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Morphometric and Immunohistochemical Characterization of Human Liver Regeneration

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Regeneration in human liver is characterized in part by the formation of ductular structures, so-called ductular hepatocytes in massive hepatic necrosis and bile ductules in mechanical biliary obstruction. In an attempt to characterize the liver regenerative process, we performed image analysis and immunohistochemical staining of the ductular structures in these well defined human liver disorders, 13 cases of massive hepatic necrosis and 9 cases of mechanical biliary obstruction. The proliferation index was determined and the expression of several antigens was localized by immunohistochemical staining using antibodies to α-fetoprotein, α-1-antitrypsin, albumin, and cytokeratin 19. The ductular structures in adult human liver were compared with the developing ductal plates in 11 fetal livers, ranging in age from 9 to 36 weeks of gestation. Image analysis demonstrated that the mean total area, mean nuclear area, and mean cell size of ductular hepatocytes were significantly larger than those of bile ductules ($p < 0.05$). The proliferation index of ductular hepatocytes and bile ductules was significantly higher than that of hepatocytes of normal livers ($p < 0.02$). Bile ducts, bile ductules in mechanical biliary obstruction, ductular hepatocytes in massive hepatic necrosis, and the ductal plate cells in fetal liver showed strong staining for cytokeratin 19, which characterizes intermediate filaments associated with bile duct epithelial cells. Albumin, a liver-specific protein, and α-1-antitrypsin, a protease inhibitor, were strongly expressed in ductal plate cells of fetal liver, hepatocytes, and ductular hepatocytes, whereas bile duct cells and bile ductules were negative for albumin. In summary, ductular hepatocytes demonstrate morphometric and immunophenotypic features of both hepatocytes and biliary epithelial cells, whereas bile ductules share characteristics primarily with fetal ductal plates and mature bile ducts. These findings suggest that ductular hepatocytes in massive hepatic necrosis may serve as bipotential progenitor cells, and bile ductules in mechanical biliary obstruction are related to ductal plates of fetal liver. (Am J Pathol 1995, 147:397–404)

Hepatocytes, bile duct epithelial cells, and hepatic progenitor or stem cells maintain the potential to multiply during adult life. Depending on the type of the injurious agent, the nature of liver disease or the number of hepatocytes lost, liver regeneration may occur by at least two mechanisms.[1–3] First, adult differentiated hepatocytes maintain the capability for several rounds of replication and respond quickly to liver damage associated with mild to moderate hepatocyte loss. Second, several lines of evidence suggest that liver regeneration in viral or toxin-induced massive hepatic necrosis (MHN) may originate from progenitor cells and lead to the generation of ductular hepatocytes (DH). We introduced this term to describe ductular structures composed of relatively small cells with oval nuclei and prominent nucleoli with poorly defined lumen and without basement membranes[4,5] (Figure 1A). In MHN, DH are located around portal tracts and extend into the necrotic lobules. Analogous to oval cells in the rat,[6] DH may have the capacity to differentiate into parenchymal hepatocytes or bile duct cells.[2] DH, also called neocholangioles[7] or biliary hepatocytes, are probably derived from hepatic progenitor cells. Sirica et al[8] observed DH in the furan rat liver model of severe hepatic injury

