

HEPATOCTYTIC PROLIFERATION IN CHRONIC LIVER DISEASE: A STUDY OF LIVER BIOPSIES USING IMMUNOHISTOCHEMICAL LOCALIZATION OF PROLIFERATING CELL NUCLEAR ANTIGEN

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It is well recognized that in several parts of the world, a significant proportion of chronic liver disease (CLD), consisting of chronic hepatitis and liver cirrhosis, results from chronic subclinical infection by hepatitis B and C viruses, HBV and HCV.¹ Unless reversed in time, this chronic hepatitis (CH) is a forerunner of liver cirrhosis (LC) and may further progress to hepatocellular carcinoma.^{2,3} The association of cirrhosis with hepatocellular carcinoma (HCC) is also well documented.⁴ While the sequential progression of chronic hepatitis to cirrhosis and ultimately to cancer seems to be well established, the exact mechanism of viral hepatocarcinogenesis is yet to be clearly defined.⁵ It has been suggested that the most important factor for the development of HCC is not the integration of viral DNA, but possibly the persistent liver cell necrosis and the resultant irregular regeneration.⁶ Therefore the proliferative rate of regenerating hepatocytes may be an important pathogenetic and prognostic factor in chronic liver disease.⁷

A number of markers have been used in the assessment of the proliferative status of cells, like bromodeoxy-uridine,⁸ Ki-67⁹ and DNA polymerase alpha.¹⁰ Techniques utilizing these markers are complex, however, and require fresh or snap frozen tissue, except for Ki-67, which can be used on paraffin sections. Proliferating cell nuclear antigen (PCNA), an accessory protein of DNA polymerase delta, is one of the best markers for evaluating cell proliferation in studies on retrospective material, since the antigen can be localized in routine formalin-fixed paraffin-embedded tissue.^{7,11-14}

Limited reports are available on the proliferation kinetics of normal human livers, though in recent years several studies reported on hepatocytic proliferation rates in acute

and chronic liver diseases. Normal hepatocytes are generally quiescent and divide very slowly.¹⁵⁻¹⁷ High proliferative rates have been reported in hepatocellular carcinoma, cirrhosis and acute hepatic failure.^{11,18-22} Nakamura et al.⁷ have shown that there is no significant difference in PCNA-labeling indices between chronic viral hepatitis types B and C. With increasing knowledge of the biology of hepatitis C virus infection, information on hepatocytic proliferation kinetics is emerging now.

In a recent study on the prevalence of HBV- and HCV-associated chronic liver diseases in liver biopsy material over the last decade, we observed a steady rise of HCV-associated diseases and a decline in HBV-associated ones.²³ Chronicity of HCV infection leads to CH and LC much more frequently than chronic infection by other hepatitis viruses,²⁴ and the former infection shows a higher degree of association with hepatocellular carcinoma in several parts of the world.²⁵

Therefore, we considered it worthwhile to examine for hepatocytic proliferation using PCNA-labeling in our material of chronic liver diseases and to note any differences between hepatitis B- and C-associated diseases.

Materials and Methods

A total of 56 biopsies from cases of chronic liver disease—28 of chronic hepatitis and 28 of liver cirrhosis—were studied. These were chosen from the files of the Mubarak Al-Kabeer Hospital (attached to the Faculty of Medicine, Kuwait University) at random, without initial knowledge of their hepatitis virus infection status. The majority of these biopsies were taken through percutaneous needle aspiration, while only a few were surgically removed. The histological diagnosis of CH or LC was confirmed in each case. Cases of possible drug-induced and autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver injury and associated hepatocellular carcinoma were all excluded.

