Evaluation of neoangiogenesis and cell activation in hepatic fibrosis and cirrhosis caused by chronic viral hepatitis

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INTRODUCTION

Hepatic cirrhosis and hepatocellular carcinoma (HCC) are the third leading causes of death in people over 50 years old in Europe. This has accounted for 90% of the 5,000 liver transplantations as a result of hepatitis C virus (HCV) since the 1970s. It has been shown that neoangiogenesis and abnormal vessel architecture are related to hepatic fibrosis, cirrhosis, and HCC.¹ Hepatic fibrosis is the storage of interstitial extracellular matrix after acute or chronic hepatic damage. Independent from the etiology, cirrhosis and fibrosis are caused by degeneration of hepatic architecture and changes in microvascular structure by large bands around hepatocyte nodules. These changes cause dysfunction of the liver and portal hypertension.² Liver fibrosis is a dynamic process with storage of extracellular matrix.³ Liver fibrosis progresses through stages with the activation of hepatic stellate cells (HSC) and Kupffer, migration and proliferation of HSC, remodeling of scar tissue, and HSC apoptosis.⁴⁻⁶

MATERIALS AND METHODS

Patients

Forty liver transplant patients due to viral hepatitis were accepted in our study. The liver explant tissue was embedded in paraffin. All chronic viral hepatitis liver biopsies were added. The liver tissue fibrosis and necroinflammation activity score were determined by Modified Ishak Scoring System.⁷ A total of 40

Purpose: Evaluation of hepatic stellate cell (HSC) activation due to viral hepatitis in various stages of cirrhosis and fibrosis. We aimed to determine portal hypertension development by examining neoangiogenesis and fibrosis in the vascular epithelium. Materials and Methods: A total of 80 patients with chronic viral hepatitis before treatment and 40 hepatocirrhotic transplant patients were consented for this study. A modified histology activity index was used for fibrosis and necroinflammatory activity. 40 samples from all groups: The fibrosis 0-1 group, the fibrosis 2-3-4 group, and the cirrhosis group were taken. Samples embedded in paraffin were subjected to immunohistochemistry and stained for alpha smooth muscle actin (SMA). For microvascular counting, the Chalkley method was used, and an average of three different areas was taken. Results: A significant difference in SMA staining was detected in the three different groups (P < 0.001). Interestingly, fibrosis 2-3-4 was significantly higher compared to the fibrosis 0-1 group (P = 0.007). The cirrhosis group was higher compared to the fibrosis 0-1 group (P < 0.001) and the fibrosis 2-3-4 group (P < 0.001). Neoangiogenesis was significantly different between groups (P < 0.001), and neoangiogenesis was the highest in the cirrhosis group. Discussion: In conclusion, hepatic neoangiogenesis and fibrosis development are associated with each other. We show that neovascularization and HSC activation were augmented in the more progressive disease states. Future work should be focused on developing therapies targeting the activation of vascular endothelial growth factor or HSC activation may help prevent chronic hepatic diseases.

KEY WORDS: Angiogenesis, Hepatic fibrosis, Hepatic stellate cell, Viral hepatitis
fibrosis 0-1, 40 fibrosis 2-3-4, and 40 cirrhotic liver tissue (fibrosis 5-6) were included in the study. All paraffin preparations were cut using a microtome.

**Immunohistochemical Evaluation**

The paraffin sections (2–3 μ thick) were spread on polylysine-coated slides and dried in a 50°C sterilizer. Actin was diluted to 1/100 (Spring, Mouse Anti-Human Actin, while Smooth Muscle Monoclonal Antibody [Clone 1A4]) and factor 8 was diluted to 1/50 (F8/vWF Novocastra). The fibrotic septa and the inside of the lobule were evaluated in fibrosis (0-1-2-3-4) patients and cirrhotic patients with anti-actin smooth muscle antibody stain. The grades were determined as: Grade 0 (negative): No dye or area smaller than 3% was stained; Grade+1 (mild): Area between 3% and 33% was stained; Grade+2 (mid): Area between 33% and 66% was dyed; and Grade+3 (high): Area larger than 66% was dyed.

In statistical analysis fibrosis, 0-1 group was named Group 1; fibrosis 2-3-4 group was named Group 2; and the cirrhosis (fibrosis 5-6) group was named Group 3. The hepatitis activity was leveled by the histology activity index (HAI) scoring system. For necroinflammatory activity scores were recorded as 1–4: Minimal, 5–8: Mild, 9–12: Middle, and 13–18: High. Vessel counting was done using the Chalkley method and factor 8 staining. Microvessels in three different grid areas are counted and averaged.

**Statistical Analysis**

Statistical analysis was performed by SPSS 15.0 Program. Data were shown in mean, standard deviation, and percentage (%). Variant homogeneity was tested using the Kolmogorov Smirnov Analysis. Groups were compared with ANOVA and Ki-square methods.

