Morphometric Analysis of Lymphatic and Blood Vessels in Human Chronic Viral Liver Diseases

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The purpose of this study was to elucidate the morphometric changes occurring in hepatic lymphatics in human chronic viral liver diseases and to investigate the relationship between liver fibrosis, liver inflammation, and these changes. The lymphatic vessels were stained intensely by enzyme histochemistry for 5'-nucleotidase, whereas blood vessels stained well for alkaline phosphatase. We performed a morphometric analysis to estimate the number of lymphatic and blood vessels and their areas, using computer graphics software (NIH Image). Both the number of lymphatics in the specimens and their areas were increased according to the degree of liver fibrosis, but neither showed any relationship with the degree of activity of hepatitis. Neither the number nor the areas of the blood vessels showed any obvious relationship with the degree of fibrosis or the activity of chronic hepatitis. Correlation between clinical and laboratory data and the sizes and number of the lymphatics supported these morphological data. Our results clarified that the sizes and number of lymphatics are related to the stage of fibrosis in chronic viral liver diseases. This is thought to be due to increased lymph production, which is caused by the disturbance of the microcirculation associated with liver fibrosis. (Am J Pathol 1998, 153:1131–1137)

The hepatic lymphatic system deserves detailed study, as it is an integral part of the liver microcirculation; however, there are few studies of the pathological changes that occur in lymphatic vessels in human liver diseases, especially in chronic hepatitis and liver cirrhosis, which are the most common liver diseases worldwide.

Evidence from experiments in dogs indicates that about 80% of the lymph formed in the liver leaves via hilar lymphatics and enters the thoracic duct at or near the cisterna chyli.1 Several studies of lymphatic vessels have been performed using routine light and electron microscopy.2–4 Liver tissue from human patients and animal models with cirrhosis or obstructive jaundice has been thoroughly analyzed by transmission and scanning electron microscopy, and dilatation of the lymphatic vessels has been reported.2,5 Lymphatic vessels on the liver surface have also been studied, macroscopically during laparoscopy, and dilatation of these superficial lymph vessels in several diseases has also been reported.6

Blood vessels can be identified by several immunohistochemical methods. It has been reported that staining for α smooth muscle actin, factor VIII-associated antigen, and alkaline phosphatase (ALPase) is strongly positive in blood vessels but negative or only weakly positive in lymphatic vessels.7,8 These staining methods have therefore been used to differentiate between blood and lymphatic vessels. On the other hand, 5'-nucleotidase (5'-Nase) activity has been reported to be higher in lymphatic vessels than in blood vessels.9,10 Kato11 developed a histochemical staining technique for identifying 5'-Nase and succeeded in staining the lymph vessels of the rat stomach. The use of combined staining for 5'-Nase and ALPase has allowed lymph vessels and blood vessels to be identified more accurately. Lymphatic vessels in human dental humps have been identified and described using this method.12

Vollmar et al8 studied the expansion of lymph vessels and their function in the development of CCl 4-induced hepatic fibrosis and cirrhosis in rat liver using intravital fluorescence microscopy. This involved the use of high molecular weight fluorescein isothiocyanate-labeled dextran as a fluorescent marker, which allowed simultaneous assessment of blood hepatocytic macromolecular exchange from the both the sinusoidal microvasculature and the hepatic lymph system. In this previous study, a quantitative analysis of lymph vessels was carried out for the first time, and marked increases in lymph vessel density and area were observed in rat liver with fibrosis and cirrhosis. No such quantitative analysis of lymph vessels has yet been reported for human livers, especially livers obtained from patients with chronic hepatitis and cirrhosis. Therefore, it is still unknown whether the data regarding lymphatic vessel area found by Vollmar et al8 are applicable to the human liver at various stages of chronic hepatitis.