Immunohistochemical staining for PCNA was done in all 56 biopsies on formalin-fixed paraffin-embedded tissue sections by the standard peroxidase-antiperoxidase (PAP)

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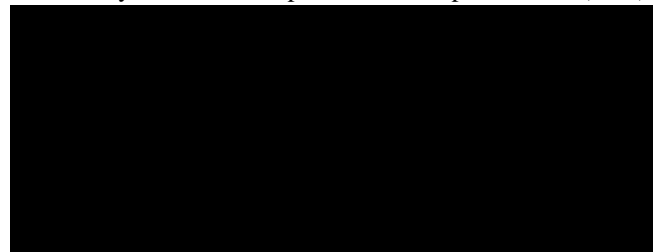


FIGURE 1. A case of chronic hepatitis with two hepatocytes showing strong to moderate staining for PCNA (arrow). A multinucleate hepatocyte in the right upper corner is negative for PCNA (Hematoxylin counterstain 240x).

technique. Mouse monoclonal antibody against PCNA (clone PC 10; M/s Dakopatts, Denmark) was used in a dilution of 1:250. The magnitude of liver cell proliferation was assessed in each case by determining the PCNA-labeled fraction (PLF) expressed as a percent, among at least 500 hepatocyte nuclei. Since labeled nuclei were almost always randomly distributed in the hepatic lobules, the eyepiece field was reduced to approximately one-eighth by black paper discs, and several areas of the tissue section were examined. Only moderately to strongly stained nuclei were counted as positive when agreed upon by two observers simultaneously viewing the material. Three histological parameters that may be relevant to hepatocyte regeneration, namely piecemeal necrosis (PN), fibrosis (FI) and portal inflammation (PI), were semiquantitatively assessed in hematoxylin-eosin stained tissue sections of each biopsy. Each individual parameter was classified into one of four categories—absent, mild, moderate and severe—on criteria similar to those described by us earlier.²³ The assessment was made on a subjective semiquantitative basis depending on the number of cells and structures involved and the extent of distribution.

Serological data on HBV and HCV infection were then looked for in all 56 cases from the files. Information was available in 28 cases. Serodiagnosis of HBV infection was available only on HBsAg testing, while for HCV infection it was available on tests for anti-HCV antibodies by the second-generation ELISA test (Abbott, USA) against C100-3 and C-33 structural and against core regions of the virus. Ground glass hepatocytes were looked for in hematoxylin-eosin stained slides and staining for hepatitis B surface antigen (HBsAg) was carried out by Orcein and the standard immunoperoxidase technique. PLF values of

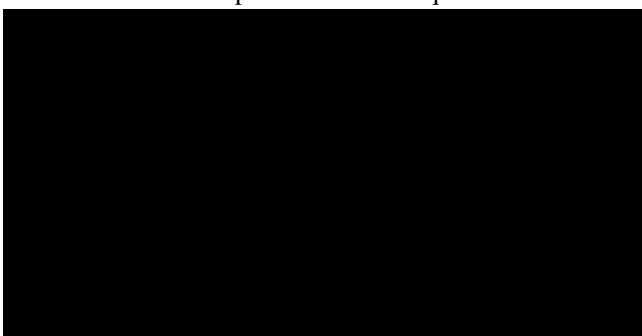


FIGURE 2. Chronic hepatitis. Few hepatocytes with positive PCNA staining (arrows) are seen away from the area of piecemeal necrosis in the right upper part (Hematoxylin counterstain 400x).

CH and LC cases associated with HBV and HCV infection were compared.

Statistical analysis was performed using non-parametric tests (Mann-Whitney).

Results

Peroxidase-labeling for PCNA immunoreactivity was confined to cell nuclei. The staining pattern varied from a few faint nuclear granules to strong densely granular staining filling the whole nucleus. Only moderately to strongly stained nuclei were taken as positive (Figure 1). In cases with background staining, immunoreactivity was considered as positive only if the nuclear signal was significantly stronger than that of the cytoplasm. As stated earlier, labeled hepatocytes were randomly distributed in the parenchyma. Neither the location nor the intensity of nuclear staining was related to foci of liver cell necrosis and degeneration (Figure 2).

