Pathways for movement of fluid and cells from hepatic sinusoids to the portal lymphatic vessels and subcapsular region in rat livers*

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Summary. It has long been a mystery how fluid and migrating cells in the hepatic sinusoids reach lymphatic vessels in the portal tract. Here we describe previously-unknown channels that connect the space of Disse with the portal tract in the rat liver. Transmission electron microscopy was performed on livers injected with either horseradish peroxidase (HRP) or lipopolysaccharide, and scanning electron microscopy was carried out on livers macerated with KOH. Transmission electron microscopy revealed the presence of channels with collagen fibers traversing the limiting plate. A tracer study showed that HRP was in the channels as well as along inlet venules. Dendritic cells in the hepatic sinusoids or between hepatocytes of the limiting plate were also observed extending their pseudopodia through the channels in the limiting plate to the interstitial space of the portal tract. Scanning electron microscopy further showed that many channels (1–3 μm in diameter) penetrated through the limit-

ing plate independently of blood vessels and connected the space of Disse with the interstitial space of the portal tract. In addition, the portal tract possessed prelymphatic vessels that were lined with fibroblast-like cells and frequently contained dendritic cells. The initial segment of the portal lymphatic vessels opened to the interstitial tissue space. These results indicate that fluid and dendritic cells in the hepatic sinusoids probably pass through both the space of Disse and the channels traversing the limiting plate, enter the interstitial space of the portal tracts, and finally move from the prelymphatic vessels to the portal lymphatic vessels.

Introduction

The liver produces a large volume of lymph estimated at 25 to 50% of the lymph flowing through the thoracic duct (Barrowman, 1991). The hepatic lymphatic system divides into three categories: portal, sublobular, and capsular lymphatic vessels (Lee, 1923; Comparini, 1969; Trutmann and Sasse, 1994). It is suggested that 80% or more of the hepatic lymph drains into portal lymphatic vessels, while the remainder drains through sublobular and capsular lymphatic vessels (Popper and Schaffer, 1957; Ritchie *et al.*, 1959; Yoffery and Courtice, 1970). As the protein content of the portal lymph is about 80% of the plasma concentration (Yoffery and Courtice, 1970; Courtice *et al.*, 1974), it must originate in a very permeable capillary bed (Gammel and Heath, 1972; Wright *et al.*, 1983; Wisse *et al.*, 1985). Considering the large volume of portal lymph in connection with the structure of the portal capillaries, the portal tracts themselves do not appear to be a major source of portal lymph; indeed, Heath and Lowden (1998) reported that the space of Disse is continuous with the interstitial space of the portal tracts at the origin of the sinusoids. However, there seem to be other routes that allow fluid in the space of

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Fig. 1. Transmission electron micrographs of intact rat livers showing the ultrastructure of the portal tract. Processes of fibroblast-like cells (F) form spaces (*) that mimic lymphatic vessels, in which migrating cells (presumably dendritic cells and/or lymphocytes) can be seen (**a**, **b**). In **b**, there is a longitudinally cut space (**) lined incompletely with extremely thin cells that appear to be lymphatic endothelial cells. At the initial segment of the sinusoid (S), is a space (arrowheads) with collagen fibers between sinusoidal endothelial cells and limiting plate hepatocytes (H) (**a**). There is also a space with collagen fibers between limiting plate hepatocytes (**b**); this space is continuous with the space of Disse (D) on the one side, and with the interstitial tissue space of portal tract (or the space of Mall) (arrows) on the other side as indicated by arrowheads. Bd: bile ductule, iv: interlobular vein. a: $\times 3,000$, b: $\times 4,850$

Disse to enter the interstitial space of the portal tract because inlet venules exist only in small portal tracts.

It is known that dendritic cells, bone-marrow-derived, professional antigen presenting cells exist in the hepatic sinusoids and in the portal tracts. They capture and process antigens and enter the draining lymphatic vessels (Matsuno and Ezaki, 2000). Some researchers suggested that rat dendritic cells can undergo a blood-lymph translocation via the hepatic sinusoids (Kudo *et al.*, 1997). Intravenous injection of lipopolysaccharide (LPS) increases the dendritic cell output in the hepatointestinal lymph (MacPherson *et al.*,

1995). It is also suggested that translocation of dendritic cells into the hepatic lymph is accelerated in pathological conditions (Matsuno and Ezaki, 2000). However, the pathways of dendritic cells from the hepatic sinusoids to portal lymphatics have not been clearly determined.

