ORIGINAL PAPER

IMMUNOREACTIVE HEPATIC STELLATE CELLS IN BIOPSY MATERIAL IN CHILDREN WITH CHRONIC HEPATITIS B. THE FIRST REPORT IN PEDIATRIC PATIENTS

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The research objective was to identify and quantify the immunohistochemically (IHC) stained hepatic stellate cells (HSCs) in children with chronic hepatitis B (CHB), including staging (S), location in the hepatic lobule, and correlation with hepatocyte count.

Retrospective morphological analysis was based on liver biopsies obtained from 70 CHB children before antiviral treatment. To determine fibrosis stage, the Batts and Ludwig scoring system was applied. Immunohistochemical examinations used monoclonal antibodies against α-SMA.

IHC observations in CHB children revealed a significant positive correlation between the mean number of α-SMA immunopositive HSCs within the hepatic lobule \( r = 0.518; p < 0.001 \) and fibrosis stage. In biopsy specimens with intensive fibrosis, most HSCs had an elongated shape and demonstrated evidently strong immunoexpression of cytoskeletal protein α-SMA. The mean counts of HSCs/100 hepatocytes (in high power field) in 4 study groups, i.e. with S-0, S-1, S-2, S-3, were 5.00, 5.98, 9.80, 12.19, respectively. Interestingly, in most groups the highest count of immunoreactive HSCs/100 hepatocytes was in the intermediate zone, indicating its high metabolic activity in liver fibrogenesis.

Immunohistochemical and statistical investigations of HSCs in children with CHB showed a close positive correlation of cell count with fibrosis intensity, which may have prognostic implications in this pathology.

Key words: hepatic stellate cells, immunohistochemistry, α-SMA, chronic hepatitis B, children, fibrosis.

Introduction

Chronic hepatitis is the main cause of liver fibrosis, including cirrhosis. Its major etiologies involve hepatotropic viral infections (HBV, HCV), excessive alcohol consumption, and metabolic and autoimmune liver disorders [1, 2, 3, 4, 5, 6, 7]. The incidence of chronic hepatitis B (CHB) in Poland has increased in recent years. Chronic hepatitis B was most frequently diagnosed among pediatric patients aged 15-19, which is most likely associated with the very high incidence observed among children under 4 years in the early 1990s [8].

The process of liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) components, which is the result of disturbances in the dynamic balance between synthesis (fibrogenesis) and degradation (fibrolysis). The course of fibrosis
Involves deposition of excessive quantities of ECM proteins in the space of Disse, which initiate collagen fibroplasia and constitute the earliest morphological form of this pathology [1, 5, 9, 10, 11, 12, 13]. It is assumed that hepatic stellate cells (HSCs), building the so-called nonparenchymal hepatic cells (NPCs) that are topographically bound to the sinusoidal wall, make the greatest contribution to fibrogenesis. It is the HSCs which being transformed to metabolically active transitional HSCs and myofibroblastic cells are responsible for the synthesis of the extracellular matrix and play a key role in this pathology [2, 13, 14, 15, 16, 17, 18].

The hepatology literature concerning the morphogenesis and development of liver fibrosis in the course of CHB refers mainly to adult patients and experimental studies [4, 5, 7, 9, 13, 15, 17]. Up to now, the microscopic picture of HSCs assessed in biopsy material obtained from large groups of pediatric patients has not been documented. Therefore, the major objective of this study was identification and quantitative evaluation of HSCs stained for immunohistochemistry (IHC) with a protein marker for these cells, α-smooth muscle actin (Monoclonal Mouse Anti-Human Alpha Smooth Muscle Actin), in biopsy material obtained from children with chronic hepatitis B. We took into consideration selected morphological parameters – stage of fibrosis, location of HSCs within the hepatic lobule, intensity of biomarker expression in the cells, and their relationship with hepatocyte count. The current study is a continuation of the morphological research conducted in our center on biopsy material obtained from children inhabiting northeastern Poland, who suffer from various liver disorders, especially those accompanied by liver fibrosis [6, 19, 20, 21, 22, 23].

