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Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis

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Recent studies disclosed that autophagy is induced during and facilitates the process of senescence. Given that biliary epithelial cells (BECs) in damaged small bile ducts in primary biliary cirrhosis (PBC) show senescent features, we examined an involvement of autophagy in the process of biliary epithelial senescence in PBC. We examined immunohistochemically the expression of microtubule-associated proteins-light chain 3β (LC3), a marker of autophagy, in livers taken from the patients with PBC ($n=37$) and control livers ($n=75$). We also examined the co-localization of LC3 with autophagy-related cathepsin D, lysosome-associated membrane protein-1 (LAMP-1), and senescent markers, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. We examined the effect of autophagy inhibitor (3-methyladenine) on the induction of cellular senescence and senescence-associated secretion (CCL2 and CX3CL1) in cultured murine BECs. The expression of LC3 was specifically seen in vesicles in BECs in the inflamed and damaged small bile ducts in PBC, when compared with non-inflamed small bile ducts in PBC and in control livers ($P<0.01$). The expression of LC3 was closely related to the expression of cathepsin D, LAMP-1, and senescent markers. In cultured BECs, oxidative stress, DNA damage, and serum deprivation induced cellular senescence, when compared with control and the inhibition of autophagy significantly decreased the stress-induced cellular senescence ($P<0.01$). Furthermore, the secretion level of CCL2 and CX3CL1 increased significantly by various stress and suppressed by the inhibition of autophagy ($P<0.01$). In conclusion, autophagy is specifically seen in the damaged small bile ducts along with cellular senescence in PBC. The inhibition of autophagy suppressed cellular senescence in cultured cells. These findings suggest that autophagy may mediate the process of biliary epithelial senescence and involve in the pathogenesis of bile duct lesions in PBC.
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Autophagy and cellular senescence are two distinct cellular responses to stress. 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).[1] Autophagy 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.[2,3] Autophagy is often associated with acute metabolic changes and rapid protein replacement. Microtubule-associated proteins-light chain 3β (LC3), a homolog of autophagy-related protein 8, which is essential for autophagy and associated to the autophagosome membranes after processing, is a widely used marker of autophagy.[4,5] 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.[1,6]

Cellular senescence is a state of stable cell arrest with active metabolism. Similar to apoptosis, senescence can be a failsafe program against a variety of cellular insults. In contrast to apoptosis, however, in which the cytotoxic signals converge to a common mechanism, senescence is typically a delayed stress response involving multiple effector mechanisms. These effector mechanisms include epigenetic regulation,[7]

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the DNA damage response,[8] and the senescence-associated secretory phenotypes (SASP).[9–11] Although autophagy can delay apoptosis, a function for autophagy in senescence was not earlier known.[1] Recent studies disclosed that autophagy is induced during and facilitates the process of senescence.[4]

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease and presents with chronic, progressive cholestasis, and liver failure.[12–14] PBC is characterized histologically as a cholangitis of small bile ducts (chronic non-suppurative destructive cholangitis, CNSDC) eventually followed by the extensive loss of small bile ducts.[13–15] Although there have been many studies on the immunopathological features,[16–18] there have been few studies on the pathogenesis of bile duct loss in PBC. We have reported cellular senescence of biliary epithelial cells (BECs) with the augmented expression of senescence-associated β-galactosidase (SA-β-gal),[19] $p16^{INK4a}$, and $p21^{WAF1/Cip1}$, and telomere shortening in damaged small bile ducts in PBC. We have suggested that the cellular senescence may be involved in the pathogenesis of progressive bile duct loss in PBC.[20–22] A possible association of oxidative stress and decreased expression of Bmi1 polycomb ring finger oncogene (Bmi1) was suggested to be involved in the pathogenesis of cellular senescence in PBC.[20–23]

Although it is well known that autophagy has important functions in clearing misfolded proteins in hepatocytes in liver diseases such as α-1 antitrypsin deficiency and hypofibrinogenemia,[24] there are few studies regarding autophagy in BECs and its relation between liver diseases to our knowledge. Given that BECs in damaged small bile ducts in PBC show senescent features, we examined an involvement of autophagy in the process of biliary epithelial senescence in PBC. Furthermore, we examined whether the inhibition of autophagy influences the induction of senescence phenotypes in cultured mouse BECs. This study suggests the involvement of autophagy in the induction of cellular senescence in BECs and may contribute to the pathogenesis of PBC.

MATERIALS AND METHODS

Human Study

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.[25] In this study, septal and interlobular bile ducts are termed as small bile ducts. Bile ductules, which are characterized by tubular or glandular structures with poorly defined lumen and located at the periphery of the portal tracts,[25,26] are not included in the small bile ducts.

Small bile ducts were histologically divided largely into 'inflamed' and non-inflamed. Inflamed bile ducts include the bile ducts showing biliary epithelial damages and being surrounded and occasionally infiltrated by inflammatory cells such as CNSDC in PBC[14] and also the bile ducts embedded in infiltrating lymphoid cells and showing mild epithelial damages in chronic viral hepatitis (CVH) (hepatitis duct lesion).

