ORIGINAL ARTICLE

Quantitative morphometric and immunohistochemical analysis and their correlates in cirrhosis – A study on explant livers

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Abstract

Background. Reproducible structural analysis was made on cirrhotic human liver samples in order to reveal potential connections between morphological and laboratory parameters. Material and methods. Large histological samples were taken from segment VII of 56 cirrhotic livers removed in connection with liver transplantation. Picro Sirus red and immunohistochemically (smooth muscle actin [SMA], cytokeratin 7 [CK7], Ki-67) stained sections were digitalized and morphometric evaluation was performed. Results. The Picro Sirus-stained fibrotic area correlated with the average thickness of the three broadest septa, extent of SMA positivity, alkaline phosphatase (ALP) values and it was lower in the viral hepatitis related cirrheses than in samples with non-viral etiology. The extent of SMA staining increased with the CK7-positive ductular reaction. The proliferative activity of the hepatocytes correlated positively with the Ki-67 labeling of the ductular cells and inversely with the septum thickness. These data support the potential functional connection among different structural components, for example, myofibroblasts, ductular reaction and fibrogenesis but challenges the widely proposed role of ductular cells in regeneration. Conclusion. Unbiased morphological characterization of cirrhotic livers can provide valuable, clinically relevant information. Similar evaluation of routine core biopsies may increase the significance of this ‘Gold Standard’ examination.

Key Words: ductular reaction, fibrosis, hepatic myofibroblasts, image analysis

Introduction

‘Cirrhosis’ is one of the most ancient terms of pathology and as it often happens with old terminology it may not be able to meet the requirements of new challenges. This leads to proposals of histological sub-classification of cirrhosis [1–4], the International Pathology Study Group even suggested that the use of the word cirrhosis should be discontinued [5].

Cirrhosis has been widely considered to be the end stage of chronic liver diseases. However, reversibility of this condition is emerging as an exciting and clinically important possibility [6,7]. It has been always puzzling that the disappearance of reliably verified cirrhosis in humans is extremely rare, while most of the experimentally induced liver cirrhoses in rodents are completely reversible. Therapeutic advances in the field of chronic liver diseases also

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raise new questions [8]. The recent tremendous progress in antiviral therapy raises the hope that, at least in certain cases, efficient treatment of hepatic viral infections could induce regression of the fibrotic process by the elimination of responsible stimuli. The pathogenesis of cirrhosis is also very far from clear, it has even been mentioned as the chronic regeneration of the liver [9]. It is not clear, however which cell population(s) contributes to the regeneration. There are wide differences in the reported proliferative activity of hepatocytes [10–14], while others claim that the senescent hepatocytes of cirrhotic livers are not able to go through the cell cycle. The participation of ductular reaction is also controversial. These cells may represent the activation of the hepatic progenitor/stem cell compartment and they would replace the lost liver parenchyma, when the senescent hepatocytes are not able to divide [15–17]. Lin et al. [18] even proposed that the cirrhotic nodules are the clonal progenies of the ductular cells.

Liver cirrhosis is defined by morphological criteria. Despite recent achievements in imaging techniques, histological examination is still the gold standard of diagnosis [19]. Therefore, histomorphological examinations also address the correlation between morphological parameters and clinical behavior. Most of these studies are based on the evaluation on standard liver biopsy specimens [20–23]. Two major drawbacks of these examinations are the small size of the biopsy specimen and the poor reproducibility of the morphological interpretation. The needle biopsy contains an average 1/50 000 of the entire hepatic tissue [24]. Heterogeneity of the fibrotic process can bring false results in such a small sample size. The subcapsular layer of liver usually contains more fibrotic tissue and macrotrabecular cirrhotic nodules can be missed in needle biopsy [25]. Systematic studies have unequivocally proved that the intra- and interobserver reproducibility is also influenced by the sample size [26]. In addition, the assessment of most of the histological slides is inevitably subjective. This subjectivity, however, could be reduced by computer-assisted morphometric analysis which enables quantitative measurement on a continuous scale [22,23,27–29].