The purpose of this study was to elucidate morphometric phenomena occurring in human hepatic lymphatic vessels during the course of chronic viral hepatitis and its progression to cirrhosis and to investigate the relation-

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ship between liver fibrosis, liver inflammation, and these changes in lymphatic vessels, using liver specimens obtained from patients with chronic hepatitis and cirrhosis. Using a combination of staining for 5′-Nase to identify lymph vessels and for ALPase to identify blood vessels, we were able to distinguish between these vessels in human liver. We also report the first quantitative analysis of lymphatic vessels within the portal tract and liver tissue in human chronic viral liver diseases, which clarifies the changes in size and number of these vessels at various stages of chronic hepatitis and cirrhosis.

**Materials and Methods**

**Materials**

Liver specimens were obtained by needle biopsy and surgical wedge biopsy or autopsy from 62 patients (36 men and 26 women; from 18 to 75 years old; needle biopsy in 55, surgical wedge biopsy in 5, and autopsy in 2). The breakdown of the 62 patients by disease was as follows: 41 had chronic viral hepatitis (hepatitis B virus in 14 and hepatitis C virus in 27), 17 had liver cirrhosis (associated with hepatitis B virus in 6 and with hepatitis C virus in 11), 2 had fatty metamorphosis, and 2 had normal livers. The two liver samples with normal histological findings were obtained from patients with cholelithiasis who had previously shown mild transient liver function abnormalities. A histological diagnosis of chronic hepatitis was determined in accordance with the classification reported by Desmet et al. Grading and staging were carried out by means of conventional histological stains. The minimum requirements are hematoxylin and eosin for general assessment and a connective tissue preparation for assessment of fibrosis and structural alterations. Histological findings were scored according to a scoring system, and the histological activity index was the sum of the three separate scores for the different components of the necroinflammatory lesions (maximum score = 18). A separate score was obtained for each of the following: portal inflammation, periportal/piece-meal necrosis, and lobular activity. Grading was recorded separately as follows: normal (score 0), minimal (scores 1 to 3), mild (scores 4 to 8), moderate (scores 9 to 12), and severe (scores 13 to 18). The histological stage of the disease was recorded separately as follows: normal, no fibrosis; mild, enlarged fibrotic portal tracts; moderate, periportal fibrosis; severe, septal and/or bridging fibrosis; and cirrhosis, definite cirrhosis.

Informed consent was obtained from each patient after the nature of the study was explained.

**Methods**

**Tissue Preparation**

For histochecmy, the liver specimens were cut into two pieces; one was used for routine microscopic observation and the other for histochemical staining of lymphatic and blood vessels. The liver specimens were fixed in acetone at 4°C for 2 days and then in a 2% paraformaldehyde solution at 4°C for 3 days. They were then frozen at a temperature of −80°C after embedding in an embedding matrix (Shandon, Pittsburgh, PA).

**Enzyme Histochemistry**

Frozen sections, 5 μm in thickness, were cut on a cryostat (Bright 5030 microtome, Huntingdon, UK), mounted on albumin-coated slides and air dried. The sections were washed in 0.1 mol/L cacodylate buffer solution with 7% sucrose, and then 5′-Nase staining was performed according to the method described by Kato, with some modifications. Briefly, the sections were reacted with a solution containing L-tetramisole (2 mmol/L) at 37°C for 60 minutes using the lead method and then immersed in 1% ammonium sulfide solution for 1 minute. To detect ALPase activity, the sections were reacted at room temperature for 20 minutes using the azo dye method. Each stained section was observed by light microscopy.

For 5′-Nase staining before transmission electron microscopy, each section was washed in 0.1 mol/L cacodylate buffer solution with 7% sucrose. The sections were postfixed in 2% osmium for 90 minutes and then embedded and cut into ultrathin sections. The sections were observed by transmission electron microscopy (Hitachi H-800, Tokyo, Japan).