Thus, the present study was carried out firstly to elucidate fluid pathways from the hepatic sinusoids to the portal lymphatic vessels by light and transmission electron microscopy of rat livers either with or without injection of horseradish peroxidase (HRP) and by scanning electron microscopy of rat livers treated with the KOH-maceration

Fig. 2. A transmission electron micrograph of the LPS-injected rat liver showing a presumable dendritic cell (Dc) that extends its long pseudopodium into the space in the portal tract, while retaining its nuclear portion between limiting plate hepatocytes (H). Bd: bile ductule, iV: expanded interlobular vein. $\times 4,000$

technique, which allows us to observe the organization of cellular elements. Secondly, we examined the traffic routes of dendritic cells by transmission electron microscopy of rat livers injected with lipopolysaccharide (LPS). Our results show hithertounknown channels for the transport of fluid and cells from the hepatic sinusoids through the limiting plate (i.e., a layer of the outermost hepatocytes) to the portal lymphatic vessels.

Materials and Methods

Eleven adult male Wistar rats weighing 150–200g were used in this study. The animals were kept in an air-conditioned room, and given laboratory chow and water *ad libitum*. All animals were treated in strict compliance with the policy of the National Institute of Health on the care of humans and laboratory animals.

HRP-injection/transmission electron microscopy

Type II HRP was purchased from Sigma (St. Louis, MO, USA), and 100 mg was dissolved in 1.0 ml of 0.9 % saline. Under anesthesia with pentobarbital sodium, three rats were injected with 0.3 mg/g body weight of HRP through the tail vein. Two rats were used for the control: one was injected with 0.9% saline equivalent volume of HRP injection and the other was left intact without any injections. All the rats were euthanized with an intraperitoneal injection of an overdose of pentobarbital sodium 15 min after the injection. After a brief perfusion with saline through the aorta, the rats were perfused with 2.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.4). The excised livers were cut into blocks (approximately $10 \times 8 \times 3$ mm³), and further immersed in the same fixative for 3 h. Sections of 100 μ m thickness were obtained with a microslicer (Dosaka, Osaka), and incubated in a tris-HCl buffered (pH 7.4)

Fig. 3. A transmission electron micrograph of an LPS-injected rat liver. A portal lymphatic vessel (L) appears to be expanded and contains a lymphocyte and a plasma cell. A wide gap (*) between lymphatic endothelial cells opens to the interstitial tissue space. H: hepatocyte, M: mast cell. $\times 4,000$

diaminobenzidine- H_2O_2 solution for 15 min. They were then postfixed in 0.1% OsO_4 in a phosphate buffer for 2 h, dehydrated through ascending concentrations of ethanol, and embedded in Epon 812 using aclar film (Nisshin EM, Tokyo). Semithin sections were stained with toluidin blue and observed with a light microscope. Ultrathin sections treated either with or without staining with uranyl acetate and lead citrate were examined under a JEOL 200 CX transmission electron microscope (JEOL, Tokyo).

LPS-injection/transmission electron microscopy

LPS from *Escherichia coli*, Serotype 0111:B4 was purchased from Sigma (Lot 70K 4108). Under anesthesia with pentobarbital sodium, two rats were each injected with 50 μg LPS in 0.5 ml saline through the tail vein. One rat was injected with 0.5 ml saline for the control study. All the rats were euthanized 8 h after the injection. Their excised livers were cut into small pieces, immersed in 2.5 % glutaraldehyde

for 2 h, and postfixed in 0.1% OsO_4 for 2 h. After dehydration through ascending concentrations of ethanol, the specimens were embedded in Epon 812. Semithin sections were stained with toluidin blue and observed with a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in the transmission electron microscope.

