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Abnormal Distribution of Nerve Fibers in the Liver of Biliary Atresia

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Iwami, D., Ohi, R., Nio, M., Shimaoka, S., Sano N. and Nagura, H. *Abnormal Distribution of Nerve Fibers in the Liver of Biliary Atresia.* Tohoku J. Exp. Med., 1997, **181** (1), 57-65 —— We investigated changes in the pattern of hepatic innervation in liver specimens from 15 infants with biliary atresia and 4 age-matched controls by immunohistochemical methods. In the control, nerve fibers identified by immunoreactivity for neural cell adhesion molecule (NCAM) and S100 protein were present around the branches of hepatic arteries, portal veins and bile ducts in the portal areas and the hepatic lobules. In biliary atresia, NCAM and S100 positive nerve fibers were increased in the vicinity of the hepatic arteries and the portal veins in the enlarged portal areas, while no nerve fibers were observed around bile ducts and periportal ductules which became NCAM positive. No innervation in the lobules was seen in any cases regardless of the histological alteration. These findings may suggest that the abnormal innervation in the liver with biliary atresia does not occur as a result of structural changes in liver architecture caused by portal fibrosis and inflammation, but is associated with immaturity or malformation of hepatic innervation in the patients. ——
biliary atresia; hepatic innervation; neural cell adhesion molecule

The liver is innervated by both sympathetic fibers derived mainly from the celiac plexus and parasympathetic fibers from the vagus. These nerve fibers are distributed in the liver along portal veins, hepatic arteries and bile ducts. Autonomic innervation of the liver has been shown to play an important role in the regulation of hepatic microcirculation, glycogen and lipid metabolism and biliary secretion and flow (Lautt 1983; Friedman 1988; Bioulac-Sage et al. 1990). Therefore, it may be valuable to elucidate the distribution and density of the peripheral nerve fibers in the liver to understand the pathogenesis and clinical courses of liver diseases.

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There has been limited information on the changes of hepatic innervation in liver diseases. We previously reported that nerve fibers were absent in the regenerated lobules of cirrhotic liver (Miyazawa et al. 1988), and such an alteration of innervation may be one factor influencing the change of liver hemodynamics and metabolic activities. In some cases of biliary atresia, the hepatic disease continues to progress, and cirrhotic change with functional abnormalities develops despite the restoration of bile drainage after portoenterostomy (Ohi et al. 1990). Thus, the aim of this study was to investigate the distribution of nerve fibers in the liver with biliary atresia and to identify the cause and influence of hepatic innervation abnormalities. This is, to our knowledge, the first article on hepatic innervation in biliary atresia.

Materials and Methods

Tissue specimens

Liver tissue specimens taken at the time of corrective operation in 15 infants with biliary atresia were studied. Liver specimens of 4 age-matched infants without any hepatobiliary disorders were taken for the diagnostic purpose and served as controls. All specimens were obtained by wedge biopsy during the operation.

Antibodies

Antibodies to Leu19 and S100 protein were used in this study. The specificity of each antibody is shown in Table 1. S-100 protein is a 23,000 MW protein soluble in 100% saturated NH_4Cl found in a variety of cells, particularly those of neuroectodermal origin. It has been proven that S100 protein is always expressed by Schwann cells, and can be used as a marker for peripheral nerve fibers. Clone Leu19 is directed against an epitope of neural cell adhesion molecule (NCAM). NCAM is a membrane protein involved in the homotypic adhesion between cells of the central and peripheral neural tissues and expressed throughout the plasma membrane of neurons, including axons and dendrites.

Immunohistochemical technique

The tissue specimens were fixed in periodate-lysine-4% paraformaldehyde

Table 1. *Antibodies used in the study*

Antibody	Specificity	Source	Reactivity	Working dilution
Anti-NCAM antibody Clone Leu 19	NHK 1a epitope	Becton-Dickinson	Plasma membranes of neurons, axons and dendrites	1 : 100
Polyclonal rabbit antibody	S100 protein	DAKO	Schwann cells	1 : 1,000

(4% PLP) for 6 hr at 4°C, and then washed in increasing concentration of sucrose in phosphate-buffered saline (PBS) and finally placed in 20% sucrose. The fixed specimens were embedded in Tissue-Tek OCT compound (Miles Pharmaceutical, Naperville, IL, USA), frozen in dry-ice acetone, and sectioned at 10 μm thickness on a cryostat microtome. The sections were mounted on egg-albumin-coated slides, and dried for 30 min at the room temperature. The indirect immunoperoxidase method was used for the immunohistochemical staining. These sections were treated with methanol and then 0.03% hydrogen peroxidate before being stained in order to inhibit endogenous peroxidase activity, and then incubated for 36 hr at 4°C with the first antibodies. As a control non-immunized rabbit or mouse serum was used instead of the first antibodies. Thereafter, the sections were incubated for 18 hr with the peroxidase-labeled second antibody to the rabbit or mouse IgG. After washing with PBS, the sections were dipped in 0.025% diaminobenzidine solution containing 10 mM hydrogen peroxide and 10 mM sodium azide for 3 min at room temperature, and then they were counterstained with methylgreen.