In order to at least partially avoid these problems, we performed detailed histological analysis on large sections of cirrhotic livers removed from the patients in connection with liver transplantation. All the examined histological blocks were taken from segment VII and their area exceeded 1 cm². The traditional histological staining was completed by standardized immunohistochemical reactions. To increase the reproducibility, digital image-based morphometric studies were performed on the specimens.

We have analyzed 56 cirrhotic samples and correlated the most important morphological and clinical parameters. The area occupied by the Picro Sirius red-stained fibrotic bundles correlated with the average thickness of the three broadest septa, the extent of smooth muscle actin (SMA) positivity and alkaline phosphatase (ALP) values. The extent of cytokeratin 7 (CK7) staining, an indicator of the intensity of ductular reaction, showed significant correlation with SMA staining and the latest aspartate aminotransferase (AST) value. In parallel with the higher Ki-67 labeling of hepatocytes, we observed higher ductular cell proliferation, but lower values of septum thickness.

Methods

Patients

Clinical and histological data of 56 patients (43 males, 13 females) of Department of Transplantation and Surgery of Semmelweis University were analyzed. The patients underwent liver transplantation between 2009 and 2012. Thirty-two of the 56 patients had chronic viral hepatitis (30 HCV, 4 HBV), the other 24 patients suffered from alcoholic liver disease (ALD), autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) based or cryptogenic cirrhosis. A total of 13 livers also contained hepatocellular carcinoma (HCC; 10 HCV, 1–1 ALD, AIH and PBC). The study protocol conformed the ethical guidelines of the 1975 Declaration of Helsinki, as revised in 1983, as reflected in an a priori approval of the ethical committee of Semmelweis University (TUBEB 125-1/2010; date: 05. 05. 2011).

Clinical data

The clinical data were collected from the latest available laboratory tests before transplantation. We gathered the values of the main laboratory tests of liver (Serum bilirubin, AST, alanine aminotransferase, ALP, γ-glutamyl transferase, albumin, total protein, international normalized ratio) and kidney (creatinine) function and also the main quantitative blood count parameters (hemoglobin, white blood cell, Thr [platelet count]). The current Model for End-Stage Liver Disease score was calculated. We checked if (any degree of) ascites, hepatic encephalopathy, esophageal varices or variceal bleeding was present in the patients’ history (Table I).
Morphological analysis

Tissue samples were taken from segment VII, which is the target tissue for the usual core biopsies and embedded into paraffin by routine procedure. The area of the sections was always > 1 cm². Traditional histological staining was performed (Figure 1A). The extent of fibrosis as relative area was determined by

Table I. Clinical and morphological data of patients. Those parameters that differ significantly between the HCV/HBV and the non-viral groups are highlighted in bold font.

|                          | Average (range) (n = 56) | HCV/HBV group average (n = 34) | Non-viral group average (n = 22) |
|--------------------------|--------------------------|---------------------------------|---------------------------------|
| Age                      | 49.38 (20–61)            | 51.88                           | 45.5                            |
| SeBi (µmol/L)            | 52.79 (11–270)           | 44.7                            | 65.3                            |
| INR                      | 1.48 (1.12–4–74)         | 1.37                            | 1.65                            |
| Creatinine (µmol/L)      | 87.89 (30–9–220)         | 78.24                           | 102.81                          |
| AST (IU/L)               | 80.27 (24–236)           | 85.57                           | 72.07                           |
| ALT (IU/L)               | 59.06 (12–204)           | 64.34                           | 50.92                           |
| ALP (IU/L)               | 319.33 (82–1037.7)       | 266.65                          | 400.76                          |
| GGT (IU/L)               | 119.11 (20–575)          | 113.26                          | 128.13                          |
| TP (g/L)                 | 73.78 (59.5–96)          | 72.93                           | 75.08                           |
| Alb (g/L)                | 34.3 (19–44)             | 35.46                           | 32.55                           |
| Hb (g/L)                 | 122.98 (84–172)          | 128.77                          | 114.05                          |
| WBC (G/L)                | 5.44 (1.43–23)           | 4.88                            | 6.3                             |
| Thr (G/L)                | 91.49 (33–229)           | 80.53                           | 108.43                          |
| MELD                     | 14.57 (9–30)             | 13.26                           | 16.59                           |
| Picro Sirius red (%)     | 17.74 (8.89–29.65)       | 16.59                           | 19.5                            |
| SMA (%)                  | 16.08 (8.33–24.53)       | 15.73                           | 16.63                           |
| CK7 (%)                  | 4.64 (1–16.58)           | 5.06                            | 4                               |
| Septum thickness (µm)    | 202.76 (78.66–309)       | 291.5                           | 270.5                           |
| Ki-67 hepatocytes (%)    | 0.403 (0–1.64)           | 0.37                            | 0.43                            |
| Ki-67 ductular cells (%) | 0.632 (0–2.8)            | 0.58                            | 0.74                            |
| Ascites                  | 58.9% (33 patients)      | 58.8% (20 patients)             | 59.1% (13 patients)             |
| Encephalopathy           | 42.9% (24 patients)      | 38.2% (13 patients)             | 50% (11 patients)               |
| Esophageal varices       | 87.5% (49 patients)      | 85.3% (29 patients)             | 90.9% (20 patients)             |
| Variceal bleeding        | 39.2% (22 patients)      | 32.4% (11 patients)             | 50% (11 patients)               |