KOH-maceration/scanning electron microscopy

Specimens were prepared according to our modification (Ohtani, 1987, 1992a; Ohtani *et al.*, 1993; Shao, 1998) of the KOH-maceration technique (Ushiki and Ide, 1988). Three rats were euthanized with pentobarbital sodium and perfused with 0.9 % saline through the aorta, which was followed by a perfusion fixation with 2.5 % glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4). The liver was excised and cut into small blocks (approximately $3 \times 3 \times 5 \text{ mm}^3$), which were further immersed in the same fixative for more

Fig. 4. A light micrograph of the HRP-injected rat liver. HRP reaction products (black) can be seen in the sinusoids (S), in the space (arrowheads) between limiting plate hepatocytes (H), and in the space of Mall (arrows). iB: interlobular bile duct, Bd: bile ductule. $\times 1,200$

than a day. After a brief rinse in a phosphate buffer, the blocks were incubated in a 7N-KOH aqueous solution at 60°C for 10 min. The blocks were dehydrated through ascending concentrations of ethanol and freeze-dried using t-butyl alcohol. Some specimens were microdissected under a binocular microscope to remove structures within the portal tracts. All the specimens were mounted on specimen holders with silver paste, coated with gold, and observed in a Hitachi S-3200 scanning electron microscope (Hitachi, Tokyo) with an accelerating voltage of 15 kV.

Results

Transmission electron microscopy of the intact liver

At the initial segments of the hepatic sinusoids, there were interstitial spaces containing collagen fibers between sinusoidal endothelial cells and hepatocytes forming the limit-

ing plates (i.e., limiting plate hepatocytes) (Fig. 1a). There were also spaces or channels which penetrated through the limiting plate with collagen fibers, independent of the blood vessels (Fig. 1b). Both types of spaces were continuous with the space of Disse on one side, and with the interstitial space of the portal tract on the other.

In the connective tissue of the portal tract were many oval spaces, their shorter diameters ranging some 3 to 10 μm (Fig. 1); these spaces were incompletely lined with thin processes of fibroblast-like cells, and their abluminal surface was frequently in contact with collagen fibers. Also observed close to such spaces were apparent lymphatic vessels, which were incompletely lined with endothelial cells, and thus opened to the interstitial space. The fibroblast-like cells also extended their processes to embrace collagen fibers (Fig. 1a, b). Migrating cells, presumably dendritic cells, were frequently observed in the space lined with fibroblasts and lymphatic vessels (Fig. 1).

Fig. 5. Transmission electron micrographs of the HRP-injected rat liver. **a:** HRP can be seen in the sinusoid (S), in the complicated space of Disse (D), between hepatocytes (H), and along a collagen fiber (C). $\times 31,200$. **b:** Between limiting plate hepatocytes is a collagen fiber that connects the space of Disse (arrowheads) with the portal tract. HRP along the collagen fiber is continuous with that in the space of Mall (arrows). **iA:** interlobular artery. $\times 4,000$

Transmission electron microscopy of the LPS-injected liver

Eight hours after the injection of LPS, dendritic cells were frequently observed between the limiting plate hepatocytes as well as in the interstitial space and lymphatic vessels of the portal tract (Fig. 2). Some dendritic cells extended their long pseudopodia into the interstitial space in the portal tract, while retaining their nuclear portions in the space between the limiting plate hepatocytes (Fig. 2).

Because the interstitial space in the portal tract was edematous, the space of Mall, i.e., the interstitial space between the connective tissue of the portal tract and the periportal limiting plate (Mall, 1901), was clearly revealed by transmission electron microscopy (Fig. 3). Mast cells frequently existed in the edematous space of the portal tract (Fig. 3).

Lymphatic vessels consisted of thin and irregular endothelial cells with scattered vesicles (Fig. 3). These vessels appeared to be expanded, and frequently showed wide gaps of some $5\ \mu\text{m}$ or more in width between the endothelial cells (Fig. 3). Plasma cells and lymphocytes were also observed in the lymphatic vessels.

Light- and transmission electron-microscopy of the HRP-injected liver

By light microscopy of the HRP-injected liver, HRP reaction products were found in the sinusoids, the space of Disse, and the space of Mall. Reaction products were also frequently observed in the space between limiting plate hepatocytes; this space connected the space of Disse and the space of Mall (Fig. 4).

