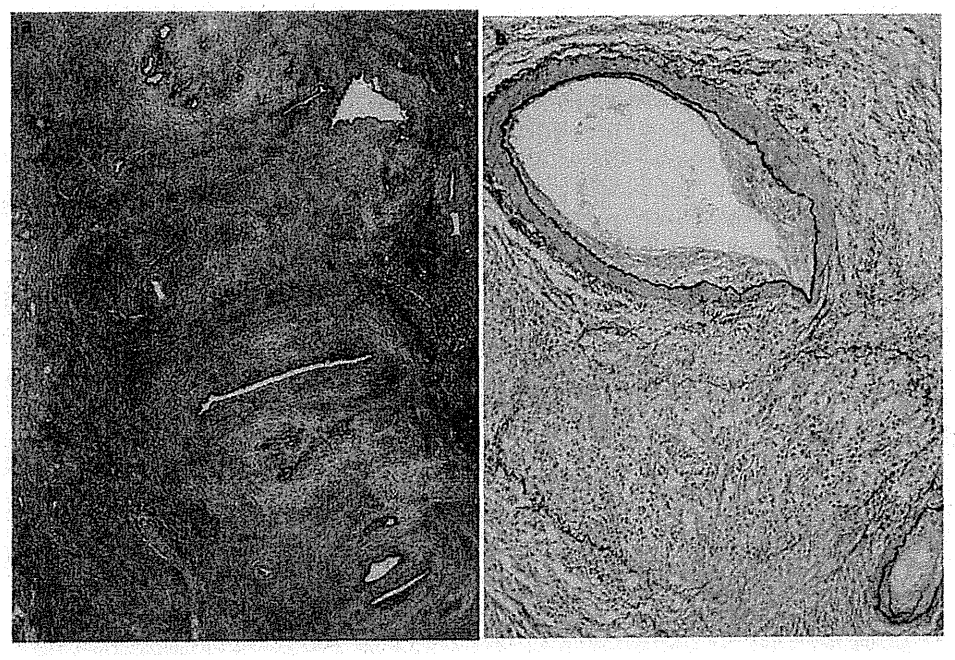

**Figure 2** (**a**) There are massive inflammation involving bile ducts, peribiliary glands and surrounding connective tissue. Hepatic inflammatory tumour of lymphoplasmacytic type belonging to IgG4-related disease. H&E. (**b**) One vein shows obliterative phlebitis, and one artery also shows focal luminal changes. Hepatic inflammatory tumour of lymphoplasmacytic type belonging to IgG4-related disease. Elastica van Gieson stain.

### IgG4-related autoimmune hepatitis

Autoimmune hepatitis (AIH) is an organ-specific autoimmune liver disease characterized by prominent interface hepatitis and a favourable response to steroid therapy.[14] Recently, several AIH cases with abundant plasma cells with much IgG4-tissue positivity, prominent lobular hepatitis and interface hepatitis, and elevated level of IgG4 in serum, have been reported.[18] These patients showed a good response to steroid therapy. High serum IgE concentrations were also pointed out. They are proposed to be called IgG4-related AIH. However, neither irregular fibrosis nor obliterative phlebitis was observed. The differences between IgG4-associated AIH and combined hepatic injury in AIP/IgG4-SC[15] are considered to be: (a) patients with IgG4-associated AIH have a much higher degree of IgG4-bearing plasma cell infiltration in the liver compared to those with classical AIH; (b) rosette formation, interface hepatitis and other features characterizing AIH are obvious; and (c) no bile duct damage or loss is found. Such patients fulfilled the revised IAIHG disease score of AIH.[14] Based on these findings, we have provisionally set the diagnostic criteria for IgG4-associated AIH as follows: (i) having definite AIH according to the IAIHG scoring system, (ii) serum IgG4 concentration $\geq$135 mg/dl, and (iii) immunostaining of IgG4 showing infiltration of $\geq$10/HPF IgG4-bearing plasma cells in the portal tract.[14] According to our survey, two patients (3.3%) met these criteria.

It remains, however, uncertain whether IgG4-associated AIH is one manifestation of AIH, belongs to IgG4-related disease, or is a manifestation of IgG4-SC. Interestingly, one patient with IgG4-associated AIH developed IgG4-SC after 5 years of follow-up. Because a bile duct biopsy was not done prior to treatment, the possibility that she had IgG4-SC at that time cannot be excluded.

### Etiopathogenesis of IgG4-related hepatobiliary diseases

While the etiopathogenesis of hepatobiliary IgG4-related disease remains enigmatic, accumulating data suggest that both disordered immunological mechanism(s) and local anatomical and physiological factor(s) specific to the hepatobiliary system may contribute to the development of these diseases.

