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Oval Cell Numbers in Human Chronic Liver Diseases Are Directly Related to Disease Severity

Kym N. Lowes,*‡ Barbara A. Brennan,† George C. Yeoh,‡ and John K. Olynyk*

From the University Department of Medicine and the Department of Histopathology,† Fremantle Hospital, Fremantle, and the Department of Biochemistry,‡ University of Western Australia, Nedlands, Western Australia, Australia*

The risk of developing hepatocellular carcinoma is significantly increased in patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C infection. The precise mechanisms underlying the development of hepatocellular carcinoma in these conditions are not well understood. Stem cells within the liver, termed oval cells, are involved in the pathogenesis of hepatocellular carcinoma in animal models and may be important in the development of hepatocellular carcinoma in human chronic liver diseases. The aims of this study were to determine whether oval cells could be detected in the liver of patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C, and whether there is a relationship between the severity of the liver disease and the number of oval cells. Oval cells were detected using histology and immunohistochemistry in liver biopsies from patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C. Oval cells were not observed in normal liver controls. Oval cell numbers increased significantly with the progression of disease severity from mild to severe in each of the diseases studied. We conclude that oval cells are frequently found in subjects with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C. There is an association between severity of liver disease and increase in the number of oval cells consistent with the hypothesis that oval cell proliferation is associated with increased risk for development of hepatocellular carcinoma in chronic liver disease. ***(Am J Pathol 1999, 154:537–541)***

Epidemiological studies indicate that hepatocellular carcinoma (HCC) is one of the most common human visceral malignancies.[1] The risk of HCC is significantly increased in chronic liver disease, especially when cirrhosis is present.[1,2] Individuals with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C infection[3–7] have a 4- to 200-fold increased risk of developing HCC compared to the general population. As cirrhosis develops, hepatocyte necrosis is followed by an attempted secondary proliferative response of mature hepatocytes,[8–11] but this proliferative response is often impaired in chronic liver disease. An alternative mechanism for hepatocyte regeneration in chronic liver disease involves stem cell proliferation and differentiation into hepatocytes. Studies in rodent models of hepatocarcinogenesis suggest that activation of a facultative stem cell compartment comprising oval cells could be the mechanism by which the liver may replace destroyed parenchyma in chronic liver disease.[8–13]

Oval cells are a bipotent cell population that can differentiate into hepatocytes and biliary epithelium.[8–14] Morphologically, oval cells are characterized by an ovoid nucleus, small size (relative to hepatocytes), and scant basophilic cytoplasm. Oval cells express phenotypic markers of both hepatocytes and biliary epithelium, including the M2-isozyme of pyruvate kinase (M2-PK), π-class glutathione S-transferase (π-GST), and cytokeratin 19 (CK 19).

Although the cellular targets for transformation in HCC have not yet been identified, several lines of evidence suggest that oval cells may be targets for transformation. Animals exposed to a variety of carcinogenic regimens display a uniform pattern of preneoplastic changes ultimately giving rise to HCC. One of the earliest cellular changes observed is the proliferation of oval cells in the periportal region of the liver, followed by infiltration of these cells into the liver lobule.[12,13] Oval cell proliferation has also been reported in rodents following chronic exposure to ethanol or iron.[15,16] In humans, oval cells have been reported in hepatitis B-associated HCC and chronic liver disease associated with ductular proliferation.[17,18] However, it is not known whether there is an association between genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C and oval cell proliferation. The aims of this study were to determine whether oval cells could be detected in the liver in genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C, and whether there is a relationship between the severity of liver disease and the number of oval cells.

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Address reprint requests to Associate Professor John K. Olynyk, M.D., University Department of Medicine, Fremantle Hospital, P.O. Box 480, Fremantle 6959, Western Australia, Australia. E-mail: jolynyk@cyllene.uwa.edu.au.

Materials and Methods

Tissue Samples

Formalin-fixed, paraffin-embedded liver biopsy specimens from 45 patients were obtained from the Department of Histopathology at Fremantle Hospital (Fremantle, Western Australia). Fifteen of these patients had been diagnosed with genetic hemochromatosis on the basis of typical clinical features, no history of secondary iron overload, liver biopsy consistent with the diagnosis, and hepatic iron index >2. Another 15 patients had a history of continuous alcohol consumption >60g/d and no evidence of other causes of liver disease on serological testing. The remaining 15 patients had been diagnosed with chronic hepatitis C (anti-hepatitis C virus-positive, PCR positive for hepatitis C virus, elevated ALT >1.5× upper limit of normal, other liver diseases excluded by standard testing). The samples were further classified by two investigators (JKO and KNL) as having mild (no fibrosis), moderate (fibrosis), or severe (cirrhosis) liver damage. Using the modified Knodell scoring system for fibrosis/cirrhosis, scores of 0 or 1 were assigned to cases of mild liver damage; scores of 2 to 5 to moderate cases; and scores of 6 to severe cases.[19] Inflammatory activity was not scored because of the difficulty of comparing it across the three different conditions. For control purposes, archival normal liver tissue was obtained from five subjects who had minimal or no abnormal liver pathology and no biochemical or serological evidence of liver disease. The study was approved by the Fremantle Hospital Ethics Committee.