**Immunohistochemistry**

Immunohistochemistry for α smooth muscle actin, factor VIII-associated antigen, desmin, and vimentin was performed using the avidin-biotin-peroxidase complex method. Sections prepared as above were incubated in absolute methanol containing 0.3% hydrogen peroxidase for 15 minutes to block endogenous peroxidase and then treated with normal goat serum for 20 minutes. They were then incubated with anti-α smooth muscle actin (Nichirei, Tokyo, Japan), anti-factor VIII-associated antigen (Nichirei), anti-desmin (Nichirei), or anti-vimentin (Nichirei) antibodies overnight at 4°C; washed in phosphate-buffered saline solution; and reincubated with anti-mouse peroxidase-conjugated immunoglobulin. The sections were incubated with diaminobenzidine-hydrogen peroxide to visualize the reaction products.

**Morphometric Analysis of Lymphatic and Blood Vessels**

The specimens obtained from all 62 patients contained more than three portal tracts. The sections were stained for 5′-Nase and ALPase for morphometric analysis.

Microscopic pictures of the stained liver specimens were taken, and positive signals were analyzed with the “freeware” image analysis program NIH Image (written by Wayne Rasband at the U.S. National Institutes of Health) using an image scanner (35-mm film scanner LS-1000, Nikon Inc., Tokyo, Japan) and a computer (Macintosh 7500AV, Apple Computers), after changing
from red-green-blue mode to cyan-magenta-yellow-black mode. The optical densities of the yellow mode were expressed as integrated densities, which were analyzed using a 256-step gray scale to identify 5'-Nase-positive signals. Using the NIH Image program, the signals from the gray scale were quantified and expressed as the number and area of the lymphatic and blood vessels. The portal and total areas of the liver specimens were quantified in the same way.

Clinical and Laboratory Data

Serum was prepared from each patient's blood on the day that the liver specimens were obtained. The serum total bilirubin concentration, aspartate aminotransferase, alanine aminotransferase, ALPase, γ-glutamyl transpeptidase, and plasma clearance rate of indocyanine green were measured and the thymol turbidity and zinc sulfate turbidity tests were performed. Ultrasonography was used to evaluate the spleen size, which was expressed as a spleen index, the product of the transverse diameter and its perpendicular diameter measured on the maximum cross-sectional image of the spleen.

Statistics

Data were expressed as the mean ± standard deviation. Group means were compared by a one-way analysis of variance. Multiple comparisons were performed using the Scheffé test. P values less than 0.05 were considered statistically significant. Linear regression analysis or Spearman’s rank-correlation test was used to calculate correlations between the different variables, as required.

Results

Immunohistochemistry

Figure 1, A and B, shows microphotographs of 5'-Nase and ALPase staining in sequential sections. Comparison of these two photographs revealed that the endothelial walls of several vessels were visualized by 5'-Nase staining (arrowheads) but were negative for ALPase. In contrast, other vessels were positive for ALPase but negative for 5'-Nase. In every section obtained from 58 of the 62 patients, both 5'-Nase-positive/ALPase-negative vessels and ALPase-positive/5'-Nase-negative vessels were identified. The exceptions were the liver specimens taken from two patients with normal livers and two with fatty livers. In these specimens, several vessels were seen in the portal tract, and all of them were positive for ALPase but negative for 5'-Nase. Thus, there was no 5'-Nase activity observed in vessels with a lumen detectable by light microscopy. Many 5'-Nase-positive vessels were observed in the cirrhotic liver (Figure 2).