Quantitation

NCAM- and S100-positive peripheral nerve fibers were counted in high-magnification fields (ocular lens $\times 10$; objective lens $\times 40$), corresponding to a surface of 0.5 mm^2 each.

Results

Clinical data and histological findings of the liver in 15 patients with biliary atresia are shown in Table 2. Data on the distribution of nerve fibers detected by the antibodies to NCAM and S100 protein are shown in Table 3.

Controls

NCAM and S-100-immunoreactive nerve fibers were seen in the portal tracts. They were found around the branches of both the hepatic arteries and portal veins, and in small numbers around bile ducts and bile ductules (Fig. 1A).

Even at 4 weeks of age, NCAM-immunoreactive nerve fibers were also found within the hepatic lobules (Fig. 1B). These fibers were detected along the sinusoidal wall and between the hepatocytes. Their distribution was uniform throughout lobules. In centrilobular areas, they were occasionally observed within the connective tissue around the central vein.

Biliary atresia

Although portal fibrosis developed earlier than 5 or 6 weeks of age, no cirrhotic change was observed. Periportal ductular proliferation associated with abundant mononuclear infiltrates was seen in all specimens. Hyperplasia of the smooth muscle of the branches of hepatic artery was present in the enlarged portal

TABLE 2. *Clinical data and histological findings of 15 patients with biliary atresia*

Case	Age at operation (weeks)	Liver fibrosis[a]	Ductular proliferation[b]	Hyperplasia and hypertrophy of arteries[c]
1	1	−	−	−
2	4	−	+	−
3	4	+	+	+
4	5	−	+	−
5	5	+	+	+
6	6	+	+	−
7	6	++	++	−
8	7	+	++	−
9	7	+	+	+
10	7	++	++	+
11	8	++	++	+
12	8	++	++	+
13	10	++	++	+
14	11	+	+	−
15	12	++	+	−

[a]degree of fibrosis: −, no visible; +, mild; ++, moderate; +++, severe.
[b]grade of proliferation: +, slight; ++, moderate; +++, marked.
[c]hyperplastic and hypertrophic change of arteries: −, absent; +, marked.

TABLE 3. *Distribution of nerve fibers of the liver with control and biliary atresia*

	(Portal areas around)		(Lobules around)	
	hepatic arteries and veins	bile duct/ductule	sinusoid	central veins
Control ($n=4$)	++[a]	+/+	+	+
Biliary atresia ($n=15$)	+〜+++	−/−[b]	−	−〜+

[a]−, absent; +, few; ++, moderate number; +++, numerous.
[b]proliferated bile ductule

areas. The ductular epithelial cells were always NCAM-positive.

In the enlarged portal areas, S100- and NCAM-positive nerve fibers were increased in close association with branches of the hepatic arteries and portal veins (Fig. 2), but were absent in the periportal zones in which NCAM-positive ductules proliferated. In some cases which were almost histologically normal without portal fibrosis, nerve fibers were almost absent in small portal tracts (Fig. 3). In these cases nerve fiber bundles were present in the larger proximal portal areas. In addition, NCAM and S100-immunoreactive nerve fibers were absent within the

