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Immunohistochemical comparative study of fibrosis and biliary ductular reaction in alcoholic and viral chronic hepatitis

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Abstract

Our study includes 102 cases of liver biopsy previously diagnosed with chronic alcoholic hepatitis and also B and C viral hepatitis. In these cases, we analyzed the extension of fibrosis with two different methods. First, we evaluated fibrosis with the subjective Knodell score; secondly, we used digital image analysis to achieve this. We also used immunohistochemical methods to mark those cells positive at Smooth Muscle Actin (SMA) and Glial Fibrillary Acidic Protein (GFAP). We have observed that the extension of fibrosis was most predominant in cases with B viral chronic hepatitis, while the number of cells responsible of fibrosis (stellate cells, myofibroblasts) was highest in C viral chronic hepatitis. These differences help clinician to divide patients into those who may be treated with interferon and those treatable with antiviral therapy. We observed ductular reaction (as shown by cytokeratin 7 immunostaining) within the lobular structure more frequently in alcohol related chronic hepatitis, whilst in C viral chronic hepatitis this reaction was more readily seen in portal spaces. We have concluded that patients with C viral hepatitis can benefit most from a correctly indicated hepatic biopsy since in these cases the lesions might be observed in an early and potentially curable phase.

Keywords: liver fibrosis, liver regeneration, image analysis, ductular reaction.

Introduction

Staging fibrosis in hepatic biopsies is mandatory, not only for patient evaluation but also for controlling the efficacy of applied treatment. Therefore, study of fibrosis in hepatic biopsies is the key factor in determining the stage of alcoholic, viral or autoimmune liver disease. It aids the examiner in appreciating severity of the destructive processes that eventually may lead to cirrhosis, hepatocellular carcinoma and liver failure.

Fibrosis and cirrhosis, in fact, represent different stages of a dynamic, bidirectional disease spectrum that can either progress or regress. Research aimed at determining fine details of liver fibrogenesis brought substantial proof that support this bidirectionality and therefore opening up new anti-fibrogenetic therapeutic ways [1–3]. The optimal moment of treatment and follow-up requires novel diagnostic methods to assess the initial and intermediate phases of fibrosis. Recognition of oval cells and myofibroblasts represents a new method in evaluating liver fibrosis. Since it is know that they play an important role in the etiology of fibrosis, their assessment brings new insights into modern therapeutic approach.

Material and Methods

In order to grade fibrosis, we determined the subjective Knodell score for 102 liver biopsy cases [4, 5], selected out of 108 cases processed in the Pathology Department of Mures Emergency County Hospital in 2007. The results were then compared with digital morphometry analysis of fibrosis performed on the same specimens.

Digital image processing system composed of a Nikon DN100 CCD camera connected to a research-level microscope, Nikon Eclipse E800 was used. Microscopic images were captured using the camera's control unit and saved to a compact flash drive, later transferred to a personal computer. Microscopic slides were stained with Masson's Trichrome method and from each case a number of three to 10 digital pictures were captured, depending on sample size, at one hundred magnification. Images were saved in 24-bit color depth [6, 7].

Digital image processing was performed using Wayne Rasban's ImageJ programme developed at the National Institute of Health, USA [8]. Processing of images consisted of measuring fibrotic tissue areas against normal liver tissue. Proportions between the two were determined using O'Brian's formula, determining the average area of fibrosis (PMF):

$$PMF = \sum_n PF/n,$$

where *n* is the number of digital images captured for each case.

We studied fibrosis by marking two types of cells: hepatic stellate cells and myofibroblasts. It is recognized that hepatic stellate cells originate from Ito cells after these are activated by various cytokines released by the

injured hepatocytes. Myofibroblasts are derived from activated stellate cells, initiators of fibrosis in liver. These cells were marked by the following antibodies: GFAP, Desmin and SMA.

Biliary ductular reaction was determined using cytokeratin 7 (CK7) expression in the mentioned chronic hepatitis types. Cytokeratin 7 is a well-known marker of cholangiocytes and recently has been proved to mark their precursor cells also, located in the Herring channels [10]. It is worth mentioning that CK7-positive cells are absent from lobular level, their presence being a pathological phenomenon. Therefore, even a minimal nuber of marked cells was considered pathological.

For immunostains we used LabVision antibodies (Table 1) and the EndVision system by LabVision. For CK7 we have performed antigen retrieval in EDTA solution, pH 9, at 100^0C. After that, the endogenous peroxidase blocking through hydrogen peroxide incubation was performed and slides were incubated with Primary Antibody for 20 minutes, Primary Antibody Enhancer for other 20 minutes and, finally, with Large Volume HRP solution for 30 minutes. In case of GFAP and SMA antibodies, we have followed same steps, except heat antigen retrieval. Development was performed with DAB (diaminobenzidine dihydrochloride) solution, which was applied for 3–5 minutes. Nuclei were stained using Mayer's Hematoxylin.