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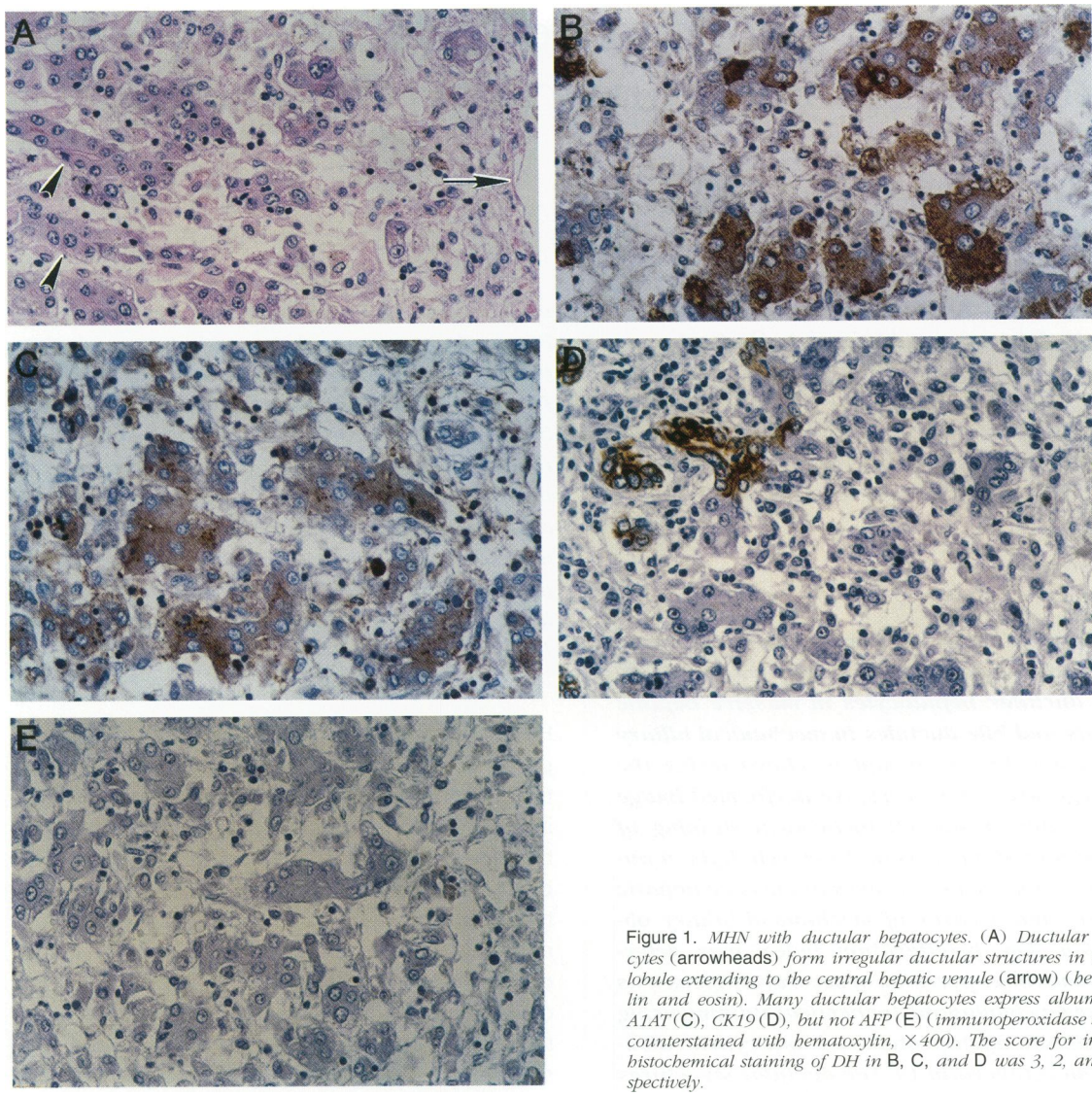

Figure 1. *MHN with ductular hepatocytes.* (A) *Ductular hepatocytes* (arrowheads) *form irregular ductular structures in necrotic lobule extending to the central hepatic venule* (arrow) (*hematoxylin and eosin*). *Many ductular hepatocytes express albumin* (B), *A1AT* (C), *CK19* (D), *but not AFP* (E) (*immunoperoxidase staining counterstained with hematoxylin,* ×400). *The score for immunohistochemical staining of DH in* B, C, *and* D *was 3, 2, and 2, respectively.*

with formation of cholangiolar-like structures composed of biliary epithelial cells and typically a single DH. They proposed differentiation of bile ductular cells to DH.

A second type of ductular structure in liver regeneration, which must be distinguished from ductular hepatocytes, is the bile ductule (BD) (Figure 1B). BD are seen in diseases of the biliary tree such as primary biliary cirrhosis, primary sclerosing cholangitis, and mechanical biliary obstruction. In these diseases, there is a marked increase in the number of bile ductules. Characteristically, they are confined to the portal tracts or marginal zone between portal tracts and liver parenchyma and often accompanied by polymorphonuclear leukocytes.[9–11] BD are characterized by small epithelial cells with round to oval nuclei attached to a basement membrane and surrounding a central lumen (Figure 2A). Desmet et al.[9] suggested that BD are probably derived from propagation of pre-existing bile ductules since mitoses may be observed in their lining cells. BD in adult liver share characteristics with ductal plate cells in fetal liver.[11]