Liver tissue preparation

A total of 112 liver tissue specimens (all were biopsied or surgically resected) were collected from the liver disease file of our laboratory and affiliated hospitals. The Ethics Committee in Kanazawa University approved this study. The liver specimens enrolled in this study were 37 PBC, 22 CVH, 22 non-alcoholic steatohepatiits, 10 livers with extrahepatic biliary obstruction (EBO), and 21 'histologically normal' livers. All PBC were from the patients fulfilling the clinical, serological, and histological characteristics consistent with the diagnosis of PBC.[14] PBC livers were staged histologically,[27] and 26 and 10 of PBC were of stages 1, 2 (early PBC) and of stages 3, 4 (advanced PBC), respectively. Eleven CVH were regarded as F0-2 and 11 as F3, 4, respectively.[28] Two and 20 of CVH cases were serologically positive for hepatitis B surface B antigen and anti-hepatitis C viral antibody, respectively. Causes of EBO were obstruction of the bile duct at the hepatic hilum or the extrahepatic bile ducts, because of carcinoma or stone, and the duration of jaundice was <1 month. 'Histologically normal' livers were obtained from surgically resected livers for traumatic hepatic rupture or metastatic liver tumor. 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.

Immunohistochemistry

We examined immunohistochemically the expression of LC3, a widely used autophagy marker. In addition, we examined immunohistochemically the expression of autophagy-related lysosomal protein, cathepsin D, lysosome-associated membrane protein-1 (LAMP-1), and senescence-associated markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$). Immunostaining was performed using the antibodies shown in Table 1, as described earlier.[21] 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° overnight. The Envision+ solution for mouse (Dako) or Histofine Simple Stain MAX-PO (G) (Nichirei, Tokyo, Japan) was then applied for 30 min at room temperature. The reaction products were visualized using 3–3′-diaminobenizidine tetra-hydrochloride (Sigma Chemica, St Louis, MO, USA) and H_2O_2. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of the control mouse or goat immunoglobulin G (Dako) was applied instead of the primary antibody as negative control. Positive and negative controls were routinely

Table 1 Primary antibodies used in this study

Primary antibody	Type (clone)	Pretreatment	Dilution	Source
LC3	Goat poly	MW-CB (95°C, 20 min)	1:50	Santa-Cruz, Santa-Cruz, CA, USA
Cathepsin D	Rabbit poly	MW-CB (95°C, 20 min)	1:200	Santa-Cruz, Santa-Cruz, CA, USA
LAMP-1	Mouse mono (E-5)	MW-CB (95°C, 20 min)	1:200	Santa-Cruz, Santa-Cruz, CA, USA
$p16^{INK4a}$	Mouse mono (JC8)	eARI–BA (121°C, 5 min)	1:100	Neomarkers, Freemont, CA, USA
$p21^{WAF1/Cip1}$	Mouse mono (70)	eARI–BA (121°C, 5 min)	1:100	BD Transduction, San Jose, CA, USA

LC3, microtubule-associated proteins-light chain 3β; 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).

included. When more than three cells showed positive signal, in a small bile duct, the bile duct was regarded as positive.

Double immunostaining

We also performed double immunostaining for LC3 with senescence markers, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. In brief, LC3 was detected as described above, followed by second staining for either of the senescence markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) using Vector Blue Alkaline Phosphatase Substrate kit (Vector Laboratories).

Culture Study

Cell culture and treatments of mouse intrahepatic BECs

Mouse intrahepatic BECs were isolated from 8-week-old female BALB/c mice and were purified and cultured as described earlier.[29] The maintenance and passage of cultured BECs was made as described earlier.[29] The cell density of the cells was $<80\%$ during experiments. In several experiments, BECs were treated with hydrogen peroxide (PO, 100 μM for 2 h), washed throughout to remove PO, and cultured in fresh medium. DNA damage was induced by a treatment with Etoposide (100 μM, Sigma Chemica). In several experiments, 3-methyladenine (3MA, 5 mM, Sigma Chemica), an inhibitor of the class III phosphatidylinositol 3-kinase (class III PI3K) complex involved in initial autophagosome formation, was used to inhibit autophagy.[30]

Immunofluorescence staining for cultured cells

The BECs growing in a Lab-Tek chamber were fixed with 5% buffered formalin and cold methanol for 20 min each. After blocking with 3% BSA, the cells were incubated with either of the primary antibodies for LC3 (described above) overnight. The cells were then incubated with Alexa-546-labeled rabbit anti-goat IgG (Molecular probes, Eugene, OR, USA) for 30 min, counterstained with 10 μg/ml 4′ and 6-diamidino-2-phenylindole, and evaluated under a conventional fluorescence microscope (Olympus, Tokyo, Japan).

Assay for cellular senescence

SA-β-gal activity was detected by using the senescence detection kit (BioVision, Mountain View, CA, USA) according to manufacturer's protocol.[19] The proportion of senescent cells in each condition was assessed at Day 4 by counting the percentage of SA-β-gal-positive cells in at least 1×10^3 total cells using light microscopy.