### Altered humoral immunity

In IgG4-related disease, levels of $\gamma$-globulin and IgG, particularly IgG4, are elevated, and nonspecific autoantibodies such as ANA and rheumatoid factor are not infrequently detected in serum, suggesting an abnormality of humoral immunity. IgG4 is reported to be non-cytotoxic and has rather self-protective antibodies, while this IgG subclass is known as a pathogenic autoantibody in the development of autoimmune skin-diseases such as endemic pemphigus foliaceus.[19] Patients with AIP reportedly exhibit high serum circulating immune complex values and decreased levels of various complements reflecting its active state. Reportedly, IgG4 subclass immune complexes lead to the formation of the membrane–attack complex in tissues by fixing the complement through the alternative pathway in membranous nephropathy. This hypothesis may be likely in IgG4-SC and also AIP because the deposition of IgG4 and complement was shown around the exocrine acini and small ducts of pancreas and also peribiliary glands of large bile ducts, suggesting the deposition of immune complexes to play a role in their pathogenesis.[20,21] A similar deposition is reported in IgG4-related interstitial nephritis.

### Predominance of Th2 reaction and disordered Treg

Recently, it was shown that IgG4-related disease is associated with over-production of helper type 2 (Th2) cytokines such as IL-4, IL-5

 

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and IL-13 and also regulatory cytokines such as IL-10, and TGF-β, suggesting that IgG4-related disease is characterized by Th2 predominance. Regulatory cytokines such as IL-10 and TGF-β are also up-regulated and the expression of Foxp3 messenger RNA, a transcription factor specific for naturally arising CD4+, CD25+ Tregs, is also significantly increased, suggesting that CD4+/CD25+ Tregs are disordered in IgG4-related disease. The presence of these cytokines can explain the histopathological features shared by IgG4-related diseases: FOXP3 regulates both IgG4 production, as a result of inducing IL-10, and the fibrosis, as a result of inducing TGF-β.[13]

### Allergy

In more than half of patients with IgG4-related disease, a history or synchronous affection of allergic disease is obtainable, and increased levels of serum IgE and eosinophilia are also pointed out,[5] suggesting that it seems likely that IgG4-related disease may have the background of an allergic disease. For example, IgG4-related hepatobiliary disease may be an allergic reaction to intrinsic or extrinsic substrates present in bile or pancreatic juice, rendering the infiltration of eosinophils and IgG4-positive plasma cells, and further studies are mandatory to clarify the participation of allergy in this disease.

### Local factors related to anatomy or physiology peculiar to individual organs involved

In AIP, the biliary tract including gallbladder is also frequently involved, and in almost all patients with hepatobiliary IgG4-related disease, AIP is present. However, the association of IgG4-sclerosing diseases of other organs is not so frequent in AIP or IgG4-related hepatobiliary diseases, suggesting that the hepatobiliary system and pancreas are closely correlated or simultaneously affected in this disease. Peribiliary glands, which are physiologically distributed around the extrahepatic and intrahepatic large bile ducts, are severely damaged in IgG4-SC[2] (Figure 3a–c). Interestingly, the pancreatic portion of the common bile duct and hilar and perihilar large bile ducts are preferentially affected in IgG4-SC. In these anatomical locations, peribiliary glands are densely and frequently distributed. Whereas the common hepatic duct and extrapancreatic portion of the common bile duct where peribiliary glands are scarce,[12] are not usually affected. Interestingly, small amounts of exocrine pancreatic acini are occasionally identifiable in these glands, and furthermore, the serous acini of the peribiliary glands contain enzymes of exocrine pancreas.[12] Taken together, it seems likely that exocrine acini of the pancreas in AIP and peribiliary glands in IgG4-SC are damaged, so these two diseases are so frequently associated in the same case, and that some antigen(s) or enzyme(s) located in pancreatic exocrine acini and peribiliary glands could be a target of immunological attack.

In patients with IgG4-related hepatobiliary disease, autoantibodies against carbonic anhydrase II, lactoferrin, amylase, and pancreatic secretory trypsin inhibitor which are located in the lining epithelial cells of the bile ducts and pancreatic ducts, are frequently detected.[5] Aoki et al. recently found that the normal epithelia of the pancreatic ducts and bile ducts reacted with IgG4 from patients' sera.[20] Such IgG4 antibodies showed decreased reactivity with these tissues after steroid therapy, suggesting that the reaction of IgG4 with the suspected antigen in these duct cells could be responsible for pancreatio-biliary lesions in IgG4-related disease. Recently, Detlefsen et al.[21] reported the deposition of IgG4 in peribiliary glands and pancreatic acini in IgG4-related cholangitis and AIP, supporting the above-mentioned suggestions.

