Decreased Hepatic Nerve Fiber Innervation in Patients with Liver Cirrhosis

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Background/Aims: Hepatic nerve innervation plays important roles in hepatic metabolism and hemodynamic mechanisms. We compared the distribution patterns of hepatic nerves between normal livers and two liver diseases to elucidate the effects of liver disease on the distribution of hepatic nerves. **Methods:** Tissue specimens were obtained by ultrasonography-guided needle biopsies from 10 normal controls, 74 patients with chronic hepatitis (CH), and 35 patients with liver cirrhosis (LC). The obtained specimens were immunohistochemically stained using antibodies for S-100 protein and α -smooth-muscle actin (α -SMA). The degree of the expression in liver tissues was quantified by manual counting of positively stained nerve fibers under light microscopy. The serum hyaluronic acid level was assayed in all subjects to evaluate hepatic fibrosis. Electron microscopy examinations were also performed. **Results:** The hepatic nerve innervation was significantly lower in LC than in normal controls, as indicated by S-100 protein staining. α -SMA and hyaluronic acid levels were higher in LC and CH than in normal controls. Electron microscopy revealed that unmyelinated nerve fiber bundles in the intralobar connective tissue coursed in the vicinity of hepatic triads. **Conclusions:** These results suggest that hepatic nerve innervation can be decreased by hepatic inflammatory responses and/or fibrotic changes in LC patients. Further study is needed to clarify this observation. (*Gut and Liver* 2007; 1:165-170)

Key Words: α -SMA; Electron microscopy; Hepatic nerve innervation; Hyaluronic acid; S-100

INTRODUCTION

The liver is innervated by sympathetic and parasympathetic nerves, which are comprised of several constituents including those of an afferent, efferent, cholinergic, adrenergic and peptidergic nature.¹⁻⁵ Hepatic innervations of those nerves have important roles in the hepatic metabolism and hemodynamic mechanisms.⁶⁻⁸ From an anatomical view point in hepatic innervations, the postganglionic sympathetic nerves are derived from the splanchnic nerves connected to the superior mesenteric and celiac ganglia, and the postganglionic parasympathetic nerves originate from the hepatic hilus ganglia. The preganglionic parasympathetic nerves are branches of the vagus nerve. These intrahepatic innervated nerves play roles in the regulation of the hepatic blood flow and hepatocellular metabolism.⁹

For the evaluation of the distribution of hepatic nerve in this study, the S-100 protein was used in detection of hepatic nerve fibers in portal area. In the present study, we compared distribution patterns of hepatic nerves between normal liver and chronic liver diseases to elucidate the role of hepatic nerves in the liver diseases. Activated but not quiescent hepatic stellate cells (HSCs) can be characterized by the expression of α -SMA in the hepatic tissues of patients with liver cirrhosis¹⁰ and a quantitative ELISA was used to determine serum hyaluronic acid for the evaluation of cirrhosis.¹¹

The comparison of the innervations of liver diseases using S-100 protein immunohistochemical method and evaluation of α -SMA and hyaluronic acid will be helpful in

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the comprehension of the relation and pathogenesis of hepatic nerve innervations and liver diseases. Electron microscopy studies (EM) were also done to further evaluate the light microscopy (LM) findings.

MATERIALS AND METHODS

1. Enrolled normal controls and patients

The subjects in this study consisted of 10 normal controls and 74 patients with chronic hepatitis (CH), 35 with liver cirrhosis (LC). CH group had 52 males and 22 females; 35 patients had chronic hepatitis B (CHB), 23 had alcoholic hepatitis, 12 had chronic hepatitis C (CHC), and 4 had hepatitis of unknown causes; mean age of CH group was 48.3 ± 12.7 years. LC group was comprised of 28 males and 7 females; 20 patients had CHB, 9 had LC of alcoholic origin, and 6 had CHC; mean age of LC group was 56.4 ± 11.6 years. Ultrasonography-guided needle biopsy was performed on the enrolled subjects with their informed consents. Normal liver specimens were obtained during the procedures of the laparoscopic cholecystectomy for gallstone ($n=5$ cases), operation of cholangiocarcinoma ($n=2$) and colon cancer liver metastases ($n=3$). The diagnoses were made from the history, laboratory test results, serologic tests, imaging studies such as ultrasonography, and/or liver biopsy. The definition of diagnostic criteria is as follows¹²; patients with chronic hepatitis showed a intermittent or sustained elevation of liver function test results and a necroinflammatory score above 4 in the liver biopsy; patients with liver cirrhosis were defined by laboratory data such as low serum albumin and platelet levels with features suggesting portal hypertension.