Transmission electron microscopy clearly showed the

Fig. 6. A transmission electron micrograph of the HRP-injected rat liver. A dendritic cell (Dc) in a sinusoid extends its pseudopodia through the space between hepatocytes (H) forming a limiting plate to the space in the portal tract. HRP can be seen along the pseudopodia as well as in the sinusoids (S), in the space of Disse, along the collagen fibers (C), and within a lymphatic vessel (L) in the portal tract. $\times 7,400$

presence of HRP reaction products in the sinusoid and the complicated space of Disse (Fig. 5a). HRP products were also found along collagen fibers in the space of Disse. Some of the collagen fibers with HRP reaction products traversed the limiting plate, and thus connecting collagen fibers in the space of Disse and those in the portal tract (Fig. 5b). Portal lymphatic vessels also contained HRP products (Fig. 6). The sinusoids occasionally exhibited dendritic cells that extended their pseudopodia through the gaps of sinusoidal endothelial cells, and the space of Disse. These cells were also found in the space between limiting plate hepatocytes and extend their processes toward the interstitial space of the portal tract (Fig. 6).

Collagen fibers running independently of blood vessels were also present between abutting hepatocytes facing the liver capsule. These fibers connected collagen fibers of the liver capsule with those in the space of Disse (Fig. 7a). HRP was observed in and around these collagen fibers (Fig. 7b).

Scanning electron microscopy of KOH-macerated liver

KOH-maceration followed by microdissection under a binocular microscope exposed the portal side surface of the limiting plate surrounding the portal tract. There were many openings of channels extending through the limiting plate (Fig. 8a). The density of the openings was approximately $1.3 \times 10^3/\text{mm}^2$. These openings of the channels were generally located in the areas where three hepatocytes met (Fig. 8b). There were sometimes undigested collagen fibers that emerged from the channels (Fig. 8a, b, 9a). Fibroblast-like cells occasionally extended their processes into the channels (Fig. 8c). Accidentally fractured limiting plates showed that the channels sometimes contained undigested collagen fibers and passed through the limiting plate to connect the space of Disse with the interstitial space of the portal tract (Fig. 9).

The KOH-maceration also exposed the cellular organiza-

Fig. 7. Transmission electron micrographs of the intact (a) and HRP-injected (b) rat liver near the capsule. Collagen fibers (C) running independently of blood vessels between hepatocytes connect those of the liver capsule (Cp) with those in the space of Disse (arrowheads). Along these collagen fibers are spaces that are filled with HRP (black). S: sinusoid. a: $\times 2,300$, b: $\times 7,400$

tion within the portal tract. Lymphatic vessels consisted of only thin endothelial cells existing in the connective tissue between the portal triads and the limiting plate (Fig. 10). There was no tendency for lymphatic vessels to run preferentially along the interlobular vein, artery, or bile duct.

Discussion

Fluid pathways from the hepatic sinusoids to the interstitial space of the portal tracts

The present study has clearly shown that the space of Disse is in continuity with the interstitial space of the portal tract or space of Mall (Mall, 1901) through the channels travers-

ing the limiting plate. The channels contain collagen fibers. Furthermore, our tracer study using HRP has shown that intravenously injected HRP was not only present in the space of Disse and that of Mall but also located along collagen fibers in the channels traversing the limiting plate. It has been established that there are numerous collagen fibers that connect those in the space of Disse with those in the portal tract (Ohtani, 1988, 1992b). In this context, fluid can flow along collagen fibers in the channels traversing the limiting plate, as speculated by Trutmann and Sasse (1994).

Recently, Heath and Lowden (1998) reported that the space of Disse is continuous with the interstitial space of the portal tract at the origin of the hepatic sinusoids (or inlet venules), which was also confirmed by the present study. However, as the inlet venules traversing the limiting plate exist only in the small portal tract, it is unlikely that

Fig. 8. Scanning electron micrographs of the rat liver treated with the KOH-maceration technique, showing surface views of the portal tract side of limiting plates. **a:** There are many openings (*arrowheads*) of channels between limiting plate hepatocytes. Occasionally collagen fibers (*arrows*) remaining undigested can be seen emerging through the openings (*arrows*). $\times 1,100$. **b:** A closer view of an opening surrounded by three hepatocytes (H). $\times 3,200$. **c:** A fibroblast-like cell (F) covers the opening between hepatocytes. $\times 2,300$

the voluminous portal lymph flows only through the space along the inlet venules.

The normal blood pressure in the portal vein is 7 mmHg, while that of the interstitial pressure of the portal tract is 5.8 mmHg (Laine *et al.*, 1979). Since the hepatic sinusoids are highly permeable and the oncotic pressure along the sinusoids is accordingly negligible, fluid in the hepatic sinusoids can flow through the channels traversing the limiting plate to the interstitial space of the portal tract in accordance with the hydrostatic pressure gradient.