### IgG4-tissue positivity in other hepatobiliary diseases

Lymphoplasmacytic infiltration by IgG4-positive plasma cells, a feature of IgG4-related disease, can be also seen in other diseases. For example, suppurative granulation tissue may contain numerous IgG4-positive plasma cells. Considerable IgG4-positive plasma cells can be also seen in pancreatic carcinomas, and some patients with pancreatic carcinoma have elevated serum IgG4 level.[22] The following hepatobiliary diseases show clinical features resembling hepatobiliary IgG4-related diseases and are also variably associated with lymphoplasmacytic infiltration and IgG4-tissue positivity, resembling IgG4-related hepatobiliary diseases. A cautious approach is thus necessary for pathologists to determine whether a condition is a IgG4-related disease, particularly in the case of small specimen.

### Primary sclerosing cholangitis (PSC)

PSC and IgG4-SC share many clinical and laboratory features, and cholangiographic studies of IgG4-SC show multiple strictures, and narrowing and obliteration of the biliary tree as seen in PSC. However, IgG4-SC responds well to steroid therapy, but PSC does not respond.

**Histological differences between PSC and IgG4-SC:** in PSC, inflammation is more pronounced on the luminal side, with erosion or ulceration of the duct lining, and cholangitis is also distributed diffusely, but unevenly, along the biliary tract. In IgG4-SC, the bile duct walls and peribiliary glands are severely affected, and erosion or ulceration of the duct epithelia or xanthogranulomatous lesions are rare.[1] PSC is a progressive and fatal disease leading to biliary cirrhosis and cholangiocarcinoma (CC) with biliary intraepithelial neoplastic lesions (BilINs) in advanced cases,[23] though such complications are rare in IgG4-SC.[24] While liver cirrhosis related to IgG4-SC is rare, this may be due to the fact that IgG4-SC is commonly resected or treated with steroids at an early stage on clinical suspicion of malignancy or IgG4-SC, thus precluding the progression of IgG4-SC.[24]

While increased serum IgG4 level and also IgG4-tissue positivity in IgG4-SC are very helpful for a differential diagnosis from PSC, they are not absolute indicators.

**PSC with IgG4-tissue positivity and/or increased serum IgG4 level:** about 5–23% of PSC show tissue IgG4-positivity and/or increased serum IgG4 level.[24] That is, according to a Mayo Clinic report, 9% of PSC patients have high serum IgG4 concentrations. In that report, the patients with elevated IgG4 level had higher total bilirubin concentration and PSC Mayo risk scores. Histologically, nearly one-quarter of explanted livers in cases of PSC also reportedly contained increased IgG4+ periductal plasma cell infiltrates. IgG4-tissue positivity in the liver strongly correlated with moderate-to-marked periductal lymphoplasmacytic inflammation, while none had storiform fibrosis or obliterative phlebitis. PSC with tissue IgG4-positivity has a more aggressive clinical course such as shorter time to transplant and higher risk of recurrence after transplant.