2. Methods

1) Immunohistochemical stainings

All liver tissues were immersed for 1 day in a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer solution at 4°C. The paraffinized tissues were deparaffinized by immersion in 60°C Xylene for 5 minutes, repeated at three times, followed by three washes with alcohol and water. The tissues were heated thrice for 15 minutes in citric acid buffer (10 mmol/L, pH 6.0) sterilization disinfectant, and washed with H₂O₂ for 10 minutes and then thrice in water for peroxidase blocking. Protein blocking was achieved by immersion on normal serum agar plates. The tissue samples were stained with the primary antibody, placed in a refrigerator overnight at 4°C and then stained with the secondary antibody at room temperature. The stained tissues were washed by phosphate buffer solutions at least thrice, and

thereafter stained with streptavidin-HRP (horseradish peroxidase) and chromogen. Hematoxylin staining was performed for control staining. The S-100 protein polyclonal antibody (Neo MARKERS, Fremont, California, USA) was used at 1 : 500 dilution. And, the anti- α -SMA antibody (Dako, Copenhagen, Denmark) was used at 1 : 100 dilution. The stained tissues were made into slides for evaluation of the immunohistochemical staining. The staining results were examined at the portal tract, periportal region and parenchyme. Extent of expressions was quantified by manually calculating the number of positively stained cells at the five different portal areas in 200x microscopic field.

2) Serum hyaluronidase testing

A quantitative ELISA was used to determine serum hyaluronic acid according to the manufacturer's instructions.

3) Electron microscopical examinations

For electron microscopical studies, specimens were perfused with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer. Liver tissue blocks were prefixed in 2% osmium tetroxide, dehydrated and embedded in Epoxy-resin. Appropriate areas of interest were selected from sections approximately 1 μ m thick stained with toluidine blue. About 60-70 nm ultrathin sections were cut using ultramicrotome made of a diamond knife. The sections were stained with 1-2% aqueous uranyl acetate, followed by 1% lead citrate. Stained sections were observed and photographed with H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

3. Statistical method

One-way ANOVA (SPSS version 10.0) was used for statistical comparison of frequency of expressions in the immunohistochemical stainings for S-100 protein and α -SMA levels in each of the enrolled groups.

RESULTS

1. Characteristics of hepatic nerve fibers

1) Normal controls

Various size and shapes of positively stained nerve fibers and bundles were observed in the entire lobules. For the S-100 protein, positive staining was detected in 94.4% of cases (Fig. 1). Stained nerve fibers in the portal tract showed round and/or oval shaped appearances. Nerve fibers around the portal tract showed spiral and/or rod-shaped findings with occasional long length fibers suggesting varicosities.

2) Chronic hepatitis

Positively stained nerve fibers were also observed in close contact with the portal tract, vessels and/or sinusoidal veins, and the bile ducts. All cases were stained for the S-100 protein (Fig. 2).

3) Liver cirrhosis

One of the characteristics of the immunohistochemical staining was 'the scantiness of staining' in the pseudolobules. Weak positive stainings in the S-100 protein were observed in the capsules surrounding the pseudolobules and the newly synthesized vessels in the lobules. 97.1% of cases showed positive staining (Fig. 3).

Fig. 1. Nerve fibers innervations of normal subjects show linear stain pattern along the wall of portal venule in the portal tract (S-100 protein, $\times 200$).

Fig. 2. Nerve fibers innervations with linear stain pattern along the portal tract in a patient with chronic hepatitis (S-100 protein, $\times 200$).

2. Comparisons of expression frequency

Almost all specimens showed positive staining. The number of expression of S-100 protein in the normal controls and chronic liver diseases revealed statistically significant decrease in liver cirrhosis compared with that in the normal controls and chronic hepatitis group (Table 1).