The interlobular bile duct is surrounded by the peribiliary capillary plexus (Ohtani and Murakami, 1978; Ohtani, 1979). The peribiliary capillary consists of endothelial cells of a continuous type (Barrowman and Granger, 1984). In accordance with the force of Starling, fluid is filtered through the peribiliary capillaries into the surrounding

connective tissue. However, Földi (1974) estimated that the peribiliary capillary plexus contributes less than 10% of the total lymph output from the liver.

Taken together, these facts show it is likely that fluid filtered out of the hepatic sinusoids into the space of Disse flows through the channels traversing the limiting plate either independently of blood vessels or along inlet venules to reach the interstitial space of the portal tract, and that fluid in the hepatic sinusoids is the major source of the portal lymph (Fig. 11).

Fig. 9. Scanning electron micrographs of the rat liver treated with the KOH maceration technique. **a:** A closer view of the surface of the portal tract side of a limiting plate showing collagen fiber (C) emerging from a channel between hepatocytes (H). $\times 10,000$. **b:** A channel with collagen fibers (arrowheads) between limiting plate hepatocytes (Lp) is continuous with the space of Disse (D) on the one side and the interstitial space of the portal tract (P) on the other. $\times 5,000$

Pathways for blood-lymph translocation of dendritic cells

The present study has shown that cells, presumably dendritic cells in the hepatic sinusoids or between limiting plate hepatocytes, extended their long pseudopodia through channels in the limiting plate to the interstitial space of the portal tract. The morphology of these cells strongly suggests that the cells migrate from the hepatic sinusoids through the channels in the limiting plate to the interstitial space of the portal tract, although the fluid and cellular pathway may not show exactly the same phenomena under physiological conditions.

According to Matsuno and Ezaki (2000), mature dendritic cells in the blood can transmigrate only from the blood vessels of the liver and spleen. Dendritic cell release is

enhanced by an intravenous injection of endotoxin (Young *et al.*, 1993; MacPherson *et al.*, 1995). In accordance with this, our study showed that cells transmigrating through the limiting plate were more frequently seen in the LPS-injected liver than in the control one. In this context, it is likely that the cells transmigrating through the limiting plate are mostly dendritic cells. As lymphocytes also translocate from the blood to the hepatic lymph, some of the migrating cells we observed may be lymphocytes. A future immunoelectron microscopic study should be able to determine the nature of the cells transmigrating through the limiting plate.

Considering the above, dendritic cells in the hepatic sinusoids probably translocate through the space of Disse and the channels in the limiting plate to the interstitial space of the portal tract, and then migrate into the portal lymphatic vessels (Fig. 11).

Fig. 10. A scanning electron micrograph of the rat liver treated with the KOH-maceration technique showing lymphatic vessels (L) in the portal tract. iA: interlobular artery with circular smooth muscles. $\times 500$

Prelymphatic and lymphatic vessels in the portal tract

The present study by transmission electron microscopy has shown many oval spaces incompletely lined with fibroblast-like cells in the portal tract. Thus, the interstitial space of the portal tract showed a porous appearance. Observations of serial sections suggest that the spaces form a tubular structure partly lined with fibroblast-like cells. The spaces in question frequently contained lymphocytes and/or dendritic cells. It is therefore likely that the spaces or tubular structures, herein termed prelymphatic vessels, serve as pathways for migrating cells such as lymphocytes and dendritic cells as well as the fluid draining into the portal tract.

Corrosion casting/scanning electron microscopy showed that the portal lymphatic networks develop around the portal triads, and extend as far distally as the terminal portal tract in rabbits, guinea pigs, and rats (Yamamoto and

Phillips, 1986; Ohtani, 1989). There were no tendencies for the portal lymphatic vessels to run preferentially along the interlobular arteries, veins, or bile ducts. These findings also seem to support the hypothesis that the portal lymph comes mainly from the hepatic sinusoids.

How do the prelymphatic vessels continue to the lymphatic vessels? In this study, we failed to demonstrate any direct transition of the prelymphatic vessels to the lymphatic vessels. As the prelymphatic vessels are only incompletely lined with fibroblast-like cells and initial lymphatic vessels open to the interstitial space, fluid and cells can probably easily move from the prelymphatic vessels to the lymphatic