Sections stained for 5'-Nase were also observed by transmission electron microscopy (Figure 3). Vessels positive for 5'-Nase activity were surrounded by anchoring filaments and lacked a tunica media and a tunica externa. From these observations, the 5'-Nase-positive vessel in Figure 3B had a typical feature of lymph vessel.
The results of immunostaining with anti-\(\mu\)/H9251 smooth muscle actin, anti-factor VIII-associated antigen, anti-desmin, and anti-vimentin antibodies are shown in Table 1. Staining with anti-\(\mu\)/H9251 smooth muscle actin produced a strong reaction in blood vessels but a weak reaction in lymphatic vessels. Anti-factor VIII-associated antigen stained strongly in vascular vessels but weakly or not at all in lymphatic vessels. A positive reaction in lymph vessels was observed more with anti-\(\mu\)-smooth muscle actin than with anti-factor VIII-associated antigen. Neither desmin nor vimentin was stained on the walls of either lymphatic or blood vessels, whereas both were positively stained on the walls of bile ducts. Staining for ALPase and factor VIII-associated antigen thus produced almost identical results. These results confirmed that a combination of \(\mu\)-Nase staining and ALPase staining was useful in differentiating between lymphatic vessels and blood vessels in liver tissue. We therefore performed staining for \(\mu\)-Nase and ALPase to identify lymphatic and blood vessels within the portal tract. Most vessels could be easily identified as lymph vessels or vascular vessels by the combination of \(\mu\)-Nase and ALPase staining, because vessels positive for both stainings or vessels negative for both stainings were scarcely seen.

### Morphometric Analysis

Quantitative analysis was performed on lymphatic and vascular vessels at the light microscopic level. In the area of the portal tract, the mean size of each lymphatic vessel and the number and total area of lymphatic or blood vessels in 0.01 mm\(^2\) of portal tract or 1 mm\(^2\) of section were morphometrically analyzed. No significant differences in the data for lymphatic or blood vessels were found between autopsy materials and those of needle and wedge biopsies (data not shown). Therefore, autopsy materials were included in this study. The histological features of liver tissues from patients with chronic hepatitis and liver cirrhosis, determined by the classification of Desmet et al., were as follows: for staging (degree of fibrosis), 15 patients showed mild fibrosis, 10 moderate fibrosis, 16 severe fibrosis, and 17 cirrhosis, and for grading (degree of activity), 6 patients exhibited minimal activity, 24 mild activity, 22 moderate activity, and 6 severe activity. The data for lymphatic and blood vessels are shown in Tables 2 and 3.

Table 2 shows that the area of each lymphatic vessel was significantly larger in cirrhosis than in vessels with other degrees of fibrosis (mild, moderate, and severe, \(P < 0.05\)). No differences between the groups with mild, moderate, and severe fibrosis were found. The number of lymphatic vessels in the portal tract showed no significant differences between groups; however, the number of lymphatic vessels in each section tended to increase in association with the progression of staging. These data indicate that the number of lymphatic vessels within the portal tract shows a strong correlation with the degree of portal tract expansion. They also suggest that the size of the lymphatic vessels does not change greatly when patients remain at the stage of chronic hepatitis (mild-severe fibrosis), but that enlargement becomes prominent in patients with cirrhosis.

The relationship between lymph vessels and the activity of hepatitis was also studied (data not shown). There

![Figure 3. Electron micrograph of a vessel stained for \(\mu\)-Nase staining. a: The lymph vessel endothelium shows strong lead reaction products of \(\mu\)-Nase (arrow). b: The lymph vessel has anchoring filaments (arrow). L, lumen; A, anchoring filaments. Magnifications: \(\times\)2,000 (a) and \(\times\)10,000 (b).](image)

Table 1. Histochemical Staining of Lymph Vessels and Blood Vessels in Liver Specimens

<table>
<thead>
<tr>
<th></th>
<th>(\mu)-Nase</th>
<th>ALPase</th>
<th>Anti-(\alpha) smooth muscle actin</th>
<th>Anti-factor VIII-associated antigen</th>
<th>Anti-desmin</th>
<th>Anti-vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph vessels</td>
<td>++</td>
<td>− (−±)</td>
<td>+</td>
<td>− (−±)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>− (−±)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

++: strongly positive; +, weakly positive; −, negative; −(−±), negative for ordinary staining but becoming positive when the section was incubated with visualizing solution for a longer time (more than double the time of ordinary staining).
were no differences between the sizes and number of the lymphatic vessels and various hepatitis activity groups.