Enzyme-linked immunosorbent assay of CCL2 and CX3CL1

Recent studies suggest that senescent cells modulate microenvironment by expressing SASP, including inflammatory cytokines and chemokines, such as CXCL8/interleukin (IL)-8, CCL2/monocyte chemotactic protein-1 (MCP)-1, and etc.[9–11] We also found that CX3CL1/fractalkine is one of SASP (paper in submission). Therefore, we examined whether the inhibition of autophagy influence the induction of CCL2/MCP-1 and CX3CL1 as senescence phenotypes; SASP. After 1×10^4 BECs were seeded on 12-well plates for 24 h, they were treated with PO, Etoposide, or serum deprivation with or without 3MA for 4 days. The concentration of CCL2/MCP-1 and that of CX3CL1 in culture media were measured by enzyme-linked immunosorbent assay (ELISA) using an Immunoassay kits (R and D Systems), according to the manufacturer's instructions.

Immunoblotting

The cell lysate samples (15 μl) were resolved by SDS–PAGE and transferred to a nitrocellulose membrane as described earlier.[31] After transfer, the membranes were processed for immunoblotting as described earlier.[31] The primary antibody for LC3 was described above. The α-tubulin was detected by using mouse monoclonal anti-α-tubulin (clone TU-01, Zymed, South San Francisco, CA, USA).

Statistical analysis

Statistical analysis for the difference used the Wilcoxon rank sum test. When the P-value was <0.05, the difference was regarded as significant.

RESULTS

Human Study

Increased expression of autophagy marker LC3 in the damaged BECs in PBC

The expression of LC3 was specifically seen in cytoplasmic vesicles in BECs in the inflamed and damaged small bile ducts in PBC, whereas the expression of LC3 was absent or faint in normal livers (Figure 1a). The expression of LC3 was significantly more frequent and intense in the inflamed small bile ducts in PBC, when compared with non-inflamed small bile ducts in PBC and small bile ducts in control livers ($P<0.01$) (Table 2). The expression of cathepsin D and LAMP-1, autophagy-related lysosomal proteins, was also seen in cytoplasmic vesicles in the inflamed and damaged bile ducts in PBC (Figure 1b). Double immunostaining revealed that the expression of LC3 was closely co-localized with the expression of senescent markers $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in BECs in the damaged bile ducts in PBC (Figure 2). The co-expression of LC3 and $p21^{WAF1/Cip1}$ was more frequently observed when compared with the co-expression of LC3 and $p16^{INK4a}$ in the damaged bile ducts in PBC.

The vesicular expression of LC3 was observed in ductular reactions in PBC, to various degrees, whereas it was rarely seen in ductular reactions in control livers. We have reported that a part of ductular cells in ductular reaction shows cellular senescence as well as the damaged small bile ducts in PBC.[22] Taken together, it is plausible that the vesicular expression of LC3 in ductular reaction may be associated with higher rate of senescent ductular cells in PBC. Further studies are needed to clarify the relation between the cell kinetics of 'bipotential stem/progenitor cells' and autophagy and/or cellular senescence in ductular reaction. The vesicular expression of LC3 was not observed in large bile ducts in PBC and control livers examined.

Culture Study

Induction of autophagy by several factors inducing cellular senescence

We determined whether autophagy is induced by several factors that are known to induce cellular senescence in BECs.[20,21] LC3 is a widely used marker of autophagy and the lipid-conjugated form of LC3 (LC3-II) localized to the membrane of autophagosomes. It can be separated from the non-conjugated form (LC3-I) by immunoblotting. Immunoblotting showed that LC3-II was upregulated by the treatment with PO (100 μM), Etoposide (100 μM), and serum deprivation (Figure 3a). Immunofluorescence showed that vesicular cytoplasmic expression of LC3 was upregulated by serum deprivation, whereas the expression of LC3 was faint in control (no treatment) (Figure 3b). The upregulated expression of LC3 by serum deprivation was inhibited by a treatment with the autophagy inhibitor, 3MA (5 mM) (Figure 3b).

Table 2 Frequency of the prevalence of vesicular expression of LC3 in small bile ducts in primary biliary cirrhosis and control livers

Diseases	Number of patients	Type of BD	Number of BD	Number of BD with vesicular LC3 expression (%)
PBC	37	Inflamed	126	114 (90.5%)*
		Non-inflamed	109	30 (27.5%)**
CVH	22	Inflamed	98	6 (6.1%)**
		Non-inflamed	134	0
NASH	22	Inflamed	15	2 (13.3%)**
		Non-inflamed	147	1 (0.7%)
EBO	10	Inflamed	15	1 (6.7%)**
		Non-inflamed	79	0
Normal liver	21	Non-inflamed	188	0

LC3, microtubule-associated proteins-light chain 3β; PBC, primary biliary cirrhosis; CVH, chronic viral hepatitis; NOSH, non-alcoholic steatohepatitis; EBO, extrahepatic biliary obstruction; BD, bile duct.
*$P<0.01$ versus PBC, non-inflamed and other groups; **$P<0.01$ versus normal livers.