These two types of ductular structures, DH and BD, propagate in a variety of liver diseases. They can be clearly differentiated in two well defined liver disorders in man, MHN with regeneration and mechanical biliary obstruction (MBO). MHN is defined as acute loss of virtually all hepatocytes due to viral hepatitis, drugs, or toxins.[12] MBO is characterized by cholestasis due to obstruction of the large bile ducts by gallstones, strictures or neoplasms.[13] The purpose of this study was to characterize DH and BD in these two

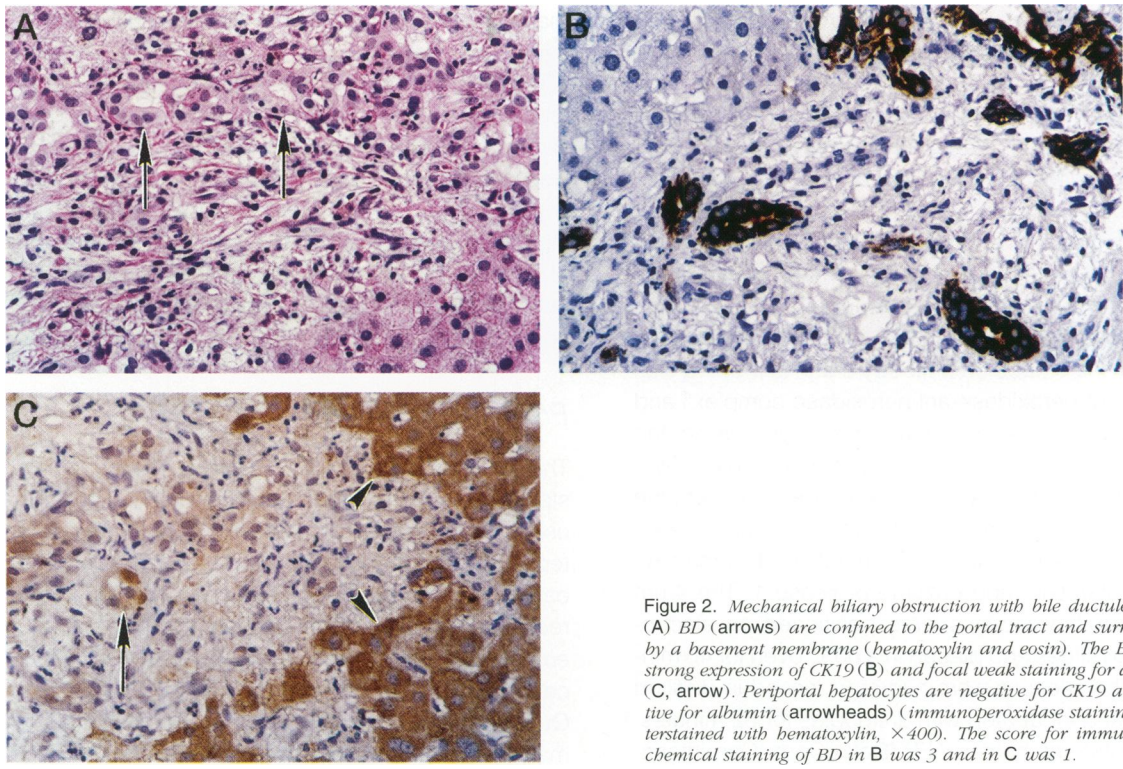

Figure 2. *Mechanical biliary obstruction with bile ductules (BD). (A) BD (arrows) are confined to the portal tract and surrounded by a basement membrane (hematoxylin and eosin). The BD show strong expression of CK19 (B) and focal weak staining for albumin (C, arrow). Periportal hepatocytes are negative for CK19 and positive for albumin (arrowheads) (immunoperoxidase staining counterstained with hematoxylin, ×400). The score for immunohistochemical staining of BD in B was 3 and in C was 1.*

liver diseases by morphometric, histochemical, and immunohistochemical methods to elucidate the relationship of these cell types in liver regeneration.