Fig. 3. Decreased nerve fibers innervations in the pseudolobules of liver cirrhosis (S-100 protein, $\times 200$). Several stainings are detected near the newly synthesized vessels in the pseudolobule.

Table 1. Expressions of S-100 protein and α -SMA positive fibers in normal controls and chronic liver diseases

Patients	Expressions of nerve fibers in portal area	
	S-100 protein	α -SMA
Normal controls (10)	28.9 $\pm$ 12.7	7.06 $\pm$ 2.00
Chronic hepatitis (74)	23.1 $\pm$ 7.52	16.48 $\pm$ 5.30*
Liver cirrhosis (35)	13.32 $\pm$ 3.23*	17.77 $\pm$ 13.07*

*, $p < 0.05$; one-way ANOVA (SPSS 10.0).

All data shown as mean $\pm$ standard deviation (SD).

Table 2. A comparison of hyaluronic acid level as liver cirrhosis marker

Patients	Hyaluronic acid (ng/mL)
Normal (10)	49.96 $\pm$ 35.88
Chronic hepatitis (74)	259.80 $\pm$ 158.10*
Liver cirrhosis (35)	362.84 $\pm$ 302.24

*, $p < 0.05$; one-way ANOVA (SPSS 10.0).

All data shown as mean $\pm$ standard deviation (SD).

Fig. 4. Direct contact between hepatic nerve (N) and hepatic arteriole (A) in portal area in a normal control ($\times 6,000$).

Fig. 6. Nerve fibers with varicosities in linear pattern adjacent to abundant collagen fibers bundles in the portal area in a patient with cirrhosis ($\times 10,000$).

Fig. 5. Multiple Schwann cells (S) and a lot of nerve fibers containing axons within the hepatic nerve plexus in a portal area in a patient with chronic hepatitis ($\times 6,000$).

3. Hyaluronic acid levels in normal controls and chronic liver diseases

Hyaluronic acid levels in chronic hepatitis and liver cirrhosis were statistically higher than that in normal control (Table 2).

4. Electron microscopical study

On electron microscopy, many unmyelinated bundles in the intralobular connective tissue ran in the vicinity of hepatic traids and bile duct cells. Multiple Schwann cells with various type exons, organelles including mitochondrias, microtubules, microfilaments, liposomes, core vesicles and empty vesicles were also found. Many vari-

Fig. 7. Direct contact between hepatic nerve (N) and hepatic arteriole (A) in a patient with cirrhosis. Extensive collagen fiber bundles can be observed ($\times 8,000$).

cosities were frequently identified. The vascular elements and bile ducts systems were innervated mainly by distant type synapses but rarely by direct contacts. However, the specific differences were not discriminated morphologically between the normal controls and chronic liver diseases except a few degenerative changes of exons in nerve fibers in necrotic tissues of portal area in chronic hepatitis and cirrhosis (Fig. 4-7).

DISCUSSION

The result showed that the nerves found to be closely placed not only in the portal tract but also along the vasculature and bile ducts. The expression of nerve fibers in chronic hepatitis and liver cirrhosis were significantly less frequent than those in the normal controls. Immunohisto-

chemical study using S-100 protein in this study was useful in detection of the distributions of innervation. α -SMA and hyaluronic acid level were higher in chronic hepatitis and liver cirrhosis group than in normal controls which was consistent with previous studies.^{10,11} Although authors had made every effort to decrease the variations, the possibility of individual variations in counting the number of nerve fibers might still exist.