Histology and Immunohistochemistry

Four-μm sections were stained with hematoxylin and eosin, Masson's Trichrome, and Perls' Prussian blue method for determination of liver morphology, fibrosis, and iron overload, respectively. Sections were evaluated by a histopathologist (BAB) and a scientist (KNL) without knowledge of the disease or category of severity. The sections being measured contained at least three portal tracts. Sections were screened on low power and areas of increased oval cell staining were determined, usually centered on portal tracts or fibrous septa. Cells were scored when they satisfied the morphological criteria for oval cells, showed cytoplasmic staining for M2-PK, π-GST, or CK 19, and did not exhibit positive staining for leukocyte common antigen (LCA). Three nonoverlapping fields were then counted using a 40× objective giving a field diameter of 0.5 mm. The variance in oval cell counts from section to section in the same subject was <10%. The average of these scores was then taken. Immunohistochemical staining was performed to detect expression of M2-PK (Schebo Tech GmbH), π-GST (Novacastra Laboratories, UK), CK 19 (Dako, Botany, NSW, Australia), and LCA (Dako) using methods previously described by us.[14,20] The sections were deparaffinized in xylene and rehydrated through graded alcohol. The sections were then digested with Protease (Type VIII from *Streptomyces avidinii*, Sigma, St. Louis, MO) for 4 minutes at 37°C for immunodetection of CK 19 or boiled in 10 mmol/L citrate buffer, pH 6.0, in a microwave for two treatments of 2 minutes (350 W) for M2-PK staining. Endogenous peroxidases were inactivated by immersing the sections in 3% hydrogen peroxide for 5 minutes. Sections to be used for polyclonal antisera were incubated for 2 minutes with normal swine serum in Tris-buffered saline to block nonspecific binding. The sections were subsequently incubated overnight at 4°C with the relevant antibodies. The following day, the sections were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (Dako). The sections were then incubated with peroxidase-conjugated streptavidin (Dako). The chromogenic reaction was developed with diaminobenzidine or 3-amino-9-ethyl-carbazole and all of the sections were counterstained with hematoxylin.

Statistical Analysis

Data are presented as the mean ± SEM. The Mann Whitney test was used to determine whether the differences between groups were significant. Significance was accepted when $P < 0.05$.

Results

The number of oval cells detected in normal liver controls and patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C is shown in Figure 1, a (M2-PK staining) and b (π-GST staining). Oval cells were not detected in control liver tissue obtained from normal subjects but were detected in liver tissue obtained from patients with genetic hemochromatosis (Figure 2), alcoholic liver disease, or chronic hepatitis C (Figure 3). The number of M2-PK-positive oval cells increased significantly ($P < 0.01$) as disease severity increased from mild through moderate to severe in chronic hepatitis C (12 ± 2, 74 ± 11, and 124 ± 16, respectively), genetic hemochromatosis (31 ± 3, 80 ± 9, and 133 ± 25, respectively), and alcoholic liver disease (21 ± 5, 102 ± 8, and 114 ± 16, respectively). The number of π-GST-positive oval cells also increased significantly ($P < 0.01$) as disease severity increased from mild through moderate to severe in chronic hepatitis C (5 ± 1, 13 ± 2, and 54 ± 12, respectively), genetic hemochromatosis (6 ± 1, 38 ± 5, and 56 ± 10, respectively), and alcoholic liver disease (3 ± 1, 12 ± 3, and 38 ± 2, respectively). There were no significant differences between the diseases in terms of the number of M2-PK-positive oval cells in each category of disease severity. Oval cells were located predominantly in the periportal region (Figures 2 and 3) and were occasionally observed to form ductular structures. Oval cells were often found in close association with fibrous septa, surrounding regenerative nodules, or associated with inflammatory cell infiltrates, particularly in chronic hepatitis C (Figure 3, c and d). Only 30–50% of M2-PK-positive oval cells stained positively for either π-GST (Figures 1a, 1b, and 2c) or CK 19 (Figure 2b). Some mature hepatocytes also expressed π-GST (Figure 2c).