Material and methods

Patients

In all study cases, the retrospective morphological analysis of liver biotopes was performed using biopsy material collected in the Department of Medical Pathomorphology, Medical University of Białystok.

Liver oligobiopsy specimens were obtained from 70 children with type B chronic hepatitis (46 boys and 24 girls), aged 3-17 years (mean 11), who were qualified for interferon α or lamivudine therapy before antiviral treatment in the Department of Pediatrics, Gastroenterology and Allergology, Medical University of Białystok.

The patients had a documented HBV infection (HBsAg- and HBeAg-positive) of > 6 months duration. Patients infected with HCV, with other liver disorders (autoimmune hepatitis, chosen metabolic diseases) and with liver cirrhosis were excluded from the study.

The study was approved by the Bioethics Committee, Medical University of Białystok.

Collection and preparation of tissue material

For morphological analysis (histological and IHC), liver oligobiopsy specimens were obtained from each patient by blind biopsy. The tissue material was fixed in 4% buffered formalin for 24 hours at 4°C. The sections were stained histologically (routine with Mayer’s hematoxylin and eosin [HE] and additionally for connective tissue stroma components: according to Gomori, by the Azan method according to Mallory in the Heidenhain modification, according to Masson-Goldner). A semiquantitative histologic scoring system according to Batts and Ludwig was used to determine the extent of liver fibrosis (staging – S) [24].

Immunohistochemical investigations

In order to reveal the presence, intracellular distribution and intensity of α-SMA expression within the stellate cells, liver biopsy specimens obtained from children with CHB were subjected to IHC reaction with mouse monoclonal antibody (Clone 1A4, N 1584, Dako) against epitopes of the human α-SMA molecule. Following incubation of preparations with the antibody for 20 minutes in a humid chamber, at room temperature, the reaction was performed using the detection system En Vision (K 8012/K 8013 kit). In order to visualize the antigen-antibody complex, the preparations were incubated in a 3,3-diaminobenzidine solution (chromogen DAB, Dako). Then for better visualization of the cell picture they were contrasted in hematoxylin solution for 2 min. After dehydration in ethanol at increasing concentrations and passage through a series of xylenes, the preparations were closed in a Canadian balsam and subjected to microscopic investigations.

Considering the intensity of IHC staining, showing α-SMA expression in the cytoplasm of the cells studied, HSCs demonstrated weak, moderate and strong immunoreactivity.

Statistical analysis

For statistical analysis performed in high power field (400 ×), all the α-SMA stained HSCs present within the respective zones, i.e. 1 – periportal, 2 – intermediate and 3 – central (perivenous), of three randomly chosen hepatic lobules and all hepatocytes found within the same zones were added up. In each of the lobular zones, the mean numbers of hepatocytes and HSCs were calculated for one patient and then for all study patients.

The IHC findings were subjected to statistical analysis using nonparametric tests: Kruskal-Wallis test and Mann-Whitney test; Spearman’s correlation coefficient was also calculated. The level of p < 0.05
was considered statistically significant. The calculations were based on SPSS Statistics (Statistical Package for the Social Sciences).

**Results**

The semiquantitative numerical scoring system evaluating staging according to Batts and Ludwig (24) was used in microscopic investigations to distinguish 4 study groups: group I (S-0) – 5 patients (2 boys; 3 girls) aged 8-13 years (mean 10.8 years); group II (S-1) – 27 patients (18 boys; 9 girls) aged 3-17 years (mean 11 years); group III (S-2) – 31 patients (21 boys; 10 girls) aged 6-17 years (mean 11.5 years); group IV (S-3) – 7 patients (5 boys; 2 girls) aged 4-13 years (mean 10.7 years).