Early investigators including Pfluger and Kupffer used metal impregnation methods such as gold, silver or osmium to study hepatic innervations.⁷ In the mid-20th century, Ungvary & Donath¹³ and Skarring & Bierring¹⁴ reported a more defined hepatic nerve distribution in the portal area and perisinusoidal region in animal experiments by applying fluorescence histochemistry. With introduction of immunohistochemical methods and electron microscopy, there has been remarkable improvement in the elucidation of hepatic nerve distributions in the liver. Immunohistochemical methods have also shown specific types of nerves and marked intra- and inter-species differences in the distribution.^{7,15-18} Moghimzadeh et al¹⁹ reported that the sinusoids of humans and other primates were as densely innervated as those of the guinea-pig. Amenta et al⁵ also showed acetylcholinesterase containing fibers within the portal tracts along the sinusoidal walls in human livers. Mann et al²⁰ demonstrated nonmyelinated axons supplying the hepatic parenchymal region using antibodies to neuron specific enolase and catecholamine synthesizing enzymes. Miyazawa et al⁸ and Terada & Nakamura also disclosed S-100 and neuron specific enolase positive nerve fibers close to the sinusoidal vein and parenchymal region. Many investigators have developed and used numerous variable immunohistochemical methods in the detection of hepatic nerve distribution.

Hepatic nerve functions are both efferent and afferent. Efferent nerves are involved in the hepatic cell metabolisms and the regulation of blood flow and also in sensory functions, such as osmoreception and chemoreception.⁹ Such neural influences may occur directly by stimulation of the cells comprising the vessel wall or hepatic parenchymal cells, or indirectly by affecting the release of metabolites and/or mediators that influence hepatic metabolisms and hemodynamic functions.¹⁻⁶

The S-100 protein²⁰ was used as a marker of Schwann cell and glial cell of nerves. The comparison between the normal controls and liver diseases groups for S-100 protein immunohistochemical staining revealed a more significant decrease in the immunohistochemical staining positive fibers in chronic hepatitis and liver cirrhosis than in the normal controls. From our results, the S-100 protein was a smart detection marker of innervations as it

was a marker of the Schwann cells that nearly totally surround the nerve sheaths. 94% of specimens, 100% of chronic hepatitis and 97% of liver cirrhosis from specimens showed positive expressions. These results accord with other studies. Undoubtedly, there may be several biases such as the state of the liver tissues, staining skill and/or methods and possible minor errors in the calculation of positive staining. The most important potential bias might arise from errors in quantifying positively stained fibers. However, attempts were made to minimize those biases by enrolling many cases of liver tissues, by repeating the calculations of the staining results for three times, and by repeated pre-examinations of the antibodies.

The decrease of nerve innervations in liver cirrhosis and chronic hepatitis may suggest that the nerves have been damaged by progressive fibrosis within parenchymal region, which in turn, deregulated the hepatic cellular metabolisms and/or hemodynamics. Therefore, deregulations of each metabolism and nerve damages may have a mutual influence in the aggravation of liver diseases.

There have been several studies on alteration of hepatic innervations in liver diseases. Ungvary & Donath²¹ showed changes of intrahepatic innervations in guinea pig livers following common bile duct ligation. Miyazawa et al⁸ also reported that there were changes in the S-100 and neuron specific enolase positive nerve fibers in chronic hepatitis. Similarly, Lee et al²² demonstrated a complete absence of nerve fibers in advanced cirrhosis. Our data from specimens of established liver cirrhosis also showed remarkable decrease in the hepatic parenchymal nerve fibers, which corresponded with previous reports.

Electron microscopy is frequently used for phenotype and delineation of ultrastructures. On electron microscopy, unmyelinated nerve fibers including Schwann cells, axons, mitochondria and lysosomes ran in the vicinity of portal ducts and bile duct cells in our examination. Hepatic nerves, arteriols and/or bile ducts were contracted with nerve fibers by direct manner. However, the specific differences of phenotypical characteristics were not noticed between the normal controls and chronic liver diseases in this study. More expanded and meticulous investigations of electron microscopical examinations may discern the difference of phenotypes in the hepatic innervation.

In conclusion, from our study, the nerves were found to be present and closely placed not only in the connective tissues in the portal tracts and in the parenchymal region but also along the vasculature and bile ducts. The expressions of nerve fibers in chronic hepatitis and liver cirrhosis decreased compared with that of the normal

controls. In established cirrhosis, the intrahepatic nerves may be damaged by inflammatory responses within the parenchymal region, and in turn, the hepatic metabolism and hemodynamic mechanism may be disturbed. Hepatic nerves may be progressively lost by fibrotic changes. Further study is needed to clarify this observation.

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