Inhibition of autophagy significantly decreased cellular senescence

Percentage of cells positive for SA-β-gal was significantly higher in cultured BECs treated with H_2O_2 (SA-β-gal-labeling index, 37.0 ± 13.1), Etoposide (34.0 ± 5.7), and serum deprivation (34.2 ± 11.7), when compared with control without treatment (3.9 ± 1.4) ($P<0.01$) (Figure 4a). Percentage of cells positive for SA-β-gal was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (PO + 3MA, 5.1 ± 4.5; Etop + 3MA, 8.6 ± 2.9; Dep + 3MA, 6.9 ± 4.8) ($P<0.01$) (Figure 4a). These data suggest that autophagy may be involved in the process of cellular senescence induced by oxidative stress, DNA damage, and serum deprivation.

Inhibition of autophagy significantly decreased SASPs

ELISA showed that the secretion of CCL2, one of SASPs, was increased in BECs treated with PO (2.74 ± 0.1 pg/ml), Etoposide (2.01 ± 0.25 pg/ml), and serum deprivation (35.17 ± 1.98 pg/ml), when compared with control (1.3 ± 0.1 pg/ml) ($P<0.01$) (Figure 4b). The enhanced secretion of CCL2 was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (PO + 3MA, 0.0 ± 0.0; Etop + 3MA, 0.0 ± 0.0; Dep + 3MA, 6.64 ± 1.32) ($P<0.01$) (Figure 4b). The secretion of CX3CL1 was also increased in BECs treated with serum deprivation (553.9 ± 43.8 pg/ml), when compared with control (96.1 ± 16.5 pg/ml) ($P<0.01$). The enhanced secretion of CX3CL1 was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (Dep + 3MA, 258.3 ± 9.20; Cont + 3MA, 0.1 ± 0.1) ($P<0.01$) (Figure 4b). The autophagy inhibitor 3MA indeed acts through inhibition of