**Immunohistochemical assessment of α-SMA positive HSCs**

**Group I (S-0)**

In the patients with CHB in the S-0 group, α-SMA positive HSCs were markedly dispersed throughout the hepatic lobule, mostly in the perisinusoidal region. They showed poorly enhanced expression of the biomarker, which was manifested by a delicate cytoplasmic IHC reaction, with a tendency to microgranular submembranous accumulation of the protein (Fig. 1A). The mean number of immunoreactive HSCs in zone 1 (periportal zone) calculated per mean number of hepatocytes (147.0 ± 36.2) was 6.27 ± 2.34. In zone 2 (intermediate), the mean number of liver parenchymal cells (145.4 ± 41.78) corresponded to an average of 8.27 ± 4.28 HSCs. In zone 3 (central; perivenous), the mean number of hepatocytes (147.8 ± 45.35) was accompanied by 7.47 ± 3.48 α-SMA positive HSCs on average (Table I). The above data correlated with the mean number of HSCs/100 hepatocytes in the respective zones of the hepatic lobule. In the periportal zone, their number was 4.86 (the smallest), in the intermediate zone 6.67 (the largest), and in the central zone 6.4. On average, there were 5.98 α-SMA immunopositive HSCs per 100 hepatocytes.

**Group II (S-1)**

In children with CHB in the S-1 group, α-SMA immunoreactive HSCs were found not only in the perisinusoidal location, dispersed throughout the hepatic lobule, but also within fibrotic foci in the portal and periportal areas. Most cells demonstrated moderately or strongly positive cytoplasmic staining for protein, mainly with submembranous accumulation of the biomarker. They were more frequently found in the intermediate part of the hepatic lobule (Fig. 1B).

The number of HSCs in zone 1 was significantly lower as compared to zones 2 and 3 (p < 0.001). The mean number of hepatocytes in zone 1 was 191.14 ± 73.3, being accompanied by 9.28 ± 4.15 immunoreactive HSCs. In zone 2, the mean number of hepatocytes (191.69 ± 69.53) corresponded to the mean number of HSCs (12.79 ± 4.74). In zone 3, the mean number of hepatocytes (192.59 ± 75.65) went along with 12.32 ± 4.34 HSCs with a positive reaction to α-SMA protein (Table I). The above data correlated with the mean number of HSCs/100 hepatocytes in the respective zones of the hepatic lobule. In the periportal zone, their number was 4.86 (the smallest), in the intermediate zone 6.67 (the largest), and in the central zone 6.4. On average, there were 5.98 α-SMA immunopositive HSCs per 100 hepatocytes.

**Group III (S-2)**

Most α-SMA immunopositive HSCs in the S-2 group showed a far stronger IHC reaction to fibrosis protein. The color reaction product was located in the cell cytoplasm, with a tendency to accumulate mainly in submembranous areas. Immunopositive HSCs were found more often than in group II; some cells were elongated. They were situated both in the perisinusoidal region, mainly around the central vein of the lobule and within the intermediate zone of the hepatic lobule (Fig. 1C), and within the foci of intensive periportal fibrosis or single porta-portal bridges. The study showed a significantly lower number of HSCs in zone 1 of the hepatic lobule as compared to zones 2 and 3 (p < 0.001). In zone 1, the mean number of immunopositive hepatic stellate cells was 14.6 ± 7.26 per mean number of 180.7 ± 65.76 hepatocytes. However, in zone 2, the mean number of HSCs was 18.99 ± 7.83 per mean number of 179.45 ± 67.3 hepatocytes. In turn, in zone 3, the mean number of hepatocytes was 179.27 ± 63.3 per mean number of 19.25 ± 6.86 HSCs (Table I). The above data correlated with the mean number of HSCs/100 hepatocytes, being the smallest in zone 1 (8.08), whereas in zone 2 it was 10.58, and it was slightly higher in zone 3 (10.74). With reference to the whole hepatic lobule, the mean number of immunopositive HSCs/100 hepatocytes was 9.8.