Table 1 – *Antibodies used*

No.	Antibody	Clone	Dilution	Company	Note
1.	GFAP	6F2	1:75	Dako	stellate cells myofibroblasts
2.	SMA	1A4	1:2000	LabVision	myofibroblasts
3.	CK7	OVTL12/30	1:100	LabVision	cholangiocytes

GFAP – Glial Fibrillary Acid Protein, SMA – Smooth Muscle Actin, CK – Cytokeratin.

⊟ Results

Beside sex, age, liver disease type and Knodell score (evaluated by two pathologists), to each case, another parameter has been quantified, namely the average fibrosis area (PMF – percentage of fibrotic tissue). This latter value was calculated using digital morphometry (Table 2). As shown in Table 2, necrotic and inflammatory processes, expressed by histologic activity, fibrosis,

Knodell score and PMF value were most readily seen in chronic B viral hepatitis. Alcohol related liver disease was characterized by intermediate values, while C viral hepatitis had the lowest values of histologic activity and fibrosis with lowest scores of fibrosis. In the same table, the concordance of fibrosis with inflammation and necrosis as shown by histological activity average, can be observed.

Table 2 – *Average values of fibrosis*

H	Average age [years]	Urb/Rur	Histologic activity	Fibrosis 1	2	PMF%
Al	55	8/7	7.8	2.2	2.2	34.00
B	39	20/9	8.87	1.8	2.2	36.74
C	46	35/23	4.5	2	1.7	26.26

H (hepatitis): Al – alcoholic, B – viral B, C – viral C; Urb – urban, Rur – rural, 1 – Knodell score by first pathologist; 2 – Knodell score by second pathologist; PMF – average fibrosis percentage.

Results of immunohistochemical studies were presented in Table 3. They further characterize the differences in pathological changes observed in the three types of hepatitis (H).

**Table 3 – *Percentage of GFAP-, SMA- and CK7-positivity***

H	No. of cases	GFAP S%	P%	I%	SMA S%	P%	I%	CK7 L%	P%
Al	15	80	33.3	0	26.6	6.66	0	32	46
B	29	82.7	41.3	13.7	62	37.9	6.8	24	64
C	58	86.2	55.1	20.6	94.8	70.6	20.6	19	72

H – hepatitis: Al – alcoholic, B – viral B, C – viral C, S% – percentage of cases with perisinusoidal positivity, P% – percentage of cases with portal positivity, I% – percentage of cases with interface positivity, L% – percentage of cases with lobular positivity.

Cells positive for GFAP and SMA, namely hepatic stellate cells and myofibroblasts, had the following localizations: perisinusoidal (Figure 1) and perihepatocytic (Figure 2), along the so-called interface or contact lines and, finally, at portal levels. Number of these cells in the three structural zones (sinusoidal, portal and interface) increased from alcohol related hepatitis, through B- to C-viral chronic hepatitis. Both of them were most abundant at sinusoidal level and also in C-viral chronic hepatitis, because they show same process of differentiation and transformation. They number was lower in alcohol-related chronic hepatitis.
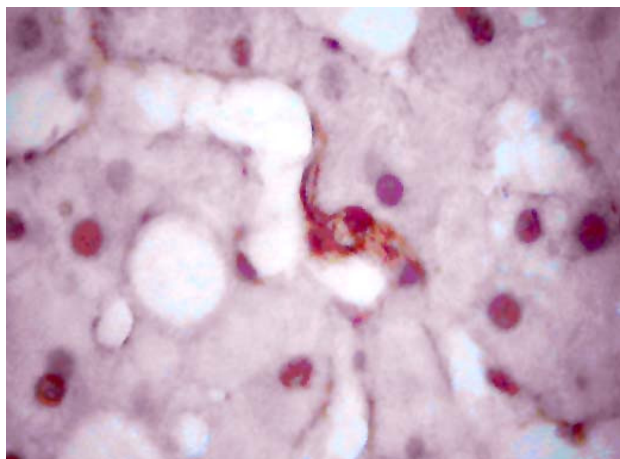
Figure 1 – *GFAP-positive perisinusoidal myofibroblast, ob. 40×.*
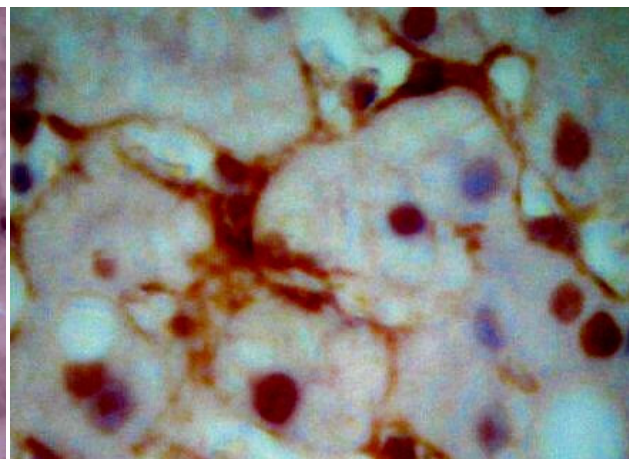
Figure 2 – *Reaction to SMA highlights fine cytoplasmic processes of myofibroblasts, ob. 100×.*