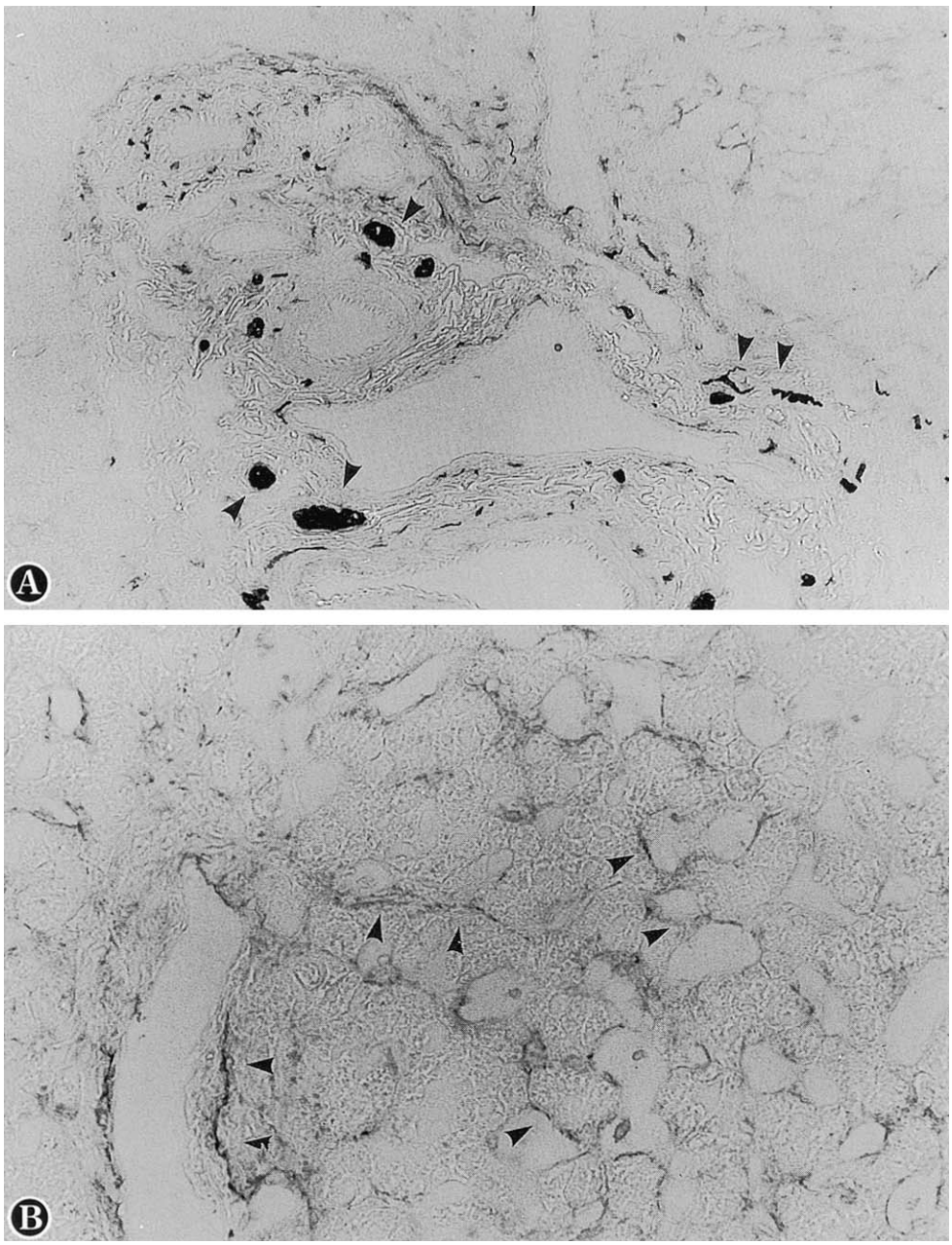

Fig. 1. (A) Small portal tract of the normal liver. NCAM immunoreactive fibers and bundles are seen around the branches of hepatic arteries, portal veins and bile ducts (arrows) (magnification ×330).
(B) Normal hepatic lobules. NCAM immunoreactive fibers are seen running along sinusoids (arrows) (magnification ×400).

hepatic lobules in all cases (Fig. 3), irrespective of the age of the patients (from 1 week to 12 weeks). Nerve fibers were also scanty around the central veins at the center of each lobule.

Discussion

This study demonstrated the abnormal innervation of liver specimens from cases of biliary atresia using immunohistocemical staining for S100 and NCAM. Complete absence of innervation was demonstrated in the lobules and around the