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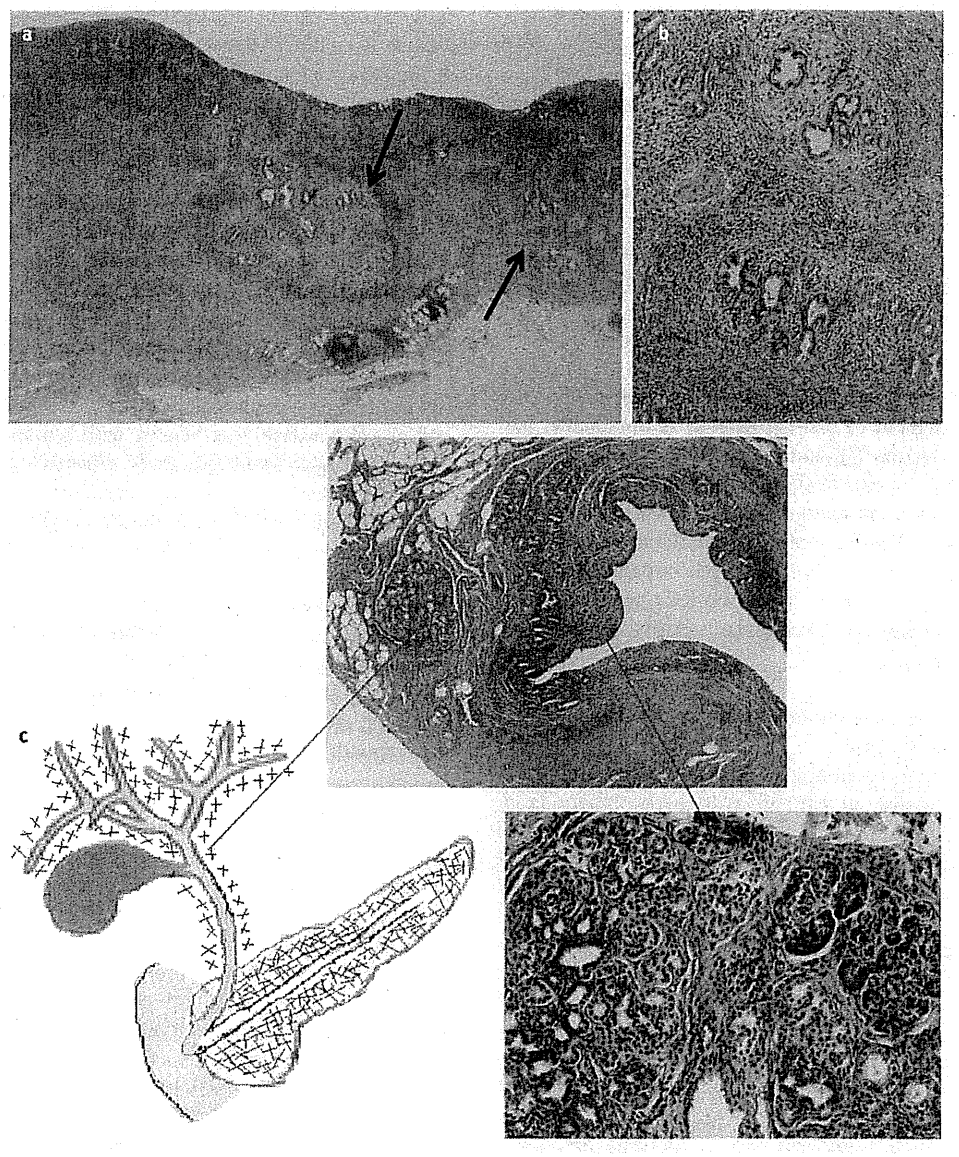


**Figure 3** (**a**) The extrahepatic bile duct shows chronic inflammatory cell infiltration and fibrosis. Inflammation is more accentuated and active around the peribiliary glands (arrows). IgG4-related sclerosing cholangitis. H&E. (**b**) Peribiliary glands show active inflammation with oedematous changes, lymphoplasmacytic infiltration and fibrosis. IgG4-related sclerosing cholangitis. H&E. (**c**) Around the extrahepatic bile duct of an apparently normal liver, there are peribiliary glands (upper part). Serous acini with a few amount of exocrine acini are found within these glands (right part). The schema shows distribution of peribiliary glands along the biliary tree (x).

Furthermore, some PSC cases show extensive infiltration of IgG4-positive plasma cells as seen in IgG4-SC. For example, Zen et al. reported that 2 (5%) of 41 cases transplanted for advanced PSC had a high number of IgG4-positive plasma cells within the large bile duct lesion, whereas those cells were scarce in the remaining cases.[24]

IgG4-positive plasma cells accumulated mainly in (xantho) granulomatous tissue within large bile ducts. Except for the presence of IgG4-positive plasma cells, there was no significant histological or clinical difference between IgG4-positive and negative PSC cases.

While a similar lymphoplasmacytic cholecystitis as seen in IgG4-SC has been reported in other cases of cholecystitis such as choledocholithiasis and PSC, the demonstration of increased IgG4-positive plasma cells by use of an immunohistochemical stain is a helpful approach for differential diagnosis.

### Cholangiocarcinoma and other biliary malignancies

As IgG4-SC or IgG4-related HIP usually presents as a mass or regional lesion with fibrosis and cholangiographic features similar to cholangiocarcinoma (CC) and IgG4-SC may mimic hilar CC more often than PSC, they should be carefully differentiated from hepatobiliary malignancies. In addition, elevated serum level of IgG4 is found in about 20% of CC patients, which makes the differentiation more complicated and difficult, clinically. In fact, until recently, many cases of IgG4-SC or IgG4-