Table 3 shows that no significant differences in blood vessel area were found between the different disease stages. The number and area of these vessels in the portal tract were higher in patients with mild fibrosis than in those with moderate and severe staging ($P < 0.05$). However, the total number and total area of blood vessels in the sections did not differ significantly between the mild fibrotic and cirrhotic stages.

The relationship between blood vessels and the activity of hepatitis was studied (data not shown). There were no differences between the sizes and number of blood vessels and various hepatitis activity groups.

The morphometric data for lymph vesels are summarized in Figure 4, which is a schema of the sizes and number of lymph vessels and the area of portal tract associated with the progression of liver fibrosis. Similar results for blood vessels are shown schematically in Figure 5.

No significant differences in the data for lymphatic or blood vessels were found between hepatitis caused by hepatitis virus B and hepatitis virus C (data not shown). Compared with the data for lymphatic vessels, the increase in the number of blood vessels is not proportional to enlargement of the portal tracts, and their enlargement is not prominent in the cirrhotic stage.

Table 4 shows the correlations between the clinical and laboratory data and the sizes and number of the lymphatic vessels. There were no significant differences in the areas of lymph vessels, number of portal tract and area in portal tract and clinical parameters. Significant correlations were noted between the number of lymphatic vessels in each section and the plasma clearance rate of indocyanine green, the results of the thymol and zinc sulfate turbidity tests, and the spleen index ($P < 0.05$). In addition, the correlations between the total area of the lymphatic vessels in each section and the plasma clearance rate of indocyanine green, and between the total area of these vessels and the zinc sulfate turbidity test results, were also significant ($P < 0.05$).

**Discussion**

We confirmed that combined staining for 5'-Nase and ALPase can be used to distinguish between lymph vessels and blood vessels in human liver. This is also the first study to analyze the sizes and number of lymphatic vessels in the liver at various stages of chronic hepatitis. To improve the accuracy of this analysis, morphometry was used. Although there have been a few reports containing quantitative analyses of lymph vessels, this is the first report of the morphometric analysis of human lymph vessels. Using these methods, changes in the sizes and numbers of lymph and blood vessels in liver disease have been clarified.

The number and areas of lymphatics did not differ significantly with the activity of hepatitis, but significant differences were associated with the degree of liver fibrosis. It has been reported that superficial lymphatic vessels can be observed more clearly in liver cirrhosis than in chronic hepatitis without marked hepatic fibrosis, and that the lymph vessels appeared to be enlarged when observed by scanning electron microscopy. Our histochemical results showed that intrahepatic lymph

**Table 3. Size and Number of Blood Vessels in Liver Tissues Obtained from Patients with Chronic Hepatitis, Associated with Fibrotic Stage**

<table>
<thead>
<tr>
<th>Staging</th>
<th>Number of specimens</th>
<th>Fibrotic rate (%) (area of portal tract/area of section)</th>
<th>Area of vascular vessel ($\mu$m²)</th>
<th>Number in portal tract (per 0.01 mm²)</th>
<th>Area in portal tract ($\mu$m²/0.01 mm²)</th>
<th>Number in section (per mm²)</th>
<th>Total area in section ($\mu$m²/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4*</td>
<td>2.3 ± 1.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mild</td>
<td>15</td>
<td>3.7 ± 1.5</td>
<td>524.1 ± 372.7</td>
<td>1.48 ± 0.79</td>
<td>314.1 ± 273.4</td>
<td>6.4 ± 3.5</td>
<td>1273.2 ± 1734.0</td>
</tr>
<tr>
<td>Moderate</td>
<td>10</td>
<td>5.9 ± 2.8</td>
<td>493.7 ± 270.2</td>
<td>0.73 ± 0.39</td>
<td>311.4 ± 173.4</td>
<td>4.9 ± 3.7</td>
<td>2073.2 ± 1734.0</td>
</tr>
<tr>
<td>Severe</td>
<td>16</td>
<td>8.4 ± 2.9</td>
<td>650.3 ± 582.1</td>
<td>0.75 ± 0.35</td>
<td>371.3 ± 185.6</td>
<td>5.9 ± 5.0</td>
<td>2727.6 ± 2008.8</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>17</td>
<td>17.5 ± 10.3</td>
<td>622.5 ± 203.7</td>
<td>0.66 ± 0.69</td>
<td>306.9 ± 136.7</td>
<td>9.3 ± 10.8</td>
<td>4064.2 ± 2473.9</td>
</tr>
</tbody>
</table>

Multiple comparisons were performed using the Scheffe test. Bracketed pairs: $P < 0.05$. ND, not detected.

