Bile Ductular Cells Undergoing Cellular Senescence Increase in Chronic Liver Diseases Along With Fibrous Progression

Motoko Sasaki, MD, PhD, Hiroko Ikeda, MD, PhD, Junpei Yamaguchi, MD, PhD, Masami Miyakoshi, PhD, Yasunori Sato, MD, PhD, and Yasumi Nakanuma, MD, PhD

Key Words: Ductular reaction; Chronic liver disease; Cellular senescence; p16INK4a; p21WAF1/Cip1

Abstract

We investigated the pathologic significance of ductular reactions in chronic liver diseases with respect to cellular senescence. The expression of senescence-associated markers (p16INK4a and p21WAF1/Cip1), cell proliferation, cell cycle markers (cyclin D and cyclin A), and neural cell adhesion molecule (NCAM) was examined immunohistochemically in primary biliary cirrhosis (PBC, n = 37), chronic viral hepatitis (n = 39), nonalcoholic steatohepatitis (n = 25), and control normal livers (n = 12). The expression of p16INK4a and p21WAF1/Cip1 was frequently found in ductular cells in the advanced stage of chronic liver diseases, especially in PBC (P < .05). Double immunostaining disclosed that most senescent cells expressed cyclin D (G1-phase marker). NCAM was frequently coexpressed in ductular cells showing senescence-associated markers. Some ductular cells in ductular reactions in chronic liver diseases were at G1 arrest and undergoing cellular senescence. Such senescent cells may be involved in the progression of fibrosis of these diseases, particularly in PBC.
Materials and Methods

Classification of the 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 distribution in the portal tracts. 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 a poorly defined lumen and located at the periphery of the portal tracts and are not accompanied by parallel running hepatic arterial branches. Ductular cells in DR, including intermediate hepatobiliary cells with a heterogeneous phenotype in the diseased liver, were also evaluated.

Liver Tissue Preparation

A total of 113 liver tissue specimens (all biopsied or surgically resected) were collected from the liver disease files of our laboratory and affiliated hospitals. The liver specimens included in this study were 37 PBC cases, 39 CVH cases, 25 NASH cases, and 12 “histologically normal” livers. All PBC cases were from patients fulfilling the clinical, serologic, and histologic characteristics consistent with a diagnosis of PBC. PBC cases were staged histologically. Of the PBC cases, 25 were stages 1 and 2 (early PBC) and 12 were stages 3 and 4 (advanced PBC). Of the CVH cases, 26 were regarded as fibrosis stages F0 to F2 and 13 as stages F3 and F4. Of the 39 CVH cases, 3 were serologically positive for hepatitis B surface B antigen and 36 for anti–hepatitis C viral antibody. The grade of activity and stage in the NASH cases were assessed by using the criteria proposed by Brunt et al and 12 NASH cases were regarded as stages 1 and 2 and 13 as stages 3 and 4. 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 were processed for immunohistochemical analysis. The Committee of Ethics, Kanazawa University, Kanazawa, Japan, approved this study.

Immunohistochemical Analysis

We examined immunohistochemically a cell proliferation marker (Ki-67 antigen), senescence-associated markers (p16INK4a and p21WAF1/Cip1), cell cycle markers (G1 phase, cyclin D; S phase, cyclin A), CK7, NCAM, and HepPar1. Immunostaining was performed using the antibodies shown in Table 1, as described previously. In brief, after pretreatment for antigen retrieval as described in Table 1 and blocking endogenous peroxidase, the sections were incubated with the primary antibody at 4°C overnight. The EnVision+ solution (DAKO, Carpinteria, CA) was then applied for 30 minutes at room temperature. The reaction products were visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, MO) and