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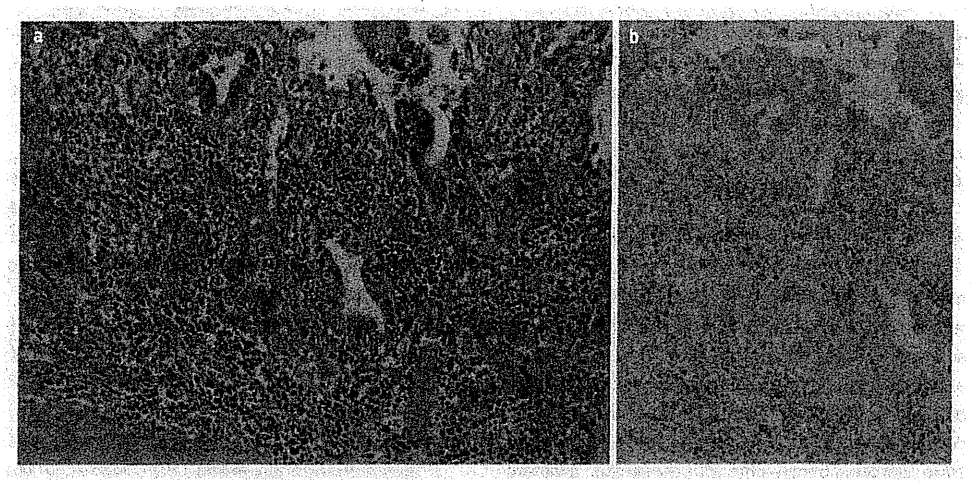

**Figure 4** (**a**) Cholangiocarcinoma of the extrahepatic bile duct. There are many lymphoplasmacytic infiltration in the stroma. H&E. (**b**) There are many IgG4-positive plasma cells in chronic inflammatory cell infiltration of the cholangiocarcinoma of the extrahepatic bile duct. Immunostaining for IgG4 and haematoxylin.

related HIP were misdiagnosed as CC, and these patients underwent unnecessary surgical resection.

To pathologists, the differentiation of CC from IgG4-related HIP or SC is not an easy task in smaller specimens, because CC itself and its surrounding biliary mucosa is not infrequently associated with lymphoplasmacytic infiltration, and there are also foci of IgG4-positive plasma cell infiltration in CC and surrounding tissue including the biliary mucosa (Figure 4a,b). According to our study, about 30% of CC showed variable infiltration of IgG4-positive plasma cells in the cancerous mucosa including BilIN lesions and more frequently in the invasive areas, when examined in surgically resected specimens (Kimura et al., in submission). However, storiform fibrosis or occlusive phlebitis was not evident, suggesting that the mechanism of IgG4-positive plasma cell infiltration in CC is different from that in IgG4-related diseases. Tregs are known to be increased in number in carcinomas and precancerous lesions and also in peripheral blood, and are involved in immune escape from the host. It seems conceivable that IgG4-positive cells may infiltrate and proliferate in response to chemokines released from Tregs. A similar phenomenon was also already pointed out in pancreatic carcinoma.[25]

The identification of evident carcinomas in specimens excludes a diagnosis of IgG4-SC. However, it is difficult to differentiate IgG4-poistive plasma cells in the surrounding mucosa of a carcinoma from IgG4-SC, and recently, biliary carcinomas and BilIN lesions arising in IgG4-SC have been occasionally reported. These cases may be a burden for pathologists. Of course, an exact diagnosis may help prevent unnecessary surgery for IgG4-related disease.

### IgG4-tissue positivity in autoimmune hepatitis

AIH and AIP share several clinical and pathological features such as high serum levels of immunoglobulin G and non-organ-specific autoantibodies and lymphoplasmacytic infiltration. So far, little is known about the possible involvement of IgG4, a hallmark of AIP, in ordinary AIH. Recently, Chung et al. recently reported that 9 of 26 patients with AIH showed more than five IgG4-positive plasma cells (/HPF) in the liver. Interestingly, the patients with PBC and with HCV hepatitis showed no or less than four IgG4-positive plasma cells in the liver. These patients with IgG4-positive AIH also showed increased serum levels of IgG, while serum IgG4 level was not elevated in these patients. This type of AIH showed very good response to steroid therapy in comparison with IgG4-negative AIH. While more studies are mandatory for this type of AIH, this AIH seems to be different from IgG4-related AIH reported by Umehara et al.[18]

### Localized fibro-inflammatory lesions including hepatic inflammatory pseudotumors

There have been no systematic studies on the prevalence of IgG4-positive plasma cells and their significance in miscellaneous localized fibro-inflammatory lesions. In our experience, there is occasional clustering of IgG4-positive plasma cells in miscellaneous hepatobiliary diseases such as *hepatolithiasis*, bacterial cholangitis and biliary diseases with granulomatous changes, though their significance remains unclear. HIP, particularly fibrohistiocytic HIP, is characterized by xanthogranulomatous inflammation, multinucleated giant cells, and neutrophilic infiltration. This type seems to be heterogeneous in its pathogenesis, and some cases showed variable amounts of IgG4-positive plasma cells.