Abbreviations: ALP = Alkaline phosphatase; ALT = Alanine transaminase; AST = Aspartate transaminase; CK7 = Cytokeratin 7; GGT = γ-glutamyl transferase; Hb = Hemoglobin; INR = International normalized ratio; MELD = Model for end-stage liver disease; SeBi = Serum bilirubin; SMA = Smooth muscle actin; TP = Total protein.

Figure 1. Hematoxylin and eosin staining (A), Picro Sirius red staining (B) and SMA immunohistochemistry (C) of a HCV-related cirrhotic sample; septal width was assessed on Picro Sirius red-stained sections by measuring the longest distance (arrows) between two cirrhotic nodules. CK7 immunostaining (D) outlines the ductular reaction. Scale bar for Figure 1: 200 µm.
Picro Sirius red staining (Figure 1B) by means of the image analyzing system Olympus Cue-2. The septal thickness was defined as the distance between adjacent nodules, the width of the three thickest septa was measured on digitalized images and the average was calculated [23]. SMA (Figure 1C), CK7 (Figure 1D), Cytokeratin 19 (CK 19), CD 34, CD 45 and Ki-67 (data not shown) immunostaining was performed using an automated Leica Bond immunostainer, with the Leica Bond Polymer refine detection system and 3,3’diaminobenzidine as the chromogen. The applied primary antibodies were the following: SMA: Dako M0851 (clone: 1A4) dil.: 1:200; CK7: Biogenex MU255-UC (clone: OV-TL 12/30), dil.:1:100; CK19: Dako M0772 (clone: BA17), dil.: 1:50; CD34: Dako M7165 (clone: QBEnd 10), dil.: 1:200; CD45: Dako M0701 (clone: 2B11 + PD7/26), dil.: 1:100; Ki-67: Dako M7240 (clone: Mib-1), dil.: 1:400. The immunostained sections were digitalized and the SMA and CK7-positive area was measured with the Olympus Cue-2 image analyzing system. The proliferative activity of different cell compartments was characterized by Ki-67 staining. Five thousand hepatocytes and 500 ductular cells were counted, the percentage of Ki-67 labeled nuclei is given as result (Table I). Three healthy liver was also examined (Supplementary Figure 1).

Statistical analysis

Statistical analysis was performed with StatSoft Statistica software (StatSoft, Inc., version 8.0). The deviation from Gaussian distribution of variables was tested with Kolmogorov–Smirnov and Lilliefors’ method. Correlation between variables was tested using Spearman rank correlation test. Spearman correlation coefficients were determined and correlations were visualized on graphs. Continuous variables in different groups (viral/non-viral, tumorous/non-tumorous) were compared with Mann–Whitney U test, 2*1 sided exact p values were used. Results were considered significant at a p value £ 0.05.

Results

The investigated clinical and morphological parameters are shown in detail on (Supplementary Table 1).