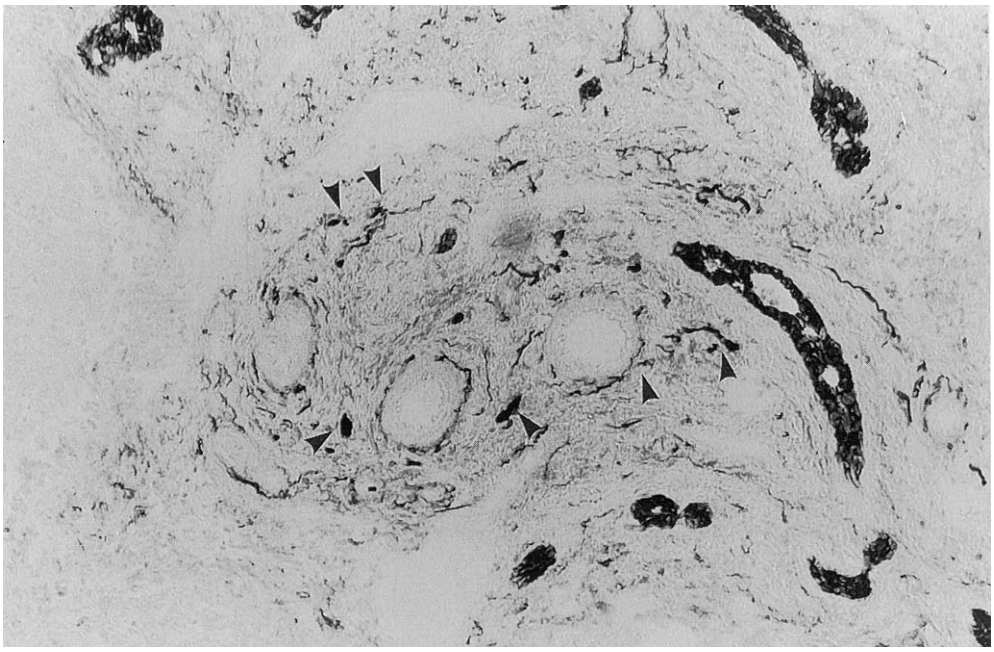

Fig. 2. Biliary atresia(case7): NCAM immunoreactive fibers and bundles are proliferated and lie in close contact with branches of the hepatic arteries and portal veins (arrows). No nerve fibers are visible in their periportal zones in which NCAM-positive ductules are proliferated (magnification $\times 660$).

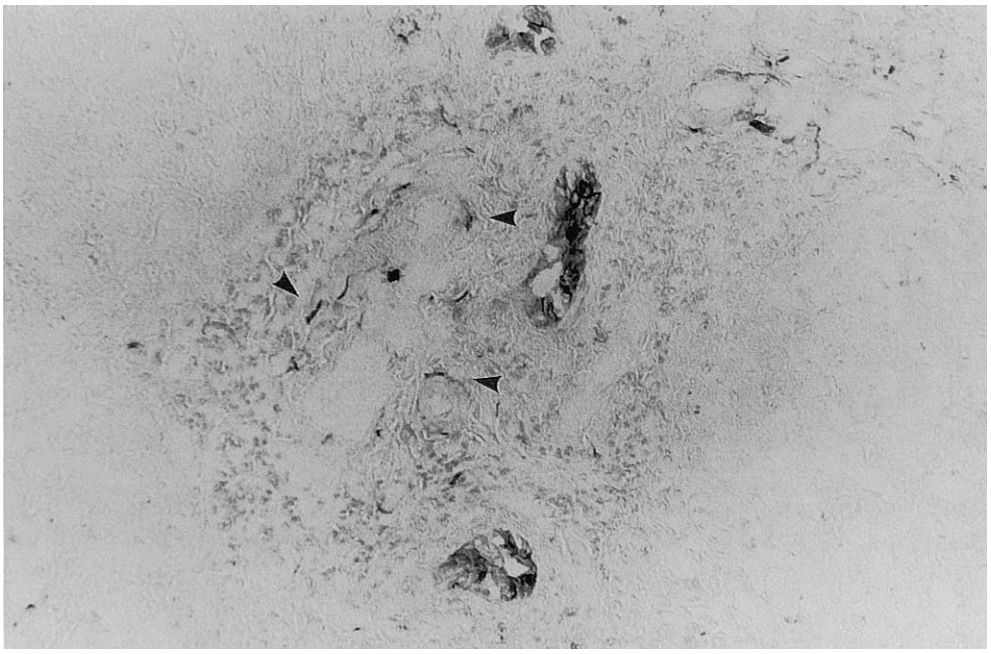

Fig. 3. Biliary atresia(case 1): A few NCAM immunoreactive fibers are seen in the portal area which is almost histologically normal without fibrosis (arrows). Nerve fibers are absent within the hepatic parenchyma (magnification $\times 400$).

proliferated bile ductule in the periportal zone, while nerve fibers around hepatic arteries and veins were increased in number.