<table>
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<tr>
<th>Primary Antibody Type (Clone)</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>p16INK4a Mouse monoclonal (JC2)</td>
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<td>1:100</td>
<td>NeoMarkers, Fremont, CA</td>
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<td>p21WAF1/Cip1 Mouse monoclonal (70)</td>
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<td>BD Transduction, San Jose, CA</td>
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<td>Immunotech, Marseille, France</td>
</tr>
<tr>
<td>Cyclin D Rabbit monoclonal (SP4)</td>
<td>eARI-BA (121°C, 5 min)</td>
<td>Prediluted</td>
<td>Nichirei, Tokyo, Japan</td>
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<td>Cyclin A Mouse monoclonal (6E6)</td>
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<td>Novocastra, Newcastle upon Tyne, England</td>
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<tr>
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<td>MW-CB (95°C, 20 min)</td>
<td>1:400</td>
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</tr>
<tr>
<td>NCAM Mouse monoclonal (1B6)</td>
<td>MW-CB (95°C, 20 min)</td>
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<td>HepPar Mouse monoclonal (OCH1E5)</td>
<td>MW-CB (95°C, 20 min)</td>
<td>1:100</td>
<td>DAKO</td>
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BA, 0.05 mol/L boric acid buffer (pH 8); CB, 0.05 mol/L citric buffer (pH 6); eARI, electronic antigen retrieval instrument (Pascal, DAKO); MW, microwave; NCAM, neural cell adhesion molecule.
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hydrogen peroxide. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of control mouse IgG (DAKO) was applied instead of the primary antibody as a negative control. Positive and negative control samples were routinely included.

Assessment of Immunostaining

All fields of each liver specimen were observed under a light microscope for the evaluation of immunohistochemical expression of CK7, NCAM, p16INK4a, and p21WAF1/Cip1.

Extent of DRs

The extent of ductular cell proliferation was semiquantitatively assessed as follows: 1+, focal, more than 5 CK7+ ductules detected in one third or fewer portal tracts; or 2+, extensive, more than 5 CK7+ ductules detected in more than one third of portal tracts.

Extent of NCAM, p16INK4a, and p21WAF1/Cip1 Expression in DRs

The extent of expression was evaluated as follows: 1+, focal, positive cells detected in one third or fewer portal tracts; or 2+, extensive, positive cells detected in more than one third of portal tracts.

Ki-67 Labeling Index in DRs and Hepatocytes

The Ki-67 labeling index (LI) was regarded as a marker for cell proliferation activity. The Ki-67 LI was assessed in 5 or more portal tracts with more than 100 ductular cells in DRs and 5 representative foci composed of more than 100 hepatocytes in each specimen.

Double Immunostaining

We also performed double immunostaining for NCAM, CK7 with senescence markers (p16INK4a and p21WAF1/Cip1), and cell cycle markers (cyclin D and cyclin A) to characterize ductular cells in DRs in the selected sections, as described previously. In brief, either of the senescence markers (p16INK4a and p21WAF1/Cip1) or cell cycle markers (cyclin D and cyclin A) was detected by first using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, CA), followed by second staining for NCAM, CK7, or HepPar1 with the use of Alexa Fluor 488-labeled antimouse IgG (Molecular Probes, Eugene, OR). The Vector Red reaction product is highly fluorescent, with rhodamine excitation and emission filter systems. The slides were then counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes) and coverslipped.

Statistical Analysis

Statistical analysis for the difference used the Wilcoxon rank sum test. The correlation coefficient of 2 factors was evaluated by using the Spearman rank correlation test. When the value was less than .05, the difference was regarded as significant.

Results

Extent of CK7+ Ductular Cells in DR

Ductular cells in DR frequently expressed CK7 and p21WAF1/Cip1, and the extent of CK7+ ductular cells is shown in Figure 1. The number of CK7+ ductular cells increased along with the progression of histologic stage in CVH and NASH (P < .05) and to a lesser degree in PBC. The number of CK7+ cells increased more in CVH and NASH at stages 1 and 2 and stages 3 and 4, respectively, than PBC at individual stages (P < .05).