We have determined biliary ductal reaction, using CK7-antibody, in two distinct zones: the portal and lobular areas. Ductular reaction was most readily observable at portal level in alcohol related chronic hepatitis, since it has the greatest number of CK-posi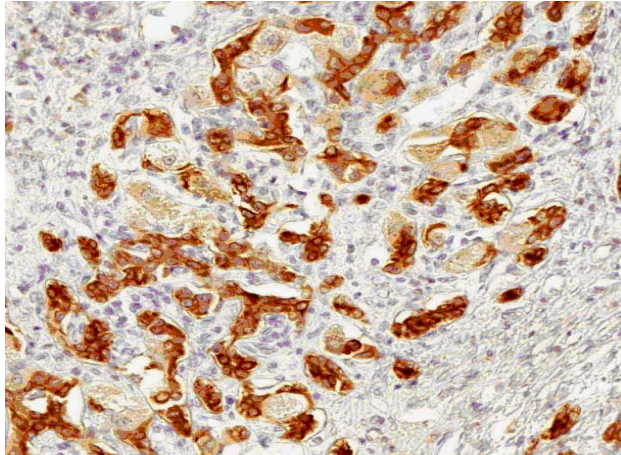tive cells in this area (Figure 3), as opposed to B- and C-viral chronic hepatitis (Table 3) where ductular reaction is mostly present in lobules. It seems obvious that in various inflammatory processes cells at the periphery of the lobule alter their immuno-phenotype, proved by their positivity for CK7 (Figure 4).
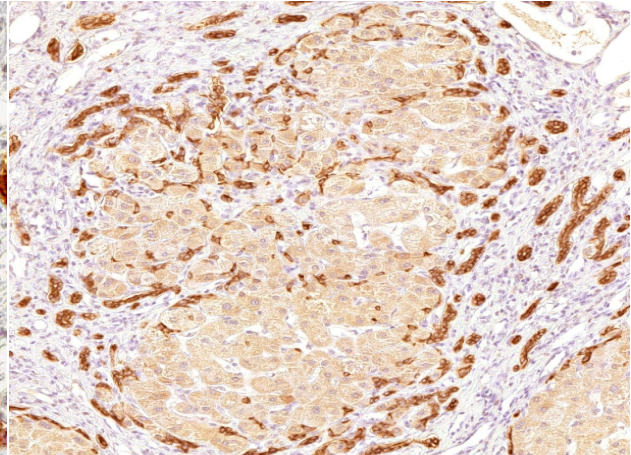

Figure 3 – *Trabecula of CK7-positive cells, ob. 10×.*

Figure 4 – *Hyperplasia of CK7-positive cells disintegrates the structure of the pseudo-lobule, ob. 4×.*

Discussion

Liver tissue regeneration is based on two mechanisms: one lead by hepatocytes and the other by hepatic progenitor cells (stem cells or oval cells). These mechanisms are mutually exclusive, the former mechanism being responsible for regeneration processes seen in acute lesions, while the latter in chronic hepatocyte lesions [11]. Fibrosis, an omnipresent process of the human body, is a generic tissue response to injury, shock or tissue destruction and its main purpose is restitution of tissue and organ integrity. If the factors that initiate fibrosis appear suddenly and are of short time, healing occurs via *per primam intentionem*. In cases where healing is delayed or the initiating factor persists over a longer period of time, fibrosis will eventually develop leading to the accumulation of extracellular matrix components, scar tissue foci and functional impairment [12].