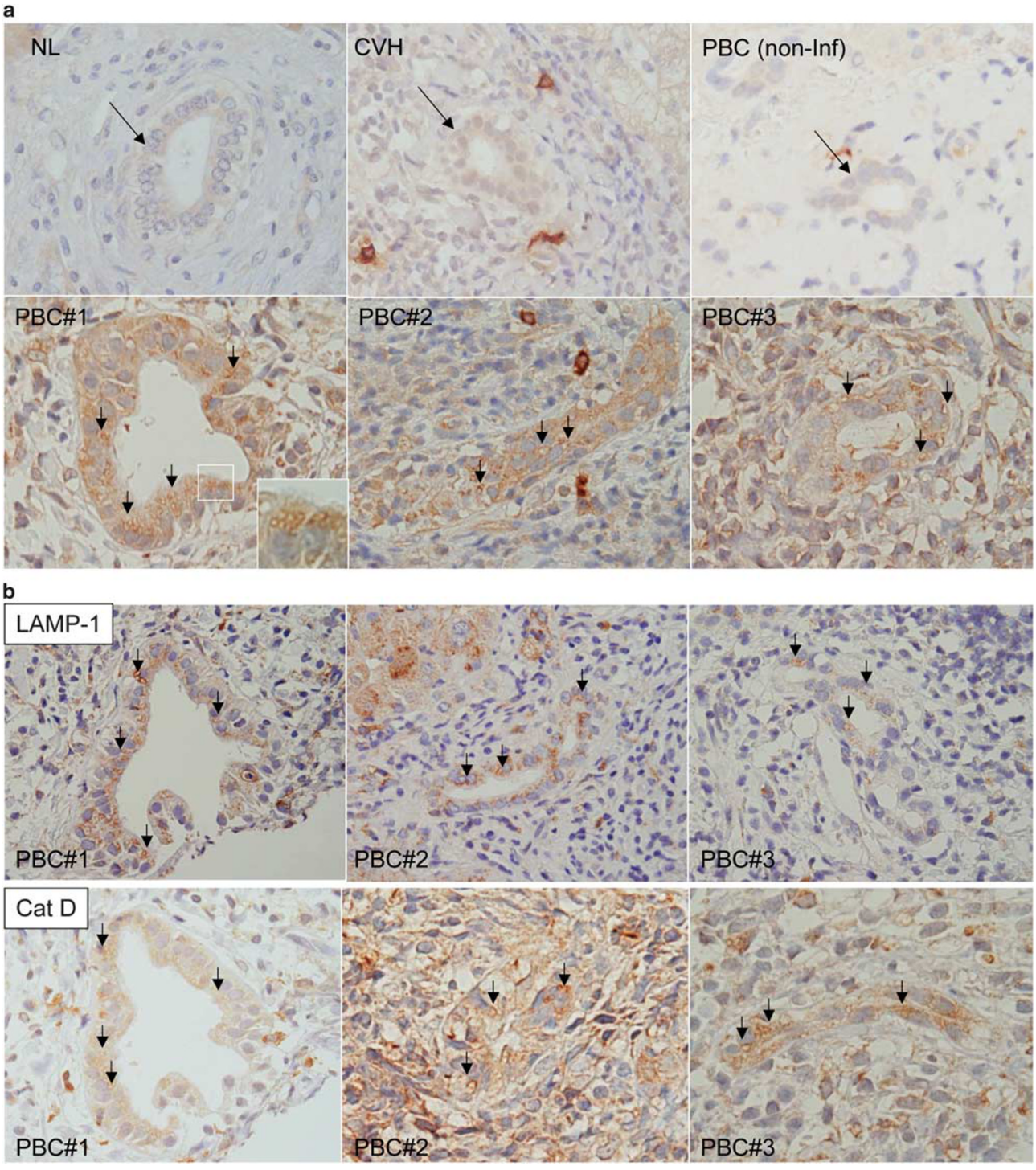

Figure 1 Increased vesicular expression of autophagy marker LC3 in the damaged small bile ducts in PBC. (**a**) The expression of LC3 was absent in BECs in small bile duct (arrow) in normal liver (NL, top left), CVH (top middle) and non-inflamed bile duct in PBC (top right). The expression of LC3 was detected in intracytoplasmic vesicles (short arrows) around nuclei in the damaged small bile duct in PBC (bottom). PBC cases #1–3 (stage 2). Immunostaining for LC3. Original magnification, $\times$400 (Inset, $\times$1000). (**b**) Top: the expression of lysosome-associated membrane protein (LAMP)-1 was detected in intracytoplasmic vesicles (short arrows) in the damaged small bile duct (arrows) in PBC. PBC cases #1–3 (stage 2). Immunostaining for LAMP-1. Original magnification, $\times$400. Bottom: the expression of autophagy-related lysosomal protein: cathepsin D (Cat D) was detected in intracytoplasmic vesicles (short arrows) in the damaged small bile duct (arrows) in PBC. PBC cases #1–3 (stage 2). Immunostaining for cathepsin D. Original magnification, $\times$400.

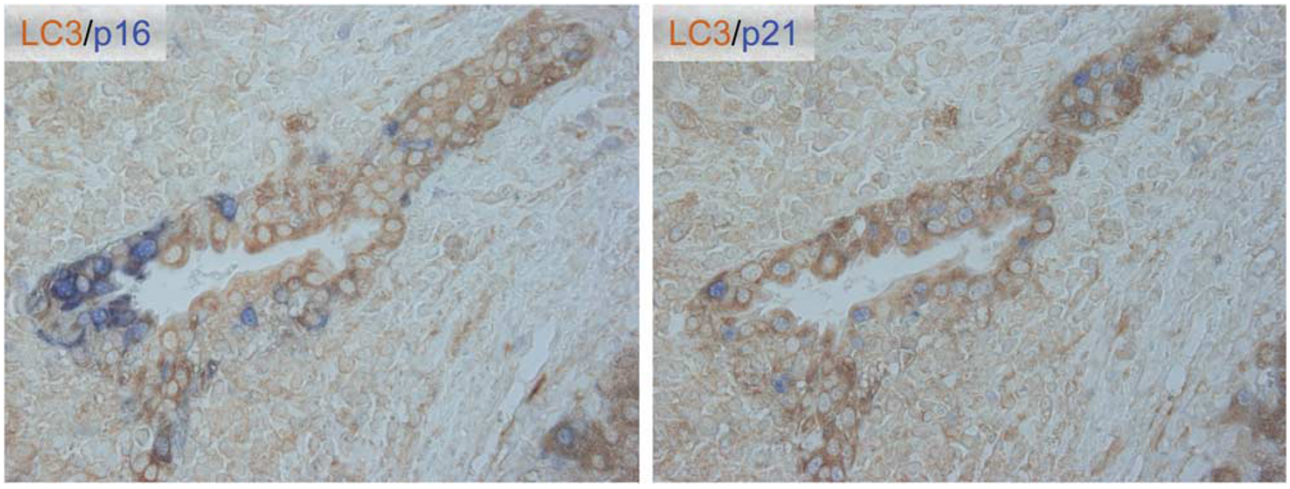

Figure 2 Co-localization of LC3 with senescent markers $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in the damaged small bile ducts in PBC. Nuclear and cytoplasmic expression of senescent marker $p16^{INK4a}$ was seen in a part of BECs showing cytoplasmic vesicular expression of LC3 in the damaged bile ducts in PBC (left). Nuclear expression of senescent marker $p21^{WAF1/Cip1}$ was also seen in a part of BECs in the same damaged bile ducts in PBC (right). PBC, stage 2. Double immunostaining LC3 (blown) and $p16^{INK4a}$ or $p21^{WAF1/Cip1}$ (blue). Original magnification, $\times 400$.