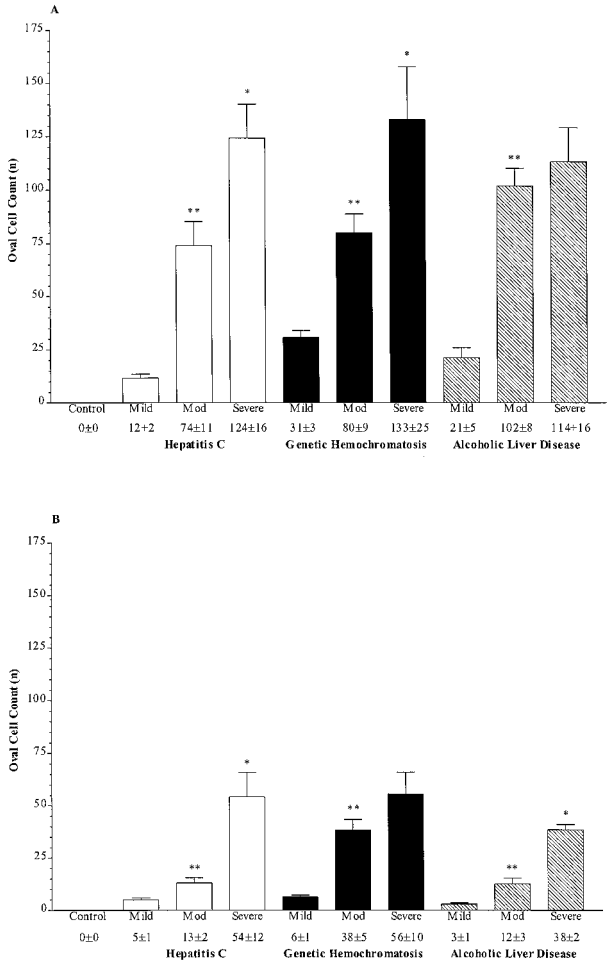

Figure 1. Oval cell numbers in control subjects and those with chronic hepatitis C ($n = 15$), genetic hemochromatosis ($n = 15$), or alcoholic liver disease ($n = 15$) who had mild ($n = 5$ in each group), moderate (mod; $n = 5$ in each group), or severe ($n = 5$ in each group) liver disease. A: Oval cell numbers determined from staining for M2-PK. B: Oval cell numbers determined from staining for π-GST. Numbers below the x axis represent the mean ± SEM for each group. $P < 0.01$ compared with mild disease. $P < 0.05$ compared with moderate disease. Error bars show the SEM.