In the total material, PCNA-labeled fraction varied from 0 to 9.4%, with the vast majority (96%) below 5% (Table 1). The PLF was 0 to 9.4%, with a mean of 1.8 ± 2.0 in CH and 0 to 8.4% with a mean of 1.59 ± 1.8 in LC. The difference was not significant. On splitting into four different ranges of PLF, less than 1%, 1%-3%, 3%-5% and more than 5%, the number of cases were 14, 10, 3, 1 and 13, 10, 4, 1 in CH and LC respectively. In both categories about four-fifths (82%-86%) had PLF less than 3% (Table 1). Again, within PLF subgroups, there was no difference in proliferative rates between the two categories of chronic liver disease, CH and LC.

Correlating PLF values with varying grades of piecemeal necrosis, fibrosis and portal inflammation also did not reveal any significant association (Table 2). Most cases had mild to moderate piecemeal necrosis (PN) and fibrosis (FI) and generally less than 3% PLF. Even in the three cases with severe grade necrosis, one each had PLF values less than 1%, between 1% and 3%, and 3% to 5%.

Similarly, among 10 cases with severe portal inflammation (3 in CH and 7 in LC), PLFs were less than 1% in seven, 1% to 3% in two, 3% to 5% in one, and above 5% in none.

Information on hepatitis virus serology was available in 28 cases, 22 and 6 cases being seropositive for HCV and HBV infection respectively. Table 3 compares the various PLF values in the two categories of CLD according to the type of associated hepatitis virus infection. Difference in hepatocyte proliferation rates between CLDs associated with the two infections was not statistically significant, though there seems to be a trend of somewhat higher proportion with 1%-5% PLF in cases of HCV infection (11 of 22) than in those with HBV infection (2 of 6). Unfortunately, the number positive for HBV infection is too small. When present, PCNA labeling was not preferentially located near areas of focal necrosis or fatty change (Figure 2). Thus in our material, no correlation was found between hepatocyte proliferation rates and the grade of hepatic injury related to these three parameters.

Discussion

Synthesis of cyclin or proliferating cell nuclear antigen (PCNA) starts in the nucleus in the late G₁ phase but attains peak only in the S phase of the cell cycle.²⁶ Therefore, detection of significant amounts of this protein marker is a reliable indicator of cell replication. In our material, distinctly positive staining for PCNA was restricted to the nuclei and for estimating replicating fractions, we counted cells with only moderately and strongly stained nuclei. A recent report²⁷ suggests that methanol-fixed tissues yield more realistic PCNA-labeling indices than paraformaldehyde-fixed ones. However, several other studies on liver tissues have reported satisfactory results on routine formalin-fixed material.^{7,11,22}

Hepatocellular proliferation rates observed in our material were generally similar to those reported by others in chronic hepatitis and cirrhosis.^{7,11,22} A little less than half of our cases showed PLFs less than 1% (Table 1) and in a few no labeled nuclei were observed. Ojanguren and colleagues²² found no PCNA labeling in more than half (7 of 13) of biopsies showing cirrhosis, while Ballardini et al.¹¹ reported absence of strong labeling indicative of premitotic phase in an unspecified number of cirrhotic livers that did or did not develop hepatocellular carcinoma during follow-up. Since replicating cells are always randomly distributed, non-detection of labeling may merely be due to relatively small tissue samples obtained by biopsies, particularly on needle aspiration.

TABLE 1. PCNA-labeled fraction in the two categories of chronic liver disease (CLD): chronic hepatitis (CH) and liver cirrhosis (LC).

Category of CLD	N	PCNA labeled fraction			
		<1%	1-3%	3-5%	>5%

CH	28	14 (50%)	10 (35.7%)	3 (10.7%)	1 (3.6%)
LC	28	13 (46.4%)	10 (35.7%)	4 (14.2%)	1 (3.6%)
Total	56	27 (48.2%)	20 (35.7%)	7 (12.5%)	2 (3.6%)

*Figures within brackets indicate percentages of total.

TABLE 2. Relationship between hepatocytic proliferation (PCNA-labeled fraction) and different grades of piecemeal necrosis (PN), fibrosis (FI), and portal inflammation (PI).