The Picro Sirius red stain has affinity for type I and III collagens and correlates well with hydroxyprolin
content [30]. Therefore, the relative area stained positively is a reliable marker of the collagen content. This value correlated with the highest septum thickness. The SMA antibody decorates the hepatic myofibroblasts [31], the major sources of collagen. The ratio of SMA staining correlated with Picro Sirius red-stained area. The Picro Sirius red staining was also more expansive in patients with higher ALP values. The correlation was also significant between two of the related parameters: septum width and SMA staining (Figure 2). The Picro Sirius red-positive area was lower in viral hepatitis-related cirrhotic samples than in samples with other etiological factors, and a similar distinction could be observed on the ALP value (Figure 3).

CK7 and CK19 are expressed by the biliary cells in the liver [32]. Hepatocytes can also become positive for these 'biliary' cytokines under cholestatic conditions but due to the lower staining intensity, these cells could be excluded by the morphometric analysis. CK7 and CK19 immunostaining resulted in almost identical reactions, thus we analyzed only the CK7 data. The presented CK7 value represents the area occupied by bile ducts and the extent of ductular reaction. This value was significantly higher in the samples with higher SMA staining. Interestingly, the

Figure 3. (A) ALP values were significantly lower in the viral hepatitis-related samples (median = 253 IU/L, n = 34) than in samples with non-viral etiology (median = 360 IU/L, n = 22; p < 0.05). (B) The extent of Picro Sirius red-stained area was also significantly lower in viral hepatitis-related samples (median = 14.47%, n = 34) than in samples with non-viral etiology (median = 19.33%, n = 22; p < 0.05).
CK7-positive area was also higher with higher AST values (Figure 4). CK7 staining did not show significant correlation with the extent of fibrosis.

The proliferative activity detected by Ki-67 staining was low in the hepatocytes as well as in the ductular cells, in most cases below 1% and the staining did not show any preferential distribution. Surprisingly, there was positive correlation between the proliferation of the two cell populations. Furthermore, the Ki-67 labeling index of hepatocytes was significantly lower in the cirrhotic samples with broader cirrhotic septa (Figure 5). Transdifferentiation of hematopoietic cells into hepatic cells has been reported [33] but we could not observe any CD34 or CD45 positivity over the ductules (data not shown). We have determined the current Child–Pugh stage of our patients, but no significant difference could be observed in any of the morphological parameters between different stages (data not shown) (Supplementary Figure 2). There was also no significant correlation between morphological parameters and history of ascites, encephalopathy, esophageal varices or variceal bleeding. No statistical difference could be observed between tumorous and non-tumorous liver samples.

Finally, we were not able to classify our cirrhotic liver samples into subgroups based on the investigated parameters.

**Discussion**

We have performed morphological analysis on cirrhotic liver samples. The tissue blocks were taken from segment VII where core biopsies are usually gained.

The extent of fibrosis by the Picro Sirius red index number can be used to monitor the progression of the fibrotic process. Goodman et al. [27] proved that morphometric evaluation of fibrosis is superior to traditional semi-quantitative methods. Several studies found correlation between the extent of fibrosis and portal pressure [1,20,22,23]. Unfortunately, we could not measure the hepatic venous pressure gradient in our patients. The extent of Picro Sirius red staining
correlated with the highest septum thickness. This is another parameter which has been used to characterize the level of fibrosis [1,23]. The correlation between these two parameters on large histological sections supports that these values grow parallelly and either of them is suitable for the characterization of fibrosis. SMA decorates the hepatic myofibroblasts, the cells that are responsible for the production of most matrix components. As far as we know, this parameter has not been compared with traditional histological data. The observed correlation between the SMA value and both of the fibrotic index numbers (highest septum width, Picro Sirius red positive area) supports that the fibrosis is an active process and the amount of matrix producing cells is increased in the latest stage of the hepatic disease. If this is so, the arrest of myofibroblast activity might be a potential target of therapeutic intervention even in advanced cirrhotic livers. Our observation about the connection between the extent of fibrosis and ALP value is also a novel result and it may reflect that the fibrosis hinders bile drainage. This latter result is indirectly supported by another connection revealed by our study, namely that both ALP value and Picro Sirius red stained area were significantly higher in cirrhosis with non-viral etiology than in HCV or HBV-infected livers. Hall et al. [28] have already described, that the collagen proportionate area was lower in viral infection-related cirrhotic livers, than in cirrhoses of any other etiologies. These observations and potential consequences deserve further follow-up.