Interferon and anti-viral treatment, not until recently introduced in the therapy of viral hepatic disease, also facilitated the development of various follow-up methods of chronic hepatic disease, which includes grading of fibrosis. From these methods we used a subjective one (Knodell score) and an objective one (digital morphometry). We had to use the Knodell score since many of the hepatology clinics demand the use of this scoring system. It is worth mentioning however that Ishak score, which is based on the evaluation of six parameters, seems to be more precise in describing the grade of fibrosis [13].

The number of articles that take advantage of digital morphometry is increasing [13–15]. Next to proving that our laboratories are equipped with basic equipment to perform these quantifications, it is nevertheless important to mention that preparation of images is of utmost importance. In this regard, so-called "parasitic areas" are of great interest, since having the same color as the fibrotic areas (especially when using Masson's trichrome stain) they can potentially alter morphometry findings. Using Syrius red might resolve this issue [15]. Average fibrosis grades obtained with the two methods are very close to each other; therefore, automated quantification of liver fibrosis is a reliable method [7].

Low average values of fibrosis (as determined by the Knodell score and PMF) in C viral hepatitis is surprising; however, others have come to the same findings [16]. Changes in patient selection criteria for interferon treatment partially explain this. On the other hand, most authors also included in their studies cases with previous interferon treatment.

Recent studies have been showed that many of the hepatocytes are capable of transforming into myofibroblasts and to produce fibrosis. This includes biliary epithelial cells. Moreover, not until recently, it has been proved that even some hepatocytes may have these properties [17, 18]. The presence of an epithelial-mesenchymal transformation (EMT) factor explains this phenomenon. This process is initiated by members of the transforming growth factor beta family, which suppress epithelial protein expression (e.g. E-cadherin) and activate mesenchymal markers, such as matrix-metalloprotease-2 (MMP-2) and smooth muscle actin (alpha-SMA) [19]. Hepatic progenitor cells have been isolated, that are capable of repopulating the injured liver. These cells express both mesenchymal and epithelial markers [20] and are activating upon the depletion of accommodation reserves of normal hepatocytes [21, 22]. During this phenomenon a great number of small and oval shaped cells are accumulate in liver. They are characteristically are located near of stellate cells and extracellular matrix, which is producing mesenchymal cells. Oval cells are capable of differentiating into either hepatocytes or cholangiocytes, while

stellate cells differentiate into collagen producing myofibroblasts [17, 23]. GFAP affinity for hepatic stellate cells is well-documented [24]. It is accepted that these cells gain myofibroblastic traits proven by the expression of smooth muscle actin [25, 26]. Our results, presented in Table 3, showed an increased number of these cells in B and C viral chronic hepatitis as opposed to chronic alcohol related liver disease. These findings indicate an increased fibrogenic potential of B and C viruses, and their primary sinusoidal localization highlights the transforming effect of beta-TGF, produced by cholangiocytes and Kupffer cells [27].

The presence of GFAP and SMA expressing cells indicate various phases of fibrosis. Lower number of SMA-positive cells and presence of CK7-positive cells in portal spaces indicate and advanced fibrosis with fibrotic tissue maturation, this pattern being observed predominantly in alcohol-related chronic hepatitis. An increased number of these cell types is found in B and C viral chronic hepatitis and indicates an initial stage of, or ongoing fibrosis. These observations are in harmony with the findings of Japanese researchers who observed that decrease of epimorphine levels, which is a mesenchymal protein expressed in hepatic stellate cells, is associated with increase of SMA-positive cells in the initial phases of fibrosis. In recrudescent phases of acute and chronic liver lesions and in the regenerative phases, increase of epimorphine levels leads to decrease of SMA-positive cells [28].

Ductular reaction is responsible for the proliferation of small biliary channels and of marginal cells of the hepatic lobule, this phenomenon being extensively studied by a research team of our department from 1973 [29, 30]. It has been confirmed that these cells are in fact small cholangiocytes with secretory properties that are able to produce inflammatory cytokines, chemoattractive proteins and inhibitors of apoptosis [31]. Cytokeratin 7 is expressed in progenitor cells and cholangiocytes, but hepatocytes lack its expression [32]. CK7 and CK9 are alternatively termed biliary cytokeratins [10].

Difference between the percentages of portal CK7 positive cells reflects the severity of fibrosis. In alcohol-related chronic liver disease, the fibrosis is more advanced, with the presence of mature fibrotic tissue, whilst in B and C viral chronic hepatitis the fibrotic process is in an earlier stage or is in its ongoing phase in the Kiernan spaces.

⌗ Conclusions

Our results point out the utility of immunohistochemical methods in emphasis of those cells which play an important role in hepatic fibrogenesis (marked with SMA, GFAP and CK7), in B- and C-viral chronic hepatitis. These methods seem to have a lower significance in alcohol related chronic hepatic disease.

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