**RESULTS**

**Demographical and Etiological Results**

A total of 120 liver biopsy patients were admitted and categorized into three different groups. The mean age was similar in all groups. Based on gender, males were higher in Group 3 (75%) whereas, Group 1 (42.5%) and Group 2 (57.5%) were not statistically different. In the comparison of the Groups, 1 and 3 had a significant difference ($P = 0.003$) although no difference was detected for Groups 1 and 2 ($P = 0.184$) and Groups 2 and 3 ($P = 0.100$). Demographical data are shown in Table 1.

A significant difference was detected in the etiology ($P < 0.001$). Groups 1 and 2 had a similar etiology ($P = 0.335$), while Groups 1 and 3 ($<0.001$), Groups 2 and 3 ($<0.001$) had a different etiology. Hepatitis B virus + hepatitis D virus (HBV + HDV) was significantly greater in Group 3 ($<0.001$). Etiological data are shown in Table 1.

**Evaluation of Immunochemistry**

There was a significant difference detected between fibrous septa with smooth muscle actin (SMA) dye between groups ($P < 0.001$). Comparison between Groups 1 and 2 (0.007), Groups 1 and 3 (<0.001), and Groups 2 and 3 (<0.001) presented different dye patterns. In Group 1, the dye pattern was 0 (40%) and +1 (52.5%), while in Group 3 the dye pattern was +2 (50%) and +3 (42.5%). Groups and fibrous septa dye pattern are given in Table 2 and Figures 1-3.

**Table 1: Demographical and etiological distribution in groups**

<table>
<thead>
<tr>
<th>Etiological data</th>
<th>Group 1 ($n=40$)</th>
<th>Group 2 ($n=40$)</th>
<th>Group 3 ($n=40$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (average±SD)</td>
<td>46.2±12.6</td>
<td>52.1±12.9</td>
<td>46.6±9.9</td>
</tr>
<tr>
<td>Sex (male, %)</td>
<td>17 (42.5)</td>
<td>23 (57.5)</td>
<td>30 (75)</td>
</tr>
<tr>
<td>Etiology (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>23 (57.5)</td>
<td>20 (50)</td>
<td>11 (27.5)</td>
</tr>
<tr>
<td>HCV</td>
<td>16 (40)</td>
<td>16 (40)</td>
<td>6 (15)</td>
</tr>
<tr>
<td>HBV+HDV</td>
<td>0</td>
<td>3 (7.5)</td>
<td>22 (55)</td>
</tr>
<tr>
<td>HBV+HCV</td>
<td>1 (2.5)</td>
<td>1 (2.5)</td>
<td>1 (2.5)</td>
</tr>
</tbody>
</table>

SD: Standard deviation, n: Number of patients, HBV: Hepatitis B virus, HCV: Hepatitis C virus, HDV: Hepatitis D virus

**Table 2: The SMA staining pattern and factor 8 expression between groups**

<table>
<thead>
<tr>
<th>Groups and fibrous septa dye pattern</th>
<th>Group 1 ($n=40$)</th>
<th>Group 2 ($n=40$)</th>
<th>Group 3 ($n=40$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade of dye (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16 (40)</td>
<td>9 (22.5)</td>
<td>0</td>
</tr>
<tr>
<td>+1</td>
<td>21 (52.5)</td>
<td>18 (45)</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>+2</td>
<td>3 (7.5)</td>
<td>13 (32.5)</td>
<td>20 (50)</td>
</tr>
<tr>
<td>+3</td>
<td>0</td>
<td>0</td>
<td>17 (42.5)</td>
</tr>
<tr>
<td>Factor 8 expression (mean±SD)</td>
<td>0.09±0.23</td>
<td>0.81±0.99</td>
<td>3.5±1.4</td>
</tr>
</tbody>
</table>

Alpha-SMA expression: Groups 1 and 2 difference: $P = 0.007$, Groups 1 and 3 difference: $P<0.001$, Groups 2 and 3 difference: $P = 0.001$, Factor 8 expression: Groups 1 and 2 difference: $P<0.001$, Groups 1 and 3 difference: $P<0.001$, Groups 2 and 3 difference: $P<0.001$. SMA: Smooth muscle actin, SD: Standard deviation
Factor 8 expression levels were statistically different and are shown in Table 2 ($P < 0.001$). In Group 3, a significantly higher expression than other groups was observed [Figure 4].

There was a significant difference detected in fibrous septa dying level and sex together ($P = 0.008$). In men, there was a stronger in staining compared with women [Table 3]. In the evaluation of the etiology fibrous septa with SMA dye, there was a significant difference ($P < 0.001$). In HDV superinfection samples, there was a +3 staining density, while HBV or HCV positive patients demonstrated a (0, +1) staining density.

Factor 8 expression in the relationship between SMA dye in fibrous septa was significantly different ($P < 0.001$). Expression was increased by the level of staining [Table 3]. SMA dye was evaluated in Group 3 patients in the nodule. All patients have different levels of staining. Groups 1 and 2 patients had no SMA staining in the liver lobule.