In pathologic consideration whether these condition(s) with variable IgG4-positive plasma cells belong to IgG4-related disease with histopathological modification or novel type of IgG4-related disease, demonstration of increased serum level of IgG4 (more than 135 mg/dl) and association of well-known IgG4-related diseases in addition to considerable IgG4-positive plasma cells and obliterative phlebitis in the affected tissue(s) are necessary.

In conclusion, IgG4-related hepatobiliary diseases, which are now regarded as a part of a family of IgG4-related disease, were discussed with reference to their pathology, etiopathogenesis and differential diagnosis. This field is expanding rapidly and new information and new comer will be discovered regarding the hepatobiliary system. ◆




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## Research directions

- New member of IgG4-related disease is till being discovered every year.
- Coexistence of other member of IgG4-related disease may be mandatory for identification of new comer of this disease.
- Considerable infiltration of IgG4-positive plasma cells and elevation of IgG4 serum level should be shown.

## Practice points

These three findings are necessary to make a diagnosis of IgG4-related sclerosing cholangitis:

- Considerable lymphoplasmacytic infiltration with many IgG4-positive plasma cells.
- Storiform fibrosis or wavy fibrosis and obliterative phlebitis.
- Association of autoimmune pancreatitis.




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# Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to hepatic fibrogenesis

Kenichi Harada,[1] Mayumi Chiba,[1] Atsushi Okamura,[1] Maylee Hsu,[1] Yasunori Sato,[1] Saya Igarashi,[1] Xiang Shan Ren,[1] Hiroko Ikeda,[2] Hajime Ohta,[3] Satomi Kasashima,[4] Atsuhiro Kawashima,[4] Yasuni Nakanuma[1]

[1]Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan
[2]Division of Pathology, Kanazawa University Hospital, Kanazawa, Japan
[3]Department of Gastroenterology, National Hospital Organization, Kanazawa Medical Center, Kanazawa, Japan
[4]Department of Pathology, National Hospital Organization, Kanazawa Medical Center, Kanazawa, Japan

**Correspondence to**
Dr Kenichi Harada, Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan;
kenichih@med.kanazawa-u.ac.jp

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## ABSTRACT

**Aims** Monocyte chemoattractant protein-1 (MCP-1) is a major chemotactic factor for hepatic stellate cells (HSCs) associated with hepatic fibrosis. In this study, among several fibrogenetic factors derived from biliary epithelial cells (BECs), MCP-1 produced by the biliary innate immune system was found to be most critical in the histogenesis of hepatic fibrogenesis.

**Methods** Using cultured human BECs, the expression of five fibrogenetic factors including MCP-1 on stimulation with Toll-like receptor ligands, inflammatory cytokines or bile acids was examined. Moreover, in situ detection of MCP-1 and α-smooth muscle actin proteins was performed using sections from normal and diseased livers by immunohistochemistry.

**Results** All fibrogenetic factors were detected in BECs, but only MCP-1 expression was upregulated, by all the Toll-like receptor ligands, IL-1β, and tumour necrosis factor-alpha. Proliferating bile ductules in interface areas expressed MCP-1 in diseased livers accompanying α-smooth muscle actin-positive activated HSCs.

**Conclusions** Bile ductules proliferate in various hepatobiliary diseases, and its significance is still unknown. This study demonstrated that BECs in bile ductules could produce MCP-1, particularly, via biliary innate immunity, suggesting that MCP-1 derived from BECs plays an important role in the recruitment of HSCs to interface areas and the activation of HSCs resulting in the progression of periportal fibrosis.