Materials and Methods

Formalin-fixed, paraffin-embedded liver tissues from 11 fetuses, 13 cases of MHN with liver regeneration, 9 cases of MBO, and 5 normal livers (9 autopsy and 29 surgical pathology specimens) were selected from the files of the Department of Pathology of Tulane University Medical Center and Charity Hospital of New Orleans, LA, and the Mount Sinai School of Medicine of the City University of New York, NY. The patients with MHN presented with signs, symptoms, and biochemical and serological test results of acute hepatitis with jaundice, coagulopathy, and hepatic encephalopathy progressing to hepatic failure. The patients with MBO had epigastric pain, jaundice, and evidence of large bile duct obstruction by endoscopy or imaging methods. The estimated gestational age of the fetuses ranged from 9 to 36 weeks. The selection of cases was based on the presence of many DH in MHN and BD in MBO. The respective ductular structures in tissues of MHN and MBO as described in the introduction were identified histologically and analyzed using the CAS 200 system (Cell Analysis Systems, Elmhurst, IL). In addition, all tissues were studied immunohistochemically with antibodies to α-1-antitrypsin (A1AT), albumin cytokeratin 19 (ALB), (CK19), and α-fetoprotein (AFP), and the proliferation index (PI) was determined, using the nuclear proliferation marker Ki 67.

Morphometry by Image Analysis

The CAS 200 system is an interactive, video image cytometer complete with hardware, software and a modified optical microscope. The CAS 200 Micrometer Application Version 1.0 allowed for area and linear measurements to be derived from live images. In tissue sections, this application uses stereological measurements to calculate the appropriate parameters from each field. For this analysis, one slide for each case was stained with hematoxylin and eosin. The area of 200 ductular structures, the *x* and *y* coordinates, and the area of individual nuclei were determined using the CAS system. The number of nuclei comprising each ductular structure was counted. Approximately 11 ductular structures were measured per case. Each image of the ductular structure with corresponding measurements was printed at high resolution.

Histochemical Staining and Immunohistochemical Analysis

Five cases each of MHN and MBO were stained by periodic acid-Schiff after diastase digestion (DPAS), and Mayer Mucicarmine, or mucin (Poly Scientific, Bayshore, NY). DPAS is a histochemical reaction for carbohydrates and other substances with free hydroxyl groups except for glycogen and is used here to demonstrate basement membranes.

Three antigens (A1AT, AFP, and ALB) were localized by the unlabeled antibody enzyme method with the use of peroxidase-antiperoxidase complex[4] and diaminobenzidine (DAB) as chromogen using the Ventana 320 Immunostainer (Ventana Medical Systems, Inc., Tucson, Arizona.) All reagents, except the antibodies, were produced by Ventana: liquid coverslip, APK wash solution, DAB detection kit, hematoxylin counterstain, and enzyme protease 1. The 4-μm thick paraffin sections were positioned on electrocharged slides and placed in a 60°C oven for 45 minutes. The sections were hydrated, rinsed in distilled water, and incubated with antibodies for 32 minutes. The rabbit anti-human AFP (Dako Corp. Carpinteria, CA), at dilution of 1:15,000, did not require an enzymatic digestion and had a counterstain step. The rabbit anti-human A1AT (Dakopatts, Copenhagen, Denmark), at dilution of 1:15,000, required an enzymatic digestion with protease 1 and had a counterstain step. The mouse monoclonal anti-human ALB (Chemicon International, Inc., Temecula, CA) was diluted at 1:50 and required enzymatic digestion with protease 1. Non-immune rabbit serum, at dilution of 1:10,000 was used as a negative control.

CK19 was localized by the following procedure. Deparaffinized and hydrated sections were rinsed in distilled water and etched for 15 minutes with 0.1% aqueous hydrogen peroxide to remove endogenous peroxidase activity. After rinsing again with distilled water, the sections were covered for 30 minutes with 0.05 gm % saponin (Sigma Chemical Company, St. Louis, MO) to reduce nonspecific background staining. The sections were incubated with 10% normal horse serum for 30 minutes. For 2 hours, the sections were incubated with mouse monoclonal antibody to CK19 (Dako Corp.), at dilution of 1:100. Next, the sections were covered with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA), as secondary antiserum, for 30 minutes at room temperature. After rinsing with PBS (Incstar Corporation, Stillwater, MN), the sections were incubated with avidin-biotinylated peroxidase complex (Vector Laboratories, Inc.) for 30 minutes. The chromogenic reaction was developed by placing DAB solution (3,3′ diaminobenzidine tetrahydrochloride, PBS, and 3% hydrogen peroxide) on the sections for 1 to 2 minutes. After rinsing in distilled water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped. As controls, normal mouse immunoglobulin was substituted for the primary antiserum on consecutive sections, and normal liver was stained during the procedure for CK19. The intensity and extent of immunohistochemical staining was semiquantitated as shown on Table 1 using the given in the legend.