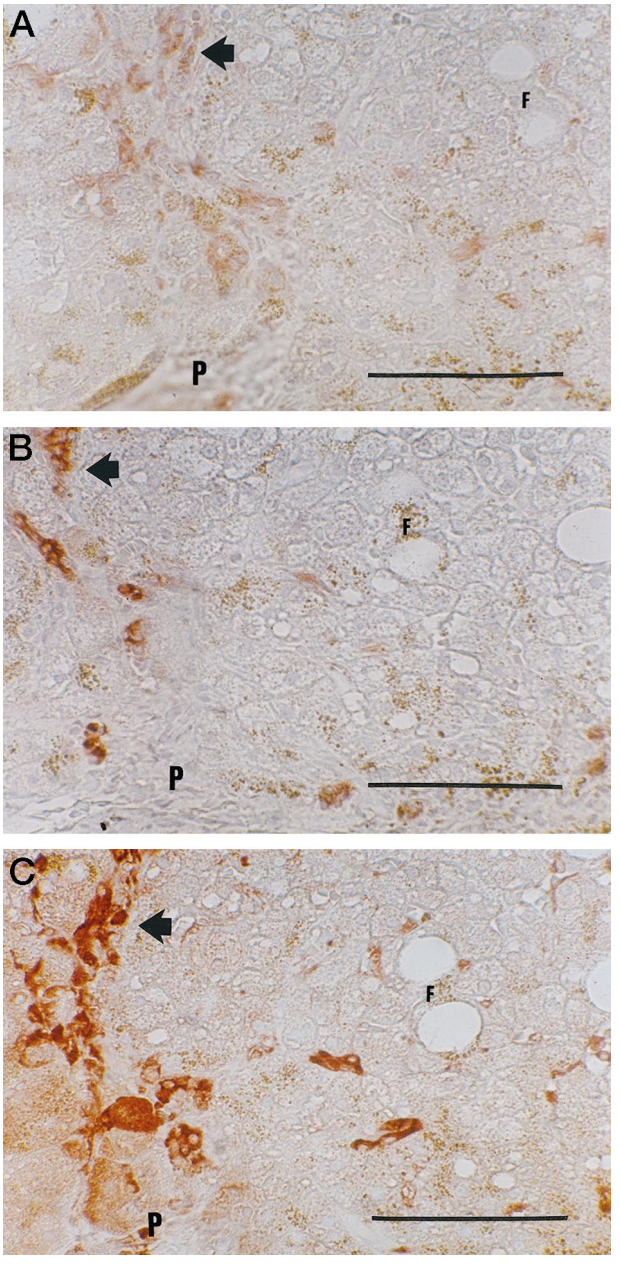

Figure 2. Immunohistochemical staining of serial sections from a patient with severe genetic hemochromatosis. A portal tract (P) and hepatocytes containing fat droplets (F) are labeled in each of the serial sections for orientation purposes. A: Oval cells (arrow) identified by M2-PK staining (red). B: Oval cells (arrow) stained with antibody to CK 19 (red). Ductular epithelium and some M2-PK positive oval cells are seen staining positively for CK 19. C: Oval cells (arrow), biliary epithelium, and some hepatocytes are seen staining positively for π-GST (red). Fewer oval cells stain positively in comparison to M2-PK. The line represents 200 μm.

Discussion

This study shows that oval cells are present in patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C. Furthermore, oval cell numbers increase significantly with progression of disease severity in each of the groups studied, suggesting that oval cell proliferation is not disease-specific but occurs in response to progressive liver injury and fibrosis. The association between severity of liver disease and increasing number of oval cells is consistent with the hypothesis that oval cell proliferation is associated with increased risk for development of hepatocellular carcinoma with advancing liver disease, particularly when cirrhosis is present.

Studies in animal models of oval cell proliferation demonstrate a close association between oval cells and infiltrating inflammatory cells within the liver.[13,20] When oval cells are isolated from animals placed on a choline-deficient, ethionine-supplemented diet, they rapidly deteriorate in culture, suggesting that exogenous factors are required for their survival and proliferation.[13] In the current human study, oval cells were often found in close association with fibrous tissue in the liver, often proliferating along the tracts from the expanded portal regions and along the limiting plates surrounding regenerative nodules. In addition, oval cells were often observed in close association with inflammatory cells, particularly in patients with hepatitis C. These observations suggest that cytokines or other factors produced by inflammatory