According to HAI scores, there was a significant difference detected (<0.001). In Group 1, scores of patients were minimal or mild although scores of Group 3 were medium or high. The distribution of HAI score is shown in Table 4.

There was a significant difference in staining levels of fibrous septa and HAI scoring (<0.001). Medium and high HAI score patients have +2 and +3 levels of dye. The distribution and percentages are shown in Table 5.

Similar to SMA dye there was a significant difference in the HAI group and expression of factor 8 ($P = 0.017$). There was a correlation between etiology and fibrous septa dying level, HAI score, and factor 8 expressions.

**DISCUSSION**

Fibrosis is a strong indicator of liver damage in chronic viral hepatitis. Fibrosis can present in several forms such as periportal, pericellular, perivenular, bridging, or others forms.[8] Proliferation and differentiation of HSCs have been related to the development of hepatic fibrosis. It has been shown in studies that HSCs express α-SMA.[9,10]

Akpolat and colleagues detected that score of fibrosis was correlated with alpha SMA expression in periportal, pericentral, and perisinusoidal areas. In addition to that, necroinflammatory activity was evident with increased alpha-SMA expression in nonfibrotic patients.[9]

Tomanovic et al. studied HSCs activation and alpha-SMA expression in chronic HCV and control group without liver disease. SMA expression and fibrosis have been correlated in portal area and fibrous septa; however, necroinflammatory activity (HAI) and SMA expression were not significantly associated. In addition, HAI score and fibrotic level were not significantly related.[11] Fibrosis degree and SMA

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Figure 1: Cirrhosis with +3 smooth muscle actin antibody staining (×40)

Figure 2: Fibrotic septa with +2 smooth muscle actin staining (×40)

Figure 3: Negative smooth muscle actin staining in the liver sinusoidal and portal areas (×40)

Figure 4: Cirrhosis with increased vascular proliferation by factor 8 staining (×40)
expression in fibrous septa were associated in our study ($P < 0.001$). In comparison with fibrosis groups, patients with cirrhosis had a significantly higher SMA expression. This represents HSCs important role in cirrhosis. There was a significant relationship in the HAI score and fibrosis. In particular, the HAI score in patients with HBV + HDV was higher. There was no SMA staining in sinusoidal cells in the lobule of minimal and mild fibrosis levels. This may be due to apoptosis in HSCs or the difference in immunohistochemistry techniques.

In the study of Chu et al., the correlation between necroinflammatory degree, fibrosis, and HSCs activation index in chronic hepatitis B and C with alpha-SMA antibody-dye were stated. However, it has been determined that HSCs activation index was higher in the HCV positive patients.

In our study, we evaluated and compared HSC activation in hepatic fibrosis stages and cirrhosis in chronic HBV, HCV, HBV+HDV, and HBV+HCV. We determined that SMA expression in chronic HBV+HDV patients were significantly higher than other groups. The reason may be related to inhomogeneity between hepatic etiologies because the groups are classified with their stage of fibrosis.

Neovascularization and abnormal vessel structure in hepatic fibrosis are markers of progression to cirrhosis and hepatic cancer. Chronic liver disease has been characterized by intravascular remodeling. Vascular remodeling has been defined as capillarization of sinusoids, fibrogenesis, and development of intrahepatic shunts. Fibrogenesis and intrahepatic shunts can cause an increase in hepatic vascular resistance (the reason for portal hypertension) and decrease in effective hepatic blood flow finally to hepatic failure.

In our study, the factor 8 antibody was used to stain, and neovascularization was counted by the Chalkley method. Neoangiogenesis was higher in the cirrhosis group, which caused increased portal blood flow resistance, development of collaterals, and complications of cirrhosis. Neoangiogenesis was correlated with HAI score and SMA expression.
Amarapurkar et al. described neoangiogenesis using a vascular endothelial growth factor (VEGF) and CD34 antibody in chronic HBV, HCV, and other cirrhotic patients. It has been determined that increased fibrosis caused significantly increased neoangiogenesis. There was no difference detected between the etiologies; however, VEGF and CD34 expression were higher in HCC.[13]

Sorafenib, a new tyrosine kinase inhibitor for cirrhosis-related HCC, has proven to be beneficial in clinical trials. It was shown that sorafenib inhibited VEGF, platelet-derived growth factor, and Ras pathway in cirrhotic and portal hypertensive rats by approximately 80% decrease in splanchnic neovascularization and portosystemic collaterals, which lowers the systemic and splenic hyperdynamic circulation.[14] In addition, hepatic neovascularization and fibrosis may be linked. The agents blocking of VEGF or HSC activation will slow the progression of chronic liver diseases which needs further studies.

CONCLUSION

We demonstrate that HSC activation and neovascularization increases by the degree of fibrosis. Neovascularization is due to vascular mediators secreted by HSCs.

REFERENCES


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