Expression of Senescence Markers (p16INK4a and p21WAF1/Cip1) in DR

p16INK4a was expressed in the cytoplasm and the nuclei of bile ductules, and p21WAF1/Cip1 was expressed in the nuclei of biliary epithelial cells, when detectable (Images 1 and 2). As shown in Figure 2, the expression of p16INK4a and p21WAF1/Cip1 was more frequent in ductular cells in the advanced stages of PBC (p16INK4a: 1+, 25%; 2+, 75%; p21WAF1/Cip1: 1+, 50%; 2+, 50%), CVH (p16INK4a: 1+, 46%; 2+, 23%; p21WAF1/Cip1: 1+, 54%; 2+, 23%), and NASH (p16INK4a: 1+, 39%; 2+, 31%; p21WAF1/Cip1: 1+, 62%; 2+, 23%) than in the early stages of PBC (p16INK4a: 1+, 76%; 2+, 4%; p21WAF1/Cip1: 1+, 80%; 2+, 4%), CVH (p16INK4a: 1+, 42%; 2+, 0%; p21WAF1/Cip1: 1+, 15%; 2+, 4%), and NASH (p16INK4a: 1+, 0%; 2+, 0%; p21WAF1/Cip1: 1+, 0%; 2+, 0%) (P < .05). The expression of p21WAF1/Cip1 and p16INK4a was more frequent in ductular cells in PBC at the early stages than in those of CVH and NASH at the early stages (P < .05). Furthermore, the expression of p16INK4a in ductular cells was significantly more frequent in advanced stage PBC in comparison with advanced stage CVH and NASH (P < .05).

Expression of NCAM in Ductular Cells

Ductular cells expressed NCAM in the cell membrane to various degrees (Images 1 and 2). As shown in Figure 3, the extent of NCAM expression in DRs was significantly increased in the advanced stage of PBC (1+, 33%; 2+, 67%) compared with the early stage of PBC (1+, 56%; 2+, 8%) and NASH (1+, 42%; 2+, 0%), CVH (1+, 62%; 2+, 4%), and normal livers (1+, 33%; 2+, 0%) (P < .05). The extent of NCAM expression in DRs was significantly increased in the advanced stage of NASH compared with the early stage of PBC, CVH, and NASH and normal livers (P < .05).
Characterization of DR Using Double Immunostaining

Cyclin D1 (G1 phase marker) and cyclin A (S phase marker) were expressed in the nuclei of ductular cells. Double immunostaining showed that ductular cells positive for the senescence markers (p16INK4a or p21WAF1/Cip1) were always positive for cyclin D1 but negative for cyclin A. Double immunostaining showed that ductular cells expressing the senescence markers (p16INK4a or p21WAF1/Cip1) were always negative for Ki-67. Double immunostaining for CK7 and NCAM disclosed that many ductular cells coexpressed CK7 and NCAM in the advanced stage of PBC, whereas a part of the ductular cells coexpressed them in CVH and NASH at advanced stages Image 3. Double immunostaining for the senescence markers (p16INK4a and p21WAF1/Cip1) and NCAM disclosed that the senescence markers were frequently coexpressed with NCAM-expressing ductular cells, especially in the advanced stage of PBC Image 4. Most ductular cells expressing NCAM expressed cyclin D (G1 phase marker), whereas few ductular cells expressed cyclin A Image 5. This finding suggests that NCAM-expressing ductular cells are at senescence and the G1 arrest state.

Cell Proliferative Activity (Ki-67 LI) in DR and Hepatocytes

Although Ki-67–labeled nuclei were scattered in hepatocytes (Ki-67 LI up to 5.6) and inflammatory cells in hepatic parenchyma, the Ki-67 LI was usually less than 1 in ductular cells in DRs Image 6. In normal livers, the Ki-67 LI was lower than 1.
Image 2: Expression of cytokeratin (CK)7 (A), neural cell adhesion molecules (NCAM; B), p16\(^{INK4a}\) (C), and p21\(^{WAF1/Cip1}\) (D) in chronic viral hepatitis, A2F4 (moderate necroinflammatory activity, cirrhosis). A part of CK7-expressing ductular cells express NCAM, p16\(^{INK4a}\) (arrows), and p21\(^{WAF1/Cip1}\) (arrowheads) (A-D, ×400).