Proliferation Index PI

The PI was determined by immunohistochemical staining for the nuclear proliferation marker Ki-67 using the monoclonal MIB-1 antibody[14] (Immunotech, Inc., Westbrook, ME) on 11 cases of MHN, 8 cases of MBO, and 5 normal livers. The procedure recommended by the manufacturer for fixed paraffin-embedded sections was followed with minor modifications, and ethyl green was used as counterstain. Quantitative assessment of the PI of DH, BD, and normal hepatocytes was performed using the CAS-200 Quantitative Proliferation Index program (QPI), application version 2.0. In tissue sections, this application uses stereological measurements to calculate the PI and is updated with each field analyzed. The image segmentation function allows ductular structures to be analyzed by measuring only specific cells within a defined area.

Statistical Analysis

Data from each specimen were recorded on separate flow sheets and entered into a Macintosh IIsi computer for analysis using Microsoft Excel 4.0 (Microsoft

Table 1. *Semiquantitative Evaluation of Immunohistochemical Staining*

Cases (no.)	Markers			
	CK19	A1AT	AFP	ALB
Fetal liver (11)				
Ductal plates	2.86	2.14	1.43	1.00
Hepatoblasts	0.30	2.10	1.10	1.90
Bile ducts	3.00	1.69	0.13	0.75
Extrahepatic biliary obstruction (9)				
Bile ductules	2.70	0.60	0.00	1.00
Hepatocytes	0.00	1.80	0.20	2.80
MHN (13)				
Bile ductules	2.85	1.62	0.00	0.23
Hepatocytes	0.00	2.18	0.00	2.45
Ductular hepatocytes	2.23	2.15	0.00	2.08

0 = no staining; 1 = focal/weak staining; 2 = diffuse/weak or focal/strong staining; 3 = diffuse/strong staining; focal = <10% of BD or DH; diffuse = >50% of BD or DH.

Corp., Redmond, WA), Exstatix 1.5 (Strategic Mapping, Inc., San Jose, CA), and Cricket Graph 1.3.2 (Cricket Software, Malvern, PA) software programs. The data were analyzed for statistical significance with Yates modification.

Results

Histochemical Analysis

The epithelial cells comprising both the DH and BD were negative for mucin. BD were surrounded by a basement membrane as demonstrated by DPAS staining, whereas DH were not attached to a DPAS-positive basement membrane. The macrophages, as well as the basement membranes and apical portions of the bile duct epithelial cells, stained positively with DPAS. The last were also mucin-positive.

Image Analysis

Using the CAS 200 Micrometer Application Version for cellular measurement, the total area and diameter of the ductular structures was determined. The total area of 97 DH in MHN, 956 ± 385 μm^2 (mean ± SD), was compared with the area of 103 BD in MBO, 749 ± 383 μm^2. The difference and direction of the required *t* value were significant at the 99.9% level. The mean area of the individual nuclei within the DH was 46 ± 13 μm^2 and was compared with the mean area of the BD nuclei of 36 ± 10 μm^2. The difference and direction of the required *t* value were significant at the 99.9% level. The mean diameter of DH was 33 ± 7 μm and was compared with the mean diameter of the BD of 29 ± 7 μm. The difference and direction of the required *t* value were significant at the 99% level. The total number of cells in each ductular structure was counted. This number divided into the total area of the ductular structure gave the approximate cellular area. The mean number of cells per DH was 8 ± 3 μm^2 and per BD 8 ± 4 μm^2. Using this calculation, the mean cellular area of DH was determined to be 129 ± 117 μm^2 and of BD 108 ± 44 μm^2 (mean ± SD). The difference and direction were statistically significant at the 95% level for the required *t* value when comparing the cellular areas of DH *versus* BD.

Immunohistochemical Analysis

Immunohistochemical staining of histological sections resulted in a brown reaction product in the cytoplasm of antigen-containing cells, whereas the background remained unstained. The results are summarized in Table 1. The controls confirmed the specificity of the reactions. Nonspecific staining was virtually absent. Table 2 summarizes the staining pattern in human liver as reported in the literature.