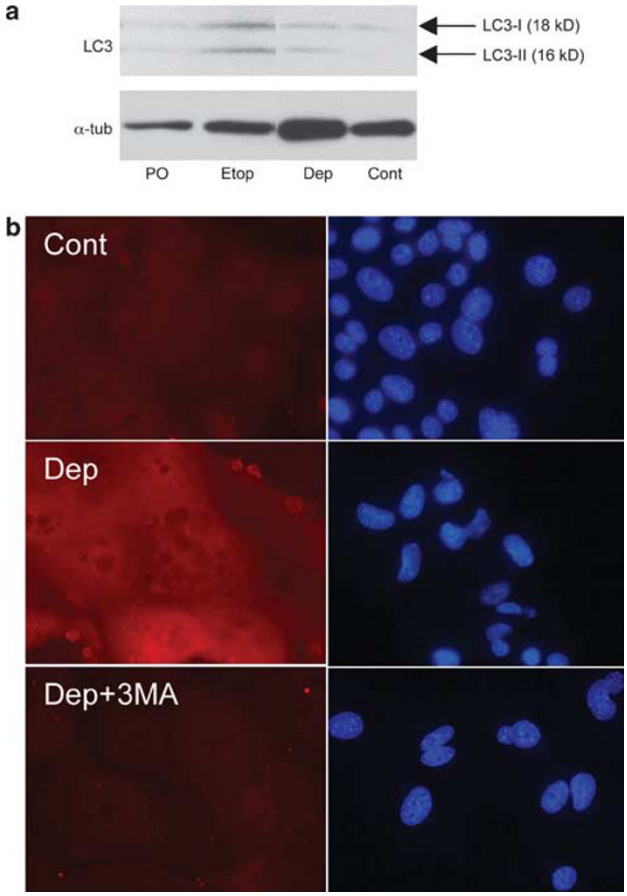

Figure 3 Increased expression of autophagy marker LC3 in cultured BECs with oxidative stress, DNA damage, and serum deprivation. (**a**) Oxidative stress and DNA damage were induced by a 2 h treatment with PO (100 μM) and a 48 h treatment with Etoposide (Etop, 100 μM). Autophagosome-associating form of LC3 (LC3-II) was upregulated by oxidative stress, DNA damage, and serum deprivation (Dep). LC3-I, a cytosolic form of LC3; α-tub, α-tubulin; Cont, control. (**b**) Immunofluorescence for the autophagy marker LC3 (left column) and a counter stain with 6-diamidino-2-phenylindole (DAPI) (right column). Vesicular cytoplasmic expression of LC3 was upregulated by serum deprivation (Dep, 48 h), whereas the expression of LC3 was faint in control (no treatment). The upregulated expression of LC3 by serum deprivation was inhibited by a treatment with 3MA (5 mM).

cellular senescence rather than be a direct general effect on chemokine release, as the treatment with 3MA did not affect CXCL1 chemokine release induced by lipopolysaccharide (Supplementary Information).

DISCUSSION

The data obtained in this study are summarized as follows: (1) The vesicular cytoplasmic expression of LC3, an autophage marker, was characteristically seen in the inflamed damaged small bile ducts in PBC. Cathepsin D and LAMP-1, the autophagy-related lysosomal proteins, was also co-expressed in the inflamed and damaged small bile ducts, suggesting that autophagy is involved in the pathogenesis of PBC. (2) The expression of LC3 was co-localized with the expression of senescent markers $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in the inflamed damaged small bile ducts in PBC. (3) Oxidative stress, DNA damage, and serum deprivation induced autophagy and cellular senescence in cultured BECs. An autophagy inhibitor, 3MA, inhibited significantly the stress-induced cellular senescence in cultured BECs. (4) Oxidative stress, DNA damage, and serum deprivation upregulated SASP, CCL2, and CX3CL1 in BECs, and a treatment with 3MA decreased the SASP in BECs treated with various stress.

This study first disclosed that autophagy may be involved in the bile duct lesion in PBC. A representative autophagy