Figure 1: The extent of ductular reaction assessed as cytokeratin 7–positive ductules in different hepatic diseases. White column, focal (1+); gray column, extensive (2+). a, \(P < .05\) vs normal liver (NL). b, \(P < .05\) vs primary biliary cirrhosis (PBC), stage (st) 1 and 2. c, \(P < .05\) vs nonalcoholic steatohepatitis (NASH), st 1, 2. d, \(P < .05\) vs PBC, st 3 and 4. CVH, chronic viral hepatitis; F, fibrosis stage.
LI was 0. As shown in Figure 4a, the Ki-67 LI was lower in ductular cells in PBC (early stage, 0.25 ± 0.43; advanced stage, 0.04 ± 0.08) in comparison with CVH (early stage, 0.50 ± 0.54; advanced stage, 0.62 ± 0.64) and NASH (early stage, 0.39 ± 0.56; advanced stage, 0.15 ± 0.25). There was statistical significance between PBC and CVH. The Ki-67 LI was significantly lower in ductular cells in the advanced stage of NASH compared with CVH (P < .05).

Discussion

The data obtained in this study are summarized as follows: (1) A majority of ductular cells in DR expressed CK7, and the number of CK7+ ductular cells was increased in PBC, CVH, and NASH at their advanced histologic stages. (2) p16INK4a and p21WAF1/Cip1 were frequently expressed in ductular cells in PBC, CVH, and NASH at advanced stages, and a proportion of ductular cells expressing these molecules were particularly frequent in PBC at advanced stages. Ductular cells expressing p16INK4a and p21WAF1/Cip1 were always positive for cyclin D but negative for cyclin A. These cells also showed no K-67 labeling. (3) NCAM was usually coexpressed in ductular cells positive for p16INK4a and p21WAF1/Cip1 and was frequently expressed in PBC, CVH, and NASH at advanced histologic stages, particularly in PBC.

CK7 is a well-known marker of biliary cell lineage, and it was found in this study that ductular cells in DR frequently expressed CK7 in any type of disease, and the number of CK7+ ductular cells increased along with the progression of histologic stage in PBC, CVH, and NASH. This finding is in accordance with previous studies, and DR could be directly or indirectly involved in the progression of these chronic liver diseases.

Cellular senescence, an irreversible cell growth arrest, is reportedly involved in the pathophysiology of various liver diseases, and senescent cells remain metabolically active. Senescent cells display several biologic characteristics, and p16INK4a and p21WAF1/Cip1 are reported as cellular senescence
It was found in this study that p16\textsuperscript{INK4a} and p21\textsuperscript{WAF1/Cip1} were frequently expressed in ductular cells in DRs in more than half of the PBC cases at the advanced stages compared with their early stages, in which fewer than 5% of PBC cases showed such overexpression of senescent markers in ductular cells. These findings confirmed our previous studies reporting the extensive expression of another senescence marker, senescence-associated β-galactosidase, in ductular cells in advanced stages of PBC\textsuperscript{11,13}. In addition, the expression of senescence markers (p16\textsuperscript{INK4a} and p21\textsuperscript{WAF1/Cip1}) was also significantly increased along with the stage of CVH and NASH. However, the proportion of ductular cells showing such senescence markers in DR was about one fourth and was lower in comparison with PBC at advanced stages. These findings suggest that cellular senescence develops gradually in ductular cells in DR along with the progression of histologic stages of PBC, CVH, and NASH, irrespective of the type of liver damage.

It is of interest that there was a difference between PBC and CVH in regard to senescence markers and NCAM expression. The difference between PBC and CVH may be based on the difference between DR in biliary disease and DR in hepatocellular disease because another biliary disease, acute extrahepatic bile duct obstruction, was more similar to PBC than CVH in regard to expression of NCAM in a preliminary study. However, the expression of senescence markers was more frequent in the advanced stages of PBC than in extrahepatic bile duct obstruction. Therefore, other factors, for example, duration of disease, also seem to be related to this difference. Further studies are needed to clarify this important point.