All fetal ductal plate and bile duct cells had diffuse, strong cytoplasmic staining for CK19. Most of these cells were positive for A1AT and negative for ALB. Anti-AFP stained many ductal plate cells, but not bile duct cells. Varying numbers of fetal hepatoblasts were positive for A1AT, AFP, and ALB, and transiently (gestational age of 9 to 16 weeks) positive for CK19. Adult hepatocytes stained for ALB and A1AT, but were negative for AFP and CK19. Many DH of MHN reacted strongly with ALB, A1AT, and CK19, but did not stain for AFP (Figure 1). By contrast, the BD of MBO had strong staining for CK19 and focal, weak staining for A1AT and ALB (Figure 2). BD were negative for AFP.

Proliferation Analysis

Immunostaining for Ki-67 was always nuclear without significant background staining. The PI of DH in MHN was 4.13 ± 0.9 and of BD in MBO 10.15 ± 2.8, both significantly higher ($P < 0.02$) than that of hepatocytes in normal liver (0.22 ± 0.1).

Discussion

A brief discussion of liver embryogenesis is necessary in order to interpret the morphological and immunohistochemical results obtained in our study. In the third to fourth week of gestation, the liver arises as the hepatic diverticulum from the foregut, a mass of immature endodermal cells, which differentiates cranially into hepatic cords and caudally into the gallbladder and extrahepatic bile ducts. The hepatic cords are composed of immature hepatocytes or hepatoblasts and grow into the mesenchyme of the septum transversum, which forms the connective tissue elements of the hepatic stroma and capsule. A capillary plexus derived from the vitelline veins in the outer margins of the septum transversum interposes between the hepatic cords and forms the primitive

Table 2. *Staining Patterns of Liver Cells*[4,11,15,26,28]

Marker	Hepatocytes	Bile duct epithelial cells
CK19	Negative	Positive
A1AT	Positive	Variable
AFP	Negative	Negative
ALB	Positive	Negative
DPAS	No basement membrane	Positive basement membrane

hepatic sinusoids. The development of the intrahepatic biliary system begins with the formation of a single layer of small epithelial cells from periportal hepatoblasts in direct contact with the mesenchyme around the portal veins. Then in the 8 mm embryo (5 to 6 weeks of gestation), a second layer of these cells transforms into the ductal plate, a cylindrical bilaminar layer of small epithelial cells surrounding portal vein mesenchyme. Finally, the ductal plates undergo transformation into tubular structures in the portal tracts, gradually maturing into intrahepatic bile ducts.[9,15–17] The process by which the primitive hepatic cords differentiate into the epithelium of the intrahepatic bile ducts supports the hypothesis that the entire intrahepatic ductal system is derived from hepatoblasts.

The liver stem cell hypothesis proposes that immature precursor cells persist in the human liver and are characterized by phenotypic markers of both hepatocytes and biliary epithelial cells [1,2,18–21] Following massive hepatocyte loss, these progenitor cells may give rise to hepatocyte and biliary epithelial cell lineages. At the present time, however, liver stem cells or progenitor cells have not been clearly defined or localized in human liver. In light of the stem cell hypothesis, we examined the ductular structures in liver regeneration by morphological, histochemical, and immunohistochemical methods.

By standard histochemical methods, DPAS was useful in distinguishing BD from ductular hepatocytes, whereas mucin staining was not. DPAS demonstrated basement membranes around BD, but not ductular hepatocytes, and therefore could be used to distinguish BD from ductular hepatocytes.

By image analysis, we found that the cell size, nuclear diameter, and total area of the ductular hepatocytes were significantly greater than the corresponding values for BD. Thus ductular hepatocytes in massive hepatic necrosis appear to be morphometrically different from the bile ductules found in such processes as mechanical biliary obstruction. Both types of ductular structures proliferate during liver regeneration following MHN and MBO as demonstrated by the PI while mature hepatocytes are dormant. PIs similar to those reported here have been documented in the literature, ie, <1% for normal hepatocytes[22] and 3 to 26% in fulminant hepatic failure.[23,24]

Immunohistochemical stains for AFP, ALB, A1AT, carcinoembryonic antigen, and CK19 have been used to differentiate the hepatocyte and biliary epithelial cell lineages. The fetal liver is the main site for AFP synthesis, and the liver is the only organ for ALB synthesis. AFP reversibly binds fatty acids with high affinity and may be both a specialized carrier of polyunsaturated fatty acids during fetal life and a factor in the transfer of these fatty acids to cells.[25] AFP staining was not seen in cases of mechanical biliary obstruction or massive hepatic necrosis. Blankenberg et al[17] found AFP intensely stained fetal hepatocytes and ductal plate cells, but not adult hepatocytes, BD, or bile duct cells. In our study, AFP was expressed in fetal ductal plate cells and hepatoblasts.

Strong ALB staining was detected in fetal hepatoblasts, adult hepatocytes, and ductular hepatocytes while focal weak staining was observed in ductal plate cells, bile duct cells, and BD. This pattern suggests that cell types such as BD and mature bile ducts follow primary differentiation along the biliary epithelial cell lineage rather than hepatocyte lineage. In agreement with our results, Blankenberg et al[17] found that both fetal and adult hepatocytes showed strong expression of ALB, while the number of positive ductal cells decreased with increasing duct size. ALB and AFP have been observed in oval cells of the rat liver.[19,20] These cells may be analogous to the putative bipotential precursor cells in the human liver. A1AT, synthesized predominantly by hepatocytes, is the primary protease inhibitor in human sera. In our study, A1AT staining pattern of fetal livers was similar to that of ALB. In the adult liver, we found similar staining patterns of A1AT and ALB, whereas Blankenberg et al.[17] detected A1AT only in macrophages. Desmet et al[9] considers A1AT to be a marker of mature hepatocytes. Thus, A1AT and ALB may have shared expression between progenitor cells and adult cells differentiated along hepatocyte lines.

Cytokeratins are the major structural proteins of intermediate filaments in epithelial cells; CK19 represents an antigenic marker for bile duct epithelial cells and BD.[26] In the fetal liver, we demonstrated transient expression of CK19 in hepatoblasts from 9 to 16 weeks gestation. In addition, there was intense cytoplasmic staining of all ductal plate cells and bile duct epithelial cells. Stosiek et al[26] found that monoclonal CK19 antibodies stained hepatoblasts, ductal plates, and the epithelial cells of the bile ducts early in gestation. Around 10 weeks gestation, fetal hepatocytes lost CK19, while ductal plate and bile duct cells continued to express the cytokeratin. In the studies of Stosiek et al[26] and Nomoto et al.,[27] and our study, mature hepatocytes failed to express CK19, while the cytoplasm of bile duct epithelial cells, BD, and many ductular hepatocytes contained the antigen. The negative staining of adult hepatocytes for CK19 most likely reflects loss of this cytokeratin as the precursor cells differentiate along hepatocyte lines. These findings support the notion that hepatoblasts

represent common progenitor cells for both hepatocytes and bile duct epithelial cells.

Ductular hepatocytes have been proposed as bipotential progenitor cells for regenerating hepatocytes and bile duct cells.[2] Ductular hepatocytes or oval cells with phenotypic properties of both hepatocytes and biliary cells have been described in cases of fulminant hepatitis,[4,7] cirrhosis,[28] furan,[8,29] D-galactosamine,[30] and bile duct-ligated/CCl_4-treated rats.[31] In our study, similarities were found between ductular hepatocytes and both adult hepatocytes and biliary epithelial cells. Many ductural hepatocytes showed intermediate to strong reactivity with antibodies to CK19, A1AT, and ALB. Thus, many ductular hepatocytes express both A1AT and ALB, markers for adult hepatocytes, as well as CK19, a marker for biliary epithelial cells. Unexpectedly, ductular hepatocytes failed to stain for AFP; this may be related to transient expression of AFP by these cells or to limits of the methodology. Taken together, these findings suggest that ductular hepatocytes are similar to oval cells in the rat and have the capacity to differentiate into parenchymal hepatocytes and bile duct cells.

In conclusion, ductular hepatocytes expressed immunohistochemical markers characteristic of biliary epithelial cells, hepatocytes, and their precursors. In contrast, BD exhibited immunophenotypic characteristics primarily of bile ducts and ductal plate cells of the fetal liver. Although in human material only static observations are possible, these findings suggest that ductular hepatocytes in massive hepatic necrosis are related to bipotential progenitor cells which may persist, in limited numbers, into adulthood.

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