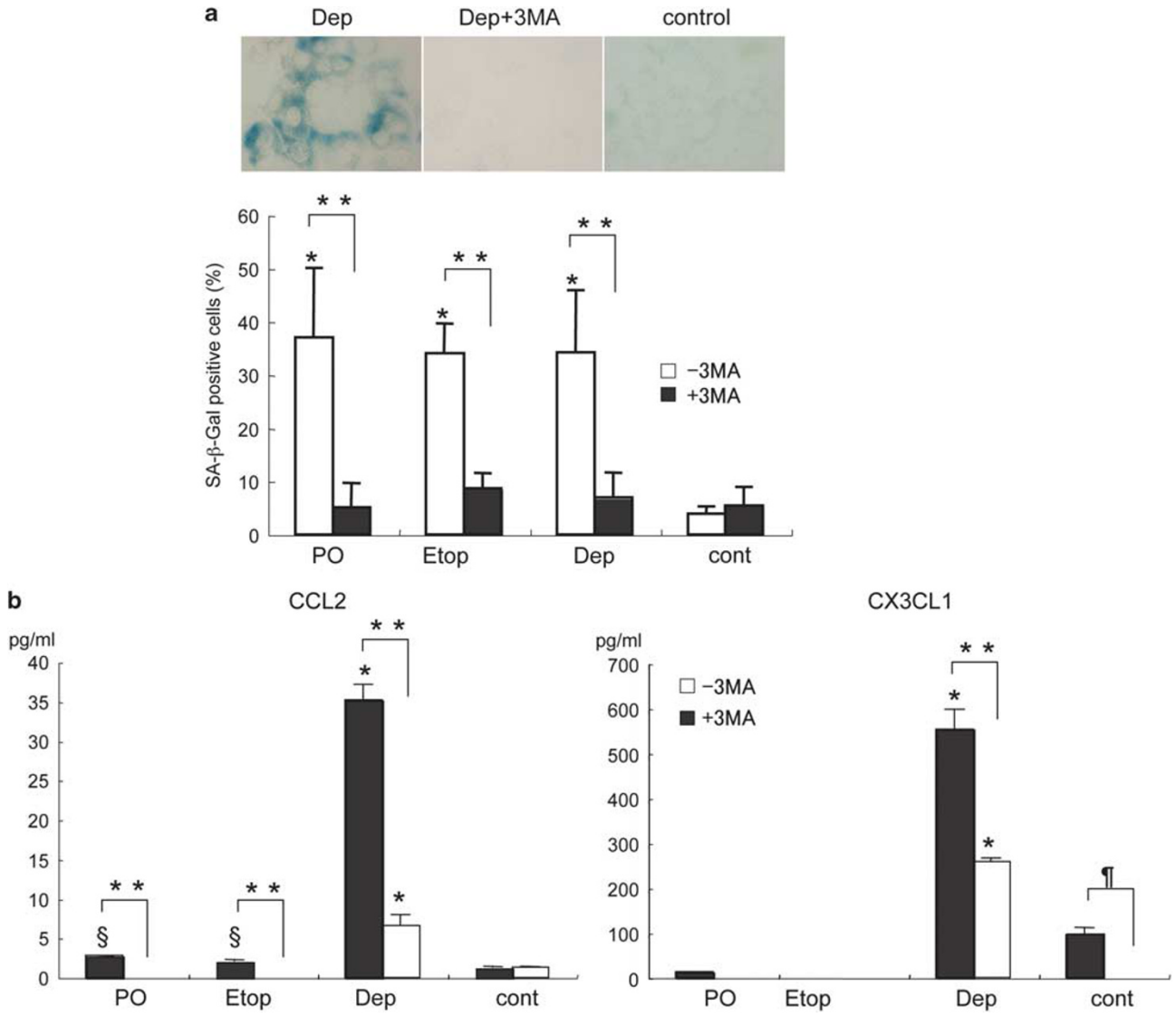

Figure 4 Inhibition of autophagy significantly decreased cellular senescence. (**a**) Cellular senescence was assessed by SA-β-gal on Day 4 after a 2-h treatment with PO (100 μM), a treatment with Etoposide (Etop, 100 μM) and serum deprivation (Dep). Percentage of cells positive for SA-β-gal was significantly higher in cells treated with PO (SA-β-gal-labeling index, 37.0 ± 13.1), Etoposide (34.0 ± 5.7), and serum deprivation (34.2 ± 11.7), when compared with control without treatment (3.9 ± 1.4). Percentage of cells positive for SA-β-gal was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (PO + 3MA, 5.1 ± 4.5; Etop + 3MA, 8.6 ± 2.9; Dep + 3MA, 6.9 ± 4.8). Data was expressed as mean ± s.d. $^{*}P<0.01$ compared with control, $^{**}P<0.01$. (**b**) Left: the secretion of CCL2, an SASP was increased in BECs treated with PO (2.74 ± 0.1 pg/ml), Etoposide (2.01 ± 0.25 pg/ml), and serum deprivation (35.17 ± 1.98 pg/ml), when compared with control (1.3 ± 0.1 pg/ml). The enhanced secretion of CCL2 was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (PO + 3MA, 0.0 ± 0.0; Etop + 3MA, 0.0 ± 0.0; Dep + 3MA, 6.64 ± 1.32). Right: the secretion of CX3CL1 was increased in BECs treated with serum deprivation (553.9 ± 43.8 pg/ml), when compared with control (96.1 ± 16.5 pg/ml). The enhanced secretion of CX3CL1 was significantly decreased by a treatment with the autophagy inhibitor 3MA (5 mM) (Dep + 3MA, 258.3 ± 9.20; Cont + 3MA, 0.1 ± 0.1). $^{*}P<0.01$ and $^{\S}P<0.05$ compared with control, $^{**}P<0.01$, $^{\P}P<0.05$.

marker LC3 was characteristically expressed in cytoplasmic vesicles in the damaged small bile ducts in PBC. Although the LC3 antibody we used in this study reacts with both a cytoplasmic and a membrane-associated form of LC3 (LC3-I and LC3-II, respectively), it is plausible that the vesicular LC3 expression exactly corresponds to a membrane-associated form of LC3 (LC3-II), which localize to the membrane of autophagosomes. The expression of LC3 was significantly higher in inflamed small bile ducts in PBC, when compared with non-inflamed bile ducts and other control livers. The expression of cathepsin D, an autophagy-related lysosomal enzyme, was coordinately expressed in the damaged bile ducts with LC3. There is no study reporting the presence and involvement of autophagy in BECs in biliary diseases, so far. In hepatocytes, it is well known that autophagy has important functions in the balance of

energy and nutrients for basic cell functions and the removal of misfolded proteins in liver diseases such as α-1 antitrypsin deficiency and hypofibrinogenemia.[24] This study indicates that autophagy is induced in BECs in diseased condition in PBC and may be related to the pathogenesis of the disease.