**Group IV (S-3)**

Among all the groups with type B chronic hepatitis, S-3 patients had the highest number of α-SMA positive HSCs, both in perisinusoidal and in the extraperisinusoidal area, i.e. situated within the foci of intensive fibrosis (bridging fibrosis with architectural disorders). Most perisinusoidal α-SMA positive HSCs were found in the intermediate part of the lobule (Figs. 1D and 1E). The quality of the IHC picture of these cells was similar to that in the S-2 patients. Most
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HSCs demonstrated evidently strong reaction for the cytoskeletal protein, showing a cytoplasmic staining pattern. These cells were often elongated (Figs. 1D and 1E). In the periportal zone of the hepatic lobule, the mean number of immunopositive HSCs was 19.67 ± 6.82 per mean number of 167.62 ± 58.4 hepatocytes. In the intermediate zone, the mean number of HSCs (23.48 ± 9.63) corresponded to that...
of hepatocytes (172.14 ± 58.37). In the perivenous zone, the mean number of HSCs was 19.38 ± 8.19 (Table I). The mean number of HSCs/100 hepatocytes was 11.73 in the periportal zone, 13.64 (the highest) in the intermediate zone and 11.21 (the lowest) in the central zone. On average, throughout the hepatic lobule, 12.19 immunoreactive HSCs were found per 100 hepatocytes. In all the patients, irrespective of the extent of fibrosis, immunoreactive HSCs were the least common near the limiting plate of the lobule. There was a significant positive correlation between fibrogenesis intensity and the increase in the mean number of immunopositive HSCs (r = 0.518; p < 0.001).

Discussion

The immunohistochemical study of α-SMA conducted on the liver biopsy material obtained from children with CHB showed a substantial effect of chronic viral infection on the number, lobular distribution and picture of the population of immunopositive HSCs.

Biopsy investigations of the liver, both histological and immunomorphological ones, have long been considered the gold standard in the determination of fibrosis progression [9, 11, 19, 25, 26, 27, 28] which, in children and adults, is the most common sequel of chronic infection caused by hepatotropic viruses, i.e. HBV and HCV. Although attempts are made worldwide, including our center, to determine noninvasive serous markers of liver fibrosis [4, 7, 29, 30, 31, 32], their diagnostic advantage over blind biopsy of the organ has not yet been proven [7, 9, 19, 25, 26, 27, 28, 29].

The current IHC observations of liver biopsy specimens from children with CHB in group I (without features of fibrosis) showed that α-SMA immunopositive HSCs were markedly dispersed throughout the lobule, being located in the perisinusoidal region and demonstrating a poorly expressed cytoplasmic expression of the fibrosis biomarker. However, the IHC pictures of HSCs, being qualitatively similar in groups II, III and IV, were characterized by strong (group II) and/or markedly strong expression of the cytoskeletal protein, α-SMA (group III and IV). The immunoreactive HSCs, frequently elongated, were located mainly perisinusoidally and within the new and already formed fibrotic foci. Most cells demonstrating evidently strong immunoreaction to the fibrosis biomarker were found in group IV. The extent of liver fibrosis in the Batts and Ludwig numerical scale [24] was proportional to the number of α-SMA immunoreactive HSCs in the biopsy material. The mean numbers of HSCs/100 hepatocytes in the study groups, i.e. with S-0; S-1; S-2 and S-3, were: 5.00; 5.98; 9.80; 12.19, respectively. Interestingly, in 3 groups, namely with S-0; S-1 and S-3, the highest percentage of HSCs/100 hepatocytes was observed in zone 2, i.e. intermediate, defined in the literature as the zone of variable metabolism. The lowest numbers of HSCs with a positive reaction to α-SMA protein were found in zone 1 of the lobule, which indicates that this area in the process of fibrosis in children with CHB is least metabolically active.

The current results of immunohistochemical and statistical analyses are consistent with our preliminary findings presented recently at the 19th Congress of the Polish Society of Pathologists [33].

The present study is the first extensive morphological work on HSCs biology in chronic hepatitis B, performed on biopsy material in pediatric patients. There are no reports concerning quantitative morphometry of these cells in adult patients, except for a study by Mak and Lieber [34] on alcohol disease, which involved a considerably smaller amount of material. In our opinion, the quantitative method applied in the current study to assess immunoreactive HSCs, although more laborious, is more objective and easier to interpret than the semiquantitative tool used in adult patients [35, 36, 37].