Immunostaining for S100 protein and NCAM allows histological

identification of nerves in tissue sections. It is well known that S100 protein is positive in Schwann cells (Vanstapel et al. 1986), and a monoclonal antibody to Leu19 which recongnizes an epitope of the NCAM (Lanier et al. 1989) has proved to be more suitable for the detection of intralobular nerve fibers than other neural cell markers, such as S100 protein, neurofilaments and neuron-specific enolase (Scoazec et al. 1993). The present study revealed that the incidence of positive nerve fibers detected by NCAM was more frequent than those by S100.

NCAM- and S100-immunoreactive nerve fibers were found around the branches of hepatic vessels in the tissue sections from both biliary atresia patients and age-matched controls. Portal and periportal fibrosis developed with increasing age in biliary atresia, and increased nerve fibers and large bundles were seen around hepatic vessels and in the connective tissues in the enlarged portal areas. Several studies have been described on the distribution of increased nerve fibers in the adult liver with hepatobiliary disorders, such as chronic active hepatitis (Miyazawa et al. 1988), toxic liver disease (Jaskiewicz et al. 1993) and hepatolithiasis (Terada and Nakanuma 1989). Thus, nerve fiber proliferation is not a specific phenomenon in biliary atresia. We and others also demonstrated that the branches of hepatic artery in portal areas showed hyperplastic and hypertrophic changes in biliary atresia (Chiba et al. 1987; Ho et al. 1993). We found that proliferating nerve fibers existed in the vicinity of the branches of these hepatic arteries. These findings suggest that the proliferation of nerve fibers closely correlate with the morphological changes of hepatic vessels. Extrinsic nerve fibers enter the liver by the same route as the portal vessels (Ueno et al. 1987) and participate in blood flow regulation (Friedman 1988). It is possible that increased nerve fibers exert an altered neuronal influence on the hepatic vascular system in biliary atresia.

Another finding of this study was an absence of an anatomic relationship between nerve fibers and proliferating bile ductules in biliary atresia. We found that no nerve fibers were seen around proliferating bile ducts and ductules, even though they were increased in number within enlarged portal areas in the vicinity of hepatic vessels. Although the functional role of bile duct innervation has not been clearly elucidated, the innervation seems to contribute to the regulation of bile flow and bile duct cell proliferation and maturation (Ding et al. 1991). It is known that there is no significant correlation between denervation around bile ducts and initial bile secretion after surgical treatments. However the liver is also innervated from the capsulular connective tissue and ligments. In addition, NCAM, expressed on the plasma membrane of neurons, might play a role for cell-to-neuron interaction and maturation of cellular functions (Cunningham et al. 1987). Interestingly, proliferated but non-innervated bile ducts were positive for NCAM, a homotypic cell adhesion molecule (Roskams et al. 1990).

This is the first report of an absence of nerve fibers in the hepatic lobules in biliary atresia. This finding was observed in all cases regardless of the

chronologic stage. In control cases, even at 4 weeks of age, NCAM-immunoreactive nerve fibers were found within the lobular parenchyma. It is interesting that there were some cases with an absence of nerve fibers in the small portal areas without fibrosis, whereas nerve fibers and large bundles were present in the proximal portal areas. These findings were seen in the case which was histologically almost normal in the earliest clinical stage. There was also a total loss of parenchymal innervation, suggesting that the parenchymal denervation does not correlate with the histologic alterations in the course of the disease.

We previously reported that no nerve fibers were detected in regenerative nodules of cirrhotic liver. Similar results were obtained with different antibodies to protein gene product 9.5 (PGP9.5) (Lee et al. 1992). A normal distribution of nerve fibers along the sinusoidal wall in various forms of acute liver injury was also observed. In our present study, no cirrhotic changes were observed in the older patients with severe portal fibrosis. Some cases have been known to develop liver cirrhosis despite successful portoenterostomy. The absence of parenchymal innervation is therefore unlikely to be related to progressive destruction of the normal structure of portal areas in biliary atresia, but the denervation within hepatic lobules may be associated with the etiology of the disease or an essential malformation. Further study of the fetal-neonatal hepatic innervation would clearly eliminate this possibility.

In summary, this paper describes the changes of hepatic innervation in patients with biliary atresia. We demonstrated the total absence of innervation in hepatic lobules and around proliferating bile ductules regardless of the severeity of the clinical stage. On the other hand, there were proliferations of nerve fibers around hepatic arteries and portal veins. The neuronal communications between the hepatic blood vessel system and hepatic nerves may be important in the regulation of hepatic blood flow, in glucose control, energy homeostasis and the functional maturation of cells in the liver. Although the functional consequences of the lack of parenchymal innervation in biliary atresia remain unclear, we speculate that these neuronal changes may be important factors in the progression of the disease.

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