It is of interest that an earlier report showed phagocytosis of apoptotic BECs in PBC.[32] In this report, the authors showed the increased BEC phagocytosis of apoptotic BECs in PBC and other liver diseases and also in cultured rat BECs.[32] They raised a possibility that phagocytosed apoptotic cells may consequently be an exogenous source of autoantigens in BECs. The phagocytosed apoptotic cells in BECs in this earlier report appear to be completely different from the increased autophagy in BECs in PBC disclosed in this study. The phagocytosed apoptotic cells were observed in healthy BECs in PBC and the size of the phagocytosed apoptotic cells was about one quarter of nucleus in BECs,[32] whereas the autophagic vesicles were observed in BECs in the damaged bile ducts in PBC in this study and their size was far smaller than 'the phagocytosed apoptotic cells.' It is possible that the autophagic vesicles may contain a special form of mitochondrial autoantigens as well as the apoptotic cells[32] and that it may influence the autoimmune reactions in PBC. Further studies are necessary to clarify the significance of increased autophagy in BECs in the autoimmune conditions in PBC.

Furthermore, this study disclosed that autophagy in BECs is closely co-localized with cellular senescence in the inflamed and damaged bile ducts in PBC. We have reported cellular senescence of BECs with the augmented expression of SA-β-gal,[19] $p16^{INK4a}$, and $p21^{WAF1/Cip1}$, and telomere shortening in damaged small bile ducts in PBC. We have suggested that the cellular senescence may be involved in the pathogenesis of progressive bile duct loss in PBC.[20–22] Taken together, it is plausible that autophagy and cellular senescence may co-ordinately have a function in the pathogenesis in PBC. The co-expression of LC3 and $p21^{WAF1/Cip1}$ was more frequently observed, when compared with the co-expression of LC3 and $p16^{INK4a}$ in the damaged bile ducts in PBC in this study. $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ work coordinately in process of the cellular senescence and $p21^{WAF1/Cip1}$ is involved in the induction of senescence, whereas $p16^{INK4a}$ is involved in maintenance of senescence, reportedly.[33] Therefore, closer co-expression of the autophagic marker LC3 and $p21^{WAF1/Cip1}$ suggest that the process of cellular senescence may follow the process of autophagy.

This study disclosed for the first time that autophagy may be involved in the process of cellular senescence in cultured BECs. Interestingly, autophagy inhibitor 3MA suppressed the induction of cellular senescence and the induction of SASP in BECs with oxidative stress, DNA damage, and serum deprivation. Recently, Young *et al*[4] identified autophagy as a new effector mechanism of senescence. Autophagy is activated during senescence and its activation is correlated with negative feedback in the mammalian target of rapamycin pathway. A subset of autophagy-related genes is upregulated during senescence: over-expression of one of those genes, ULK3, induced autophagy and senescence.[4] Furthermore, inhibition of autophagy delays the senescence phenotype, including senescence-associated secretion.[4] This study supported the earlier study by Young *et al*[4] and provides evidence that autophagy may mediate cellular senescence in the inflamed and damaged small bile ducts in PBC.

Recent data suggest that senescent cells have an important function in modulating microenvironment by expressing SASP such as cytokines (IL-6, IL-1, and etc), chemokines (IL-8, CCL2/MCP-1, and etc), growth factors, and profibrogenic factors.[9–11] In fact, studies in human with biliary disorders and in animal models of biliary fibrosis have shown that BECs can express a number of profibrogenic and chemotactic proteins such as IL-8 and CCL2/MCP-1, the latter capable of attracting and activating cells of both inflammatory and stellate cell lineage.[34] Upregulation of several cytokines and chemokines in the damaged bile ducts in PBC has been reported, so far,[35–37] and recent studies disclosed that most of these factors are known to belong to SASP.[9–11] This study clearly showed that SASP such as CCL2/MCP-1 and CX3CL1 is induced by various stress such as oxidative stress and serum deprivation in cultured BECs through autophagy followed by cellular senescence. CCL2/MCP-1 and CX3CL1 are potent chemotactic factors inducing accumulation of inflammatory cells such as monocytes and T cells. Therefore, it is plausible that SASP secreted by senescent BECs may modulate microenvironment around bile duct and may accentuate inflammation and fibrosis in PBC.

In conclusion, autophagy is specifically seen in the senescent BECs in PBC. The inhibition of autophagy suppressed cellular senescence and senescence-associated secretion of CCL2/MCP-1 and CX3CL1 in cultured BECs. These findings suggest that autophagy may mediate biliary epithelial senescence and involved in the pathogenesis of bile duct lesions in PBC.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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