The participation or role of hepatic progenitor cells in the fibrogenesis is another hot issue. Although there are several candidates, the cells constituting the ductular reaction are most widely accepted as hepatic progenitor cells [34]. The intensity of the ductular reaction assessed by a semiquantitative method correlated with the extent of fibrosis [14,35] and the number of activated hepatic stellate cells [35] in chronic HCV-related hepatitis. CK7 is one of the established markers of this cell population. We used a reproducible morphometric analysis of CK7 staining to estimate the intensity of ductular reaction. In our case, this parameter did not show significant correlation with the extent of Picro Sirius red positivity, but its level showed correlation with the extent of SMA staining. This controversy may indicate a closer, direct connection between the ductular and myofibroblastic reactions [36,37]. In fact, close relationship between the CK7 and SMA stained structures could be observed on serial sections. It may also suggest that the connection between the ductular reaction and fibrogenesis is not so closed, at least in this late stage of hepatic disease. The significant correlation between the CK7 staining and the AST value is, however, a novel and surprising result. Correlation between ductular reaction and AST value was also reported in liver-transplanted patients with recurrent Hepatitis C infection [34]. This result might imply that the ductular reaction may induce parenchymal damage or vice versa, the ductular reaction is intensified by hepatocytic damage. Serum transaminase level also coincided with progenitor cell expansion in an experimental model of fibrotic liver regeneration in mice [38].

Cirrhosis is occasionally regarded as a chronic regenerative process [15,17,39]. According to the recently popular opinion, when the hepatocytes are not able to divide due to cellular senescence, the parenchyma is maintained by the ductular reaction [13,16,17,37]. Our data do not support this view, because the lower proliferative activity of the hepatocytes was associated with smaller labeling index of the ductular cells. Eleazar et al. could not reveal correlation between the proliferative activity of any of the cell lineages in chronic hepatitis samples [15]. Interestingly, the Ki-67 positive hepatocytes were less common in samples with larger septum thickness, which is with most advanced cirrhosis. The ductular cells differentiate into hepatocytes via the so-called intermediate hepatobiliary cells [34], which can be easily recognized on CK7 stained slides. Such cells were seen only in very few of our sections. The low proliferative activity of ductular cells detected in our study may not reflect the recently proposed focal regenerative activity of ductular reaction [40], since the average proliferative activity was counted on large histological fields. Our samples represent mostly advanced, end-stage cirrhotic livers. It cannot be excluded, that in these ‘burned out’ cirrhotic livers both cell populations are approaching senescence and therefore we could not observe the compensatory role of the ductular cells. It would be important to study these parameters in earlier stages of the cirrhotic process. The morphological parameters of HCC containing and tumor-free samples were compared since in these patients HCC was the indication of transplantation and they may represent an earlier stage of the fibrotic process. Nevertheless, no difference was revealed.

We have performed morphological analysis on human cirrhotic liver sections. It was new in our study that the analysis was not performed on core biopsies but on relatively large sections and the morphometric analysis was done on digitalized images including immunohistochemically stained sections. Based on our experience the morphometric analysis of collagen proportionate area and ductular reaction (CK7 or 19 immunostaining) of routine liver core biopsies
could provide valuable and more reliable information, than the recently mostly applied semiquantitative evaluation, as also recommended by Lee et al. [41]. Naturally, the small sample size of a core biopsy means inevitable limitation. The correlations found between different morphological and laboratory parameters support the recent view [29] that cirrhosis is not a stage, but a step-wise process, which can be characterized by quantitative measurements. However, using explanted liver samples, all our specimens represented advanced hepatic diseases, what naturally limited the possibility of classification. Furthermore, the investigated parameters could not be adjusted to the prognosis of the patients. Our results support the close relationship between the extent of fibrosis and the activity of myofibroblasts and progenitor cells. With our tools, we could not confirm the regenerative role of the ductular reaction. The potential connection between the extent of fibrosis/ALP-etiology and intensity of ductular reaction-AST value should be carefully analyzed in further studies.

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Supplementary material available online

Supplementary Table 1
Supplementary Figures 1 & 2