<table>
<thead>
<tr>
<th>ZONES OF HEPATIC LOBULE</th>
<th>Mean number of HSCs in group I (S-0)</th>
<th>Mean number of HSCs in group II (S-1)</th>
<th>Mean number of HSCs in group III (S-2)</th>
<th>Mean number of HSCs in group IV (S-3)</th>
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<tr>
<td>Zone 1</td>
<td>6.27 ± 2.34</td>
<td>9.28 ± 4.15</td>
<td>14.6 ± 7.26</td>
<td>19.38 ± 8.19</td>
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<tr>
<td>Zone 2</td>
<td>8.27 ± 4.28</td>
<td>12.79 ± 4.74</td>
<td>18.99 ± 7.83</td>
<td>23.48 ± 9.63</td>
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<tr>
<td>Zone 3</td>
<td>7.47 ± 3.48</td>
<td>12.32 ± 4.34</td>
<td>19.25 ± 6.86</td>
<td>19.67 ± 6.82</td>
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<tr>
<td>I vs. II</td>
<td>NS</td>
<td>&lt;0.001</td>
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<tr>
<td>I vs. III</td>
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HSCs – hepatic stellate cells; S – stage of fibrosis; NS – not statistically significant.
A topographically similar picture of α-SMA positive HSCs in the course of chronic HBV infection in biopsy material was observed in the studies conducted by Guido et al. [35], Shi et al. [38], and Tomanović et al. [39] in adult patients. Both the current IHC study in children with CHB and analogical observations performed by the quoted researchers in adult patients indicate that the majority of perisinusoidal HSCs with strong or very strong α-SMA expression can be found in the intermediate zone of the hepatic lobule and then in its central zone. Thus, it was zone 2, intermediate, that was most metabolically active and involved in the development of changes. On the other hand, the fewest α-SMA positivestellate cells were found in zone 1, i.e. periportal [35, 38, 39].

A separate group of literature reports have discussed the biology of stimulated HSCs, with special regard to α-SMA immunoeexpression, in other types of liver diseases [28, 34, 36, 37, 40, 41, 42]. Tomanović et al. [37], who performed a semiquantitative assessment of α-SMA positive HSCs in chronic hepatitis C in adult patients, noted their increased number in all regions of the hepatic lobule studied, especially in the portal space, as well as in the fibrous septa. They observed a positive correlation between the number of activated HSCs within the portal spaces and fibrous septa and the stage of fibrosis, which they consider to play a role in the development of progressing fibrosis and cirrhosis in chronic viral HCV infection. A significant increase in the number of immunopositive HSCs in zone 1 of the lobule in patients with chronic type C hepatitis topographically differentiates this type of hepatitis from type B chronic hepatitis, where the fewest cells were found, both in adults [35, 38, 39] and in children in the current study.

Summing up, the immunohistochemical assessment of HSCs within different areas of hepatic tissue showed an increase in the number of cells with enhanced intensity of liver fibrosis, which confirms their crucial role in the development and progression of liver fibrosis, and can be a useful prognostic factor of unfavorable termination of the disease.

The highest mean number of immunoreactive HSCs/100 hepatocytes was found in the periporal zone, which indicates that this area of the hepatic lobule is most metabolically active in the process of fibrosis in children with CHB. However, the smallest count of activated HSCs was observed in the periportal zone, which suggests that this area of the hepatic lobule is the least metabolically active in the course of liver fibrogenesis.

Conclusions

The immunohistochemical assessment of HSCs within the hepatic lobule in children with CHB, combined with statistical assessment, showed a close positive correlation of cell count with fibrosis intensity. The analysis of HSC activity within specified areas of liver tissue, being the first extensive morphological work on the biology of these nonparenchymal hepatic cells in CHB pediatric patients, may lead to new perspectives in early diagnosis of liver fibrosis and have prognostic implications in this pathology.

The study may also contribute to the clinical development and validation of antifibrotic therapies in patients with chronic liver diseases.

The authors declare no conflict of interest.

References


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