*Two specimens with no abnormal findings and two with fatty liver.
vessels, ie, nonsuperficial lymph vessels, are also enlarged in patients with liver cirrhosis. This phenomenon is thought to be due to increased lymph production, which is caused by the disturbance in the drainage of vascular flow from the sinusoid to the central or terminal hepatic veins associated with lobular distortion in patients with advanced liver diseases or cirrhosis. According to our observations, the area of a lymph vessel was enlarged when cirrhosis of the liver developed. There were no significant differences in the mean number (density) of lymphatics in the portal tract between progressive disease stages; therefore the number of vessels in the sections increased with the development of liver fibrosis.

Our results also showed that the degree of fibrosis but not the activity of hepatitis related to the sizes and number of the lymphatic vessels. Correlation between clinical and laboratory data and the sizes and number of the lymphatic vessels supports these morphological data. No significant correlation was found between the sizes and number of lymphatic vessels and aspartate aminotransferase or alanine aminotransferase levels, which reflect the inflammatory activity of hepatitis, but significant correlation was found between plasma clearance rate of indocyanine green and spleen index, which reflect the stage of fibrosis and resulting disturbance of the hepatic circulation.

The analysis of blood vessels revealed that their number (density) in the portal tract was highest during the stage of mild fibrosis and that their area in the portal tract is higher in normal liver and mild fibrosis than in moderate and severe fibrosis. These data indicate that the number or area of blood vessels does not increase in parallel with enlargement of the portal area. These changes in the number and area of blood vessels were different from those seen in lymphatic vessels.

Vollmar et al.8 reported the development of a strong negative correlation between the density of the lymphatic network in the sinusoid and trans-sinusoidal macromolecular exchange and suggested that increased lymph flow is an important mechanism by which fluid can bypass increased sinusoidal/postsinusoidal resistance in animals. They reported that the lymphatic network enlarged according to fibrotic changes in rats. Our histochemical and morphometric analyses produced results in agreement with the findings of the Vollmar et al.8 study, showing that lymphatic vessels in the liver increase in size and number with the progression of chronic fibrosis. Thus, in human chronic liver diseases, lymphatic vessels increase and expand according to fibrotic changes. Vollmar et al.8 also reported that in parallel with the values for functional lymphatic density, the area of the lymphatic vessels progressively increased after 1, 2, and 4 weeks of CCl4 exposure, with no further change after CCl4 exposure for 8 and 12 weeks. The number and size of the lymphatic vessels increased until the stage of liver cirrhosis in our study. Our data for human livers do not conflict with the data of Vollmar et al.8 because our study did not include the terminal stage of liver cirrhosis, which corresponds to rat livers after CCl4 exposure for 8 and 12 weeks.

It was interesting that the changes in size and number of the lymph vessels were considerably different from those seen in blood vessels. Several growth factors, such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor, and transforming growth factor, have been reported to be involved in angiogenesis. However, noth-
ing is known about the factors that promote the proliferation of lymphatic endothelial cells or the formation of lymphatic vessels. The data in the present study suggest that the stimulatory factors causing the proliferation of lymphatic and blood vessel endothelial cells might be different. Investigation of the mechanism by which dilatation and proliferation of lymph vessels occurs was not included in this study; however, these changes are thought to be caused by the disturbance of the microcirculation associated with liver fibrosis and lobular distortion. Further study will be needed to clarify the mechanism underlying lymphatic proliferation in the liver.

Acknowledgments

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References