## INTRODUCTION

Hepatic fibrosis is a major feature of advanced liver diseases and defines the prognosis. Hepatic fibrosis spreads within portal tracts and also periportal areas in patients with hepatitic and cholestatic liver diseases. Although periportal fibrosis is thought to be associated with the accumulation and activation of hepatic stellate cells (HSCs), its mechanisms are not fully understood. HSCs undergo differentiation towards an activated phenotype, and this process is enhanced by soluble mediators such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which mediate the increase in cell proliferation and extracellular matrix production.[1 2] Then, HSCs migrate into damaged areas in response to a chemokine, secreting monocyte chemotactic protein-1 (MCP-1/CCL2) that recruits monocytes and lymphocytes.[3] Moreover, MCP-1 triggers the migration of activated, but not quiescent, HSCs in a dose-dependent manner. Experiments in vitro using HSCs isolated from normal human livers revealed that MCP-1-dependent signals were not transduced by the chemokine receptor of MCP-1, CCR2, and may be mediated by alternative chemokine receptors.[3] Recently, Ramm *et al*[4] reported that hepatocyte-derived MCP-1 induced by a hydrophobic bile acid, taurocholate, could result in the recruitment of HSCs in cholestatic liver injury as the major fibrogenesis in a paediatric cholestatic liver disease such as biliary atresia. In contrast, biliary epithelial cells (BECs) may promote fibrogenesis by a number of mechanisms including the synthesis of matrix constituents and the release of mediators such as MCP-1, PDGF-BB, TGF-β, connective tissue growth factor (CTGF) and endothelin-1.[5] The role of BECs in the pathogenesis of hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis in various hepatobiliary diseases including chronic viral hepatitis (CVH) and primary biliary cirrhosis (PBC), is speculated to be important to the disease's progression, but its precise mechanism is still unknown.

Our previous study demonstrated that human BECs possess several inflammatory cytokine receptors and Toll-like receptors (TLRs) and produced cytokines and chemokines in response to inflammatory cytokines and pathogen-associated molecular patterns (PAMPs), respectively.[6–9] In particular, bile ductules located between interlobular bile ducts in portal tracts and bile canaliculi in hepatocytes are frequently increased in number under a variety of pathological conditions of the liver and take part in various immunological responses and the pathogenesis of biliary diseases.[10–12] This ductular reaction is speculated to be closely associated with hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis, but its precise mechanism is still unknown. In this study, we examined the possibility that BECs produce MCP-1 and play a role in fibrogenesis in periportal areas via MCP-1 production.

## MATERIALS AND METHODS

### Cultured human BECs

Two human intrahepatic BEC lines were established from explanted livers with PBC as described previously.[7] These cell lines had been confirmed to be BECs by the expression of biliary-type cytokeratins.

### Stimulation

Cultured BECs were stimulated with inflammatory cytokines, PAMPs and bile acids (table 1). As an NF-κB inhibitor, isohelenin (30 μM, Calbiochem, Darmstadt, Germany) was added to the culture medium before the stimulation. We previously confirmed that BECs possess the receptors for all these cytokines and PAMPs.[6] Cell samples for the examination of mRNA as shown below were prepared 2 h after the stimulation.

### RT-PCR and real-time PCR

For the evaluation of mRNAs of MCP-1, PDGF-B, CTGF, TGF-β1, endothelin-1 and glyceraldehyde 3-phosphate dehydrogenase (internal control) in cultured BECs, total RNA was isolated from BECs, and RT-PCR and real-time quantitative PCR were performed according to a standard protocol using specific primers (table 2).

### ELISA

Cultured BECs were stimulated with lipopolysaccharide, Pam3CSK4, poly(I:C) and IL-1β for 24 h, and supernatants were tested for human MCP-1 by ELISA (Biosource International, Camarillo, California).

### Liver tissues

A total of 26 surgical or wedge liver biopsy specimens were obtained from patients with CVH (n=8, hepatitis C virus-related, male/female=5/3, average age 58-year-old., F1/F2=4/4), PBC (n=5, all female, average age 68-year-old, Nakanuma's classification[13] Stage 2/3=4/1, Scheuer's classification Stage 1/2=2/3) and congenital hepatic fibrosis (CHF, n=5, used as a case of activated HSC-poor case[14], male/female 4/1, average age 23-year-old), and six cases with no significant histopathological change ('normal liver').

### Immunohistochemistry

Deparaffinised sections were pretreated in Target Retrieval Solution (Dako, Tokyo, Japan) and incubated with a primary antibody against MCP-1 (10 μg/ml; Abcam, Tokyo, Japan) or α-smooth muscle actin (αSMA, activated HSC marker) (1 μg/ml, Dako), and then Envision-HRP (Dako) was used. No positive staining was obtained when the primary antibody was replaced with an isotype-matched, non-immunised immunoglobulin.

The simultaneous detection of combined CCR2 and αSMA, and CCR2 and CD68, was evaluated by double fluorescence immunohistochemical staining. After incubation with the antibodies for these combinations, rabbit antibody (CCR2, 1:400, Epitomics, Burlingame, California) and mouse antibodies (αSMA and CD68, Dako) were visualised by Alexa Fluor 488 and 594 (Molecular Probe, Eugene, Oregon), respectively.