Double immunostaining showed that ductular cells positive for the senescence markers (p16\textsuperscript{INK4a} or p21\textsuperscript{WAF1/Cip1}) were always positive for cyclin D1 (G\textsubscript{1} phase marker) but negative for cyclin A (S phase marker). Double immunostaining showed that ductular cells expressing the senescence markers (p16\textsuperscript{INK4a} or p21\textsuperscript{WAF1/Cip1}) were always negative for Ki-67. These data suggest that senescent bile ductules are actually arrested at the G\textsubscript{1} phase. Recent data suggest that senescent cells have an important role in modulating the microenvironment by secreting biologically active molecules such as cytokines (eg, interleukin [IL]-6 and IL-1), chemokines (eg, IL-8 and monocyte chemotactic protein [MCP]-1), growth factors, and profibrogenic factors.\textsuperscript{28-30} In fact, studies in human beings with biliary disorders and in animal models of biliary fibrosis have shown that ductal epithelium can express a number of profibrogenic and chemotactic proteins (eg, IL-1, IL-6, IL-8, and MCP-1), with MCP-1 capable of...
Immunostaining for senescence markers [p16INK4a (top left) or p21WAF1/Cip1 (bottom left)] (red) and neural cell adhesion molecule (NCAM; green) (center) and both (right) in primary biliary cirrhosis (PBC), stage 4 (A) and chronic viral hepatitis (CVH), A2F3 (moderate necroinflammatory activity, fibrosis), hepatitis C (B). The expression of senescent markers p16INK4a and p21WAF1/Cip1 is seen in NCAM+ ductular cells (arrows) in PBC, stage 4, and CVH, A2F3, C (A and B, x400).
Immunostaining for cell cycle markers (G1 phase, cyclin D [top left]; S phase, cyclin A [bottom left]) (red) and neural cell adhesion molecule (NCAM; green) (center) and both (right) in primary biliary cirrhosis (PBC), stage 4. Most NCAM+ ductular cells (arrows) express cyclin D, whereas there is no cyclin A expression in ductular reactions in PBC, stage 4 (all parts, ×400).

The expression of Ki-67 antigen (a cell proliferation marker) in primary biliary cirrhosis, stage 4. There are no Ki-67–labeled ductular cells (arrows), whereas there are several Ki-67–labeled hepatocytes (arrowheads) (×400).

Proliferation activity in ductular cells and hepatocytes in different hepatic diseases, as measured by the Ki-67 labeling index. The dots show the Ki-67 labeling index of ductular cells or hepatocytes in each case. The line shows the mean value for each group. H, hepatocytes; D, ductular cells; *P < .05 vs ductular cells in the same disease group. a, P < .05 vs primary biliary cirrhosis (PBC), stage (st) 1 and 2; PBC, st 3 and 4; and normal liver (NL). b, P < .05 vs PBC, st 1 and 2; PBC, st 3 and 4; and nonalcoholic steatohepatitis (NASH), st 3 and 4. CVH, chronic viral hepatitis; F, fibrosis stage.
attracting and activating cells of inflammatory and stellate cell lineages.\textsuperscript{31} Taken together, it is plausible that senescent ductular cells may secrete a part of these modulators in DRs and participate in regulating the microenvironment in DRs and possibly the fibrous progression of chronic liver diseases. Because the expression of senescence markers is greater in advanced disease than in early disease, it may be an alternative hypothesis that cellular senescence is a secondary phenomenon after the development of fibrosis, not a cause of fibrosis. However, a smaller number of senescent ductular cells was present even in the early stage of disease, and the increase of senescent ductular cells and the development of fibrosis may progress synergistically.