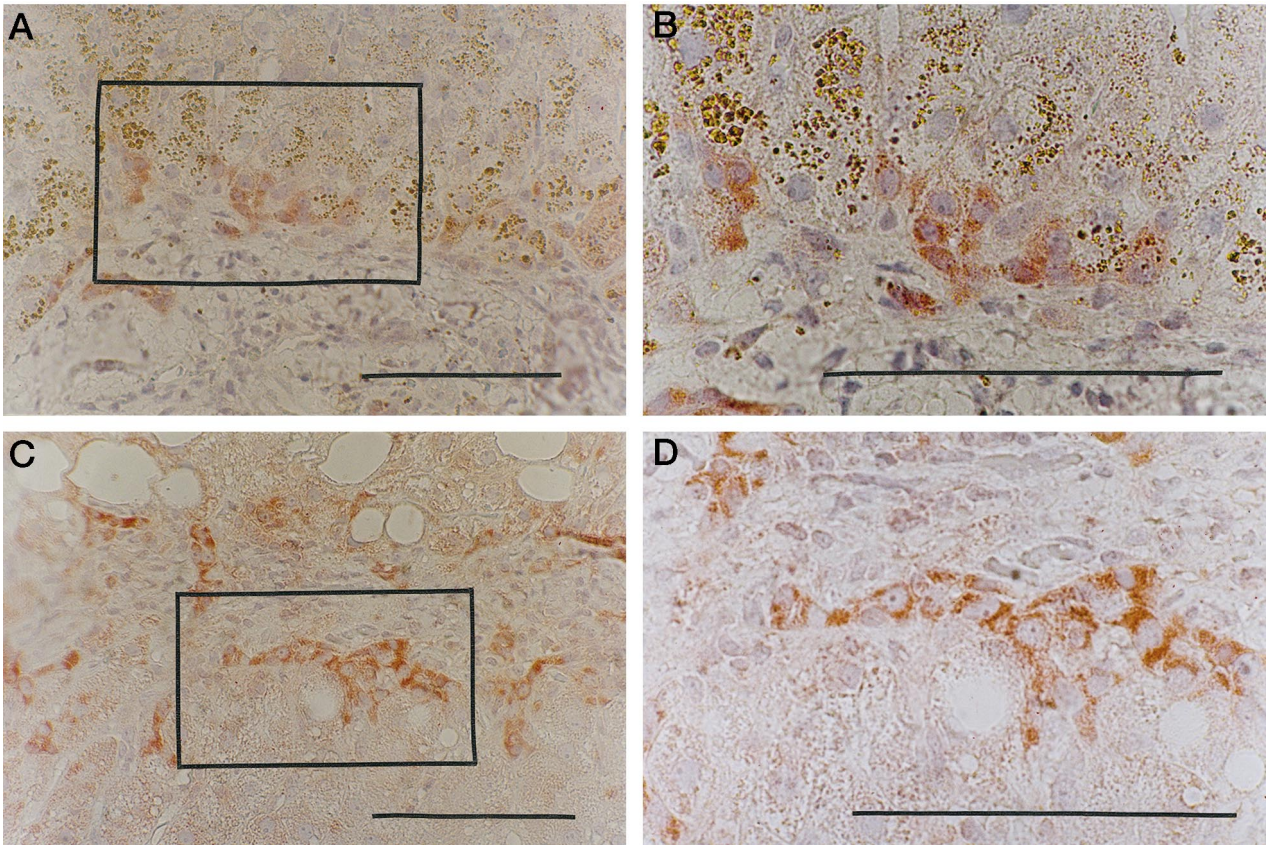

Figure 3. Oval cells identified by M2-PK staining (red). A: A portal tract from a patient with mild genetic hemochromatosis. B: The boxed portal tract area in (A) magnified to demonstrate oval cell morphology. C: A portal tract from a patient with moderate chronic hepatitis C. D: The boxed portal tract in (C) has been magnified to demonstrate oval cell morphology. More oval cells are demonstrated in the moderate than in the mild disease cases. The line represents 200 μm.

cells or cells associated with the development of fibrosis, such as hepatic stellate cells and Kupffer cells, may be required to stimulate oval cell proliferation and migration.

The demonstration of oval cells in human liver disease is based on the presence of cells with the typical histological appearance of their counterparts in rodents combined with an appropriate immunohistochemical marker. Several immunohistochemical markers have been described for oval cells including M2-PK, α-fetoprotein, π-GST, and CK 19.[13–15,20,21] The marker M2-PK has been found to be the most reliable marker for oval cells in our laboratory because it does not stain ductal cells as π-GST does and it is not expressed in some adult hepatocytes in the same manner as α-fetoprotein.[13–15] M2-PK is detected in oval cells of adult liver.[15] We have observed that infiltrating lymphocytes in chronic hepatitis C infection can also exhibit some staining for M2-PK. However, the combined use of immunohistochemistry and morphology to characterize oval cells and the exclusion of inflammatory cells on the basis of negative staining for LCA allowed us to identify and score oval cells reliably. The possibility of including lymphocytes was largely eliminated by staining serial sections to ensure that LCA-positive cells were not scored. Other markers, such as π-GST and CK 19, are less specific because they stain biliary epithelium in the adult liver.[21] A recent study which assessed α-fetoprotein as a marker of isolated oval cells found that only 40% of oval cells expressed this marker.[15] We found that π-GST and CK 19 were less specific markers for oval cells in human liver disease because only 30–50% of M2-PK-positive oval cells expressed either π-GST or CK 19, as judged from serial sections. Some mature hepatocytes also exhibited staining for π-GST, whereas biliary epithelium stained strongly for both π-GST and CK 19. These observations are similar to previous reports in which oval cells isolated from animals fed the choline-deficient, ethionine-supplemented diet demonstrated higher frequencies of positive staining for M2-PK than for π-GST or CK 19.[15] It is has been suggested that these differences may reflect the heterogeneity of the oval cell population in terms of their developmental maturity or their commitment to either the hepatocytic or biliary lineage.

The significance of the ductule-like structures formed by oval cells in various disease states is yet to be elucidated. Previous studies in rodents have demonstrated that oval cells can proliferate and form ductule-like structures during carcinogenesis and biliary obstruction.[12–14] Some of the cells in the ductule-like structures also express the adult L-pyruvate kinase isoenzyme (L-PK), with a small population coexpressing M2-PK and L-PK.[12–14] These observations suggest that some cells within the ductular structures may be capable of progressing along the hepatocyte lineage. It is unclear whether the ductule-like structures communicate with the biliary tree.

In conclusion, we have shown that oval cells are frequently found in subjects with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C. There is an association between the severity of liver disease and increasing number of oval cells, consistent with the hypothesis that oval cell proliferation is associated with increased risk of development of hepatocellular carcinoma in chronic liver disease.

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