**Table 1** Stimulants

| Stimulants | Concentration | Supplier |
|---|---|---|
| Cytokines | | |
| Interleukin-1β | 1000 U/ml | PerpoTech |
| Interleukin-6 | 1000 U/ml | PerpoTech |
| Interferon-γ | 1000 U/ml | PerpoTech |
| Tumour necrosis factor-α | 1000 U/ml | PerpoTech |
| Pathogen-associated molecular patterns | | |
| Lipopolysaccharide (TLR4 ligand) | 1 μg/ml | Invitrogen |
| Pam3CSK4 (TLR1/2 ligand) | 100 ng/ml | Invitrogen |
| Poly(I:C) (TLR3 ligand) | 25 μg/ml | Invitrogen |
| Bile acid | | |
| Taurochenodeoxycholic acid | 200 μM | Calbiochem |
| Taurocholic acid | 200 μM | Calbiochem |
| Taurodeoxycholic acid | 200 μM | Calbiochem |
| Tauroursodeoxycholic acid | 200 μM | Calbiochem |

Invitrogen, San Diego, California; PerpoTech, London.
TLR, Toll-like receptor.

**Table 2** Primers used for RT-PCR and quantitative PCR

| Transcript | Primers | Product size (bp) |
|---|---|---|
| Monocyte chemoattractant protein-1 | Forward 5′-CTGAATTTTGTTTGTTGATGTGAAA-3′<br>Reverse 5′-GCAATTTCCCCAAGTCTCTG-3′ | 128 |
| Platelet-derived growth factor-B | Forward 5′-GAGGACCTCTCAGCATAGCC-3′<br>Reverse 5′-GGGGTTTTCTCCAGTCTGTG-3′ | 135 |
| Connective tissue rowth factor | Forward 5′-GCAGGCTAGAGAAGCAGAGC-3′<br>Reverse 5′-ATGTCTTCATGCTGGTGCAG-3′ | 153 |
| Transforming growth factor-β1 | Forward 5′-CAGAAATACAGCAACAATTCCTGG-3′<br>Reverse 5′-TTGCAGTGTGTTATCCCTGCTGTC-3′ | 186 |
| Endothelin-1 | Forward 5′-CCATGAGAAACAGCGTCAAA-3′<br>Reverse 5′-AGTCAGGAACCAGCAGAGGA-3′ | 213 |
| Glyceraldehyde 3-phosphate dehydrogenase | Forward 5′-GCACCGTCAAGGCTGAGAAC-3′<br>Reverse 5′-ATGGTGGTGAAGACGCCAGT-3′ | 142 |

## RESULTS

### Detection of MCP-1 in cultured human BECs

RT-PCR revealed that all fibrogenic cytokines were detected in human BECs in the basal cultures (figure 1). Quantitative PCR revealed that only MCP-1 was upregulated following stimulation with all PAMPs and two cytokines (IL-1β and tumour necrosis factor-alpha (TNF-α); figure 2). Moreover, ELISA demonstrated that the concentration of MCP-1 protein in the supernatants was increased by these stimulants (figure 3). None of the bile acids upregulated the expression of MCP-1 (figure 2). The Pam3CSK4-induced upregulation of MCP-1 mRNA was significantly inhibited by pretreatment with the NF-κB inhibitor (figure 2).

### In situ detection of MCP-1 and activated HSCs

MCP-1 was mainly expressed in bile ducts, and the majority of proliferating bile ductules predominantly expressed MCP-1 in periportal and interface areas in cases of CVH and PBC (figure 4). Moreover, αSMA-positive cells (activated HSCs) were scattered around these bile ductules (figure 4). These characteristic findings were made in CVH and PBC accompanying interface hepatitis and ductular reaction, but rare or absent in normal livers. Periportal hepatocytes were also frequently positive for

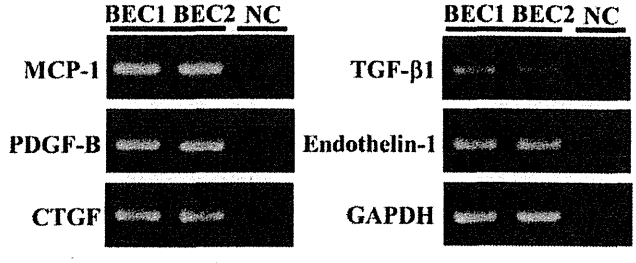


**Figure 1** Detection of fibrogenic cytokines in cultured human BECs. The two human biliary epithelial cell lines (BEC1 and BEC2) express all mRNAs of fibrogenic cytokines (monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor (PDGF)-B, connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β)1, and endothelin-1 (GAPDH)) and the internal control (glyceraldehyde 3-phosphate dehydrogenase) under basal conditions. Each PCR product showed the predicted size as a single band. Negative controls (NC) were obtained by replacing reverse transcriptase with RNase- and DNase-free water.