Evidence supporting a role for epithelial-mesenchymal transition (EMT) in repair of chronic liver injury is growing in human studies and animal model studies.\textsuperscript{32-34} For example, cells in DR expressed epithelial (CK7, CK19, and E-cadherin) and mesenchymal markers (S100A4, vimentin, and matrix metalloproteinase-2) in human diseased livers.\textsuperscript{32} The involvement of hedgehog signaling was reported in regulation of EMT during biliary fibrosis in rodents and humans.\textsuperscript{33} NCAM is a mesenchymal phenotype, not an original epithelial phenotype; therefore, the increase of NCAM-expressing ductular cells demonstrated in this study may be related to EMT coexisting with cellular senescence. Yovchev et al.\textsuperscript{35} reported that epithelial cell adhesion molecule-positive and thymus cell antigen 1-positive (Thy-1+) cells represent 2 different populations of cells in the oval cell niche in rodent models. Epithelial cell adhesion molecule-positive oval cells are bipotential adult hepatic epithelial progenitors and display a mixed epithelial-mesenchymal phenotype. Thy-1+ cells are mesenchymal cells with characteristics of myofibroblasts/activated stellate cells.\textsuperscript{35} Inflammatory and fibrogenic factors secreted by senescent ductular cells may regulate these Thy-1+ cells in the progression of fibrosis. Direct correlation between EMT and cellular senescence in DR remains to be elucidated.

NCAM is a cell surface adhesion molecule that has a role in morphogenesis, remodeling, and migration in several organs.\textsuperscript{6,36} Roskams et al.\textsuperscript{36} reported that a portion of proliferating bile ductules express NCAM, although the exact role or significance of NCAM+ bile ductules remains speculative. It was found in this study that the number of NCAM+ ductular cells was significantly increased in the advanced stages of PBC compared with early-stage PBC and with NASH and CVH in the advanced stages. Double immunostaining for CK7 and NCAM disclosed that many ductular cells coexpressed CK7 and NCAM in the advanced stages of PBC, whereas a part of the ductular cells coexpressed them in CVH and NASH in the advanced stages. Double immunostaining for the senescence markers (p16\textsuperscript{INK4a} and p21\textsuperscript{WAF1/Cip1}) and NCAM disclosed that the senescence markers were frequently coexpressed with NCAM in ductular cells. The coexpression of NCAM and senescence markers in ductular cells may suggest that ductular cells expressing NCAM may be undergoing cellular senescence in DR. The findings that most ductular cells coexpressing NCAM and senescence markers expressed cyclin D (G1 phase marker), whereas few ductular cells expressed cyclin A (S phase marker) in DR, support the aforementioned suggestion. The expression of NCAM was also correlated with the expression of p16\textsuperscript{INK4a} and cyclin D in CVH and NASH at advanced stages.

It is well known that a portion of “proliferating bile ductules” displays NCAM,\textsuperscript{1,36} and NCAM has been used as a marker of hepatic stem cells/progenitor cells (HSPCs) in several studies.\textsuperscript{6,37,38} In a model of cell lineage and marker relationship between cells composing DRs,\textsuperscript{6} bipotential stem cells were singly NCAM+, transition cells in the bile ductular lineage were NCAM+ and CK19+, and transitional cells in the hepatocellular lineage were negative for all markers. However, it was found in this study that NCAM-expressing ductular cells expressed features of cellular senescence and they failed to show active cell proliferation, particularly in PBC. In addition, most NCAM-expressing ductular cells expressed cyclin D (G1 phase marker), and few NCAM-expressing ductular cells expressed cyclin A (S phase marker), raising a possibility that a majority of NCAM-expressing ductular cells are at a G1 arrest state and failing to show the evidence of a real “proliferating” phase. Therefore, it seems likely that NCAM can be a marker of senescent ductular cells and that NCAM can have an autocrine or paracrine regulatory role in such a process of cellular senescence in bile ductules. The findings in the present study suggest that the term ductular reaction may be a more accurate term than proliferating bile ductules. However, the possibility that HSPCs are present among DRs could not be denied, and markers other than NCAM may be more suitable for the detection of HSPCs.

This study revealed that DRs are heterogeneous in cell kinetics and expression of NCAM and that at least a part of the ductular cells in DRs in PBC, CVH, and NASH are undergoing cellular senescence, and these senescent cells may be involved in the progression of these chronic liver diseases, particularly PBC. Our study raises a possibility that NCAM can be used as a marker of cellular senescence developing in DRs.

From the Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan.

Supported in part by grant-in-aid 18590325 for Scientific Research (C) from the Ministry of Education, Culture, Sports and Science and Technology of Japan, Tokyo.

Address reprint requests to Dr Nakanuma: Dept of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan.
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