CLD	Grade	PCNA-labeled fraction																	
		<1%			1-2%			3-5%			>5%			<1%->5%					
		PN	FI	PI	PN	FI	PI	PN	FI	PI	PN	FI	PI	PN	FI	PI			
CH	Absent	0	4	0	0	0	0	0	1	0	0	0	0	0	0	0	5	0	0
	Mild	9	9	6	6	9	8	1	1	1	1	1	1	1	1	1	17	20	16
	Mod.	4	0	5	3	1	2	1	1	2	0	0	0	0	0	0	8	2	9
	Severe	1	1	3	1	0	0	1	0	0	0	0	0	0	0	0	3	1	3
LC	Mild	2	1	0	2	0	0	1	0	0	0	0	0	0	0	0	5	1	0
	Mod.	10	12	9	8	10	8	3	4	3	1	1	1	1	1	1	22	27	21
	Severe	1	0	4	0	0	2	0	0	1	0	0	0	0	0	0	1	0	7

Mod.=moderate.

PLFs in chronic hepatitis and cirrhosis were no different in our material. Virtually identical PCNA-labeling indices have been reported in chronic viral hepatitis and cirrhosis, though separately, in some earlier reports.^{7,11,22} On the other hand, in a recent communication²⁷ hepatocyte proliferative activity has been reported to decrease at the stage of cirrhosis from a higher rate in active chronic hepatitis. Compared to chronic persistent hepatitis, chronic active hepatitis, particularly of the more severe types, has been reported to show higher replicative rates.^{7,27,28} Currently, however, division of chronic hepatitis into these categories is considered inappropriate and qualifying the grade of activity and the stage of fibrosis in a case of chronic viral hepatitis seem adequate for clinical purposes. Three histological parameters that are likely to be associated with chronic hepatocellular insult, namely, piecemeal necrosis, portal inflammation and fibrosis, did not correlate with the magnitude of cell proliferation in either chronic hepatitis or cirrhosis. Nakamura and colleagues⁷ found a direct relationship between hepatitis activity index (HAI) scores and PCNA-labeling indices (LI) in cases of chronic persistent hepatitis, but not in chronic active hepatitis. They suggested that in the former, the LI may be related to lobular and portal inflammation. These latter histologic features are seen more in chronic lobular hepatitis, which is considered to be a benign form of disease, whereas

TABLE 3. PCN-labeled fraction (PLF) in chronic hepatitis (CH) and liver cirrhosis (LC) according to type of associated hepatitis virus infection.

Category of CLD	N	Hepatitis virus infection			
		HBV		HCV	

PLF values	N	CH	LC	N	CH	LC
<1%	3	2	1	11	6	5
1-3%	1	1	0	9	8	1
4-5%	1	1	0	2	1	1
>5%	1	1	0	0	–	–
ALL	6	5	1	22	15	7

piecemeal necrosis is the hallmark of chronic active hepatitis. Therefore, as in our cases, their material showed no correlation between activity and hepatocyte replication. Admittedly, the number of biopsies studied by us is not large and as such, statistically relevant conclusions may not be drawn from this material. However, a trend is obvious and it appears that correlating the activity of the hepatic process in chronic viral injury with liver cell proliferation rates is not immediately apparent. This area needs to be studied further. Factors other than those that can be readily assessed morphologically may be important in determining the hepatocyte proliferation in chronic liver disease.⁷

We had very few cases of HBV-associated chronic liver disease. A temporal decline in this category and a significant increase in HCV-associated disease has been recently reported by us.²³ We believe that this may be related to the institution of control measures against HBV infection several years prior to those against HCV infection in areas such as ours. As in the study by Nakamura et al.,⁷ we did not find significant difference between PLFs in HBV- and HCV-associated diseases, though in our material there appeared to be a somewhat higher rate in the latter. Also, in terms of location, PCNA-labeling did not necessarily occur in the vicinity of piecemeal necrosis, fibrosis or portal inflammation. If this excess of PLF in HCV-associated chronic liver disease is confirmed by correcting the limitation of small numbers, it may partly explain the intriguing phenomena of some biological features of chronic HCV infection recently reported,²⁹ regarding the more frequent association with cirrhosis and quicker progression to hepatocellular carcinoma when compared to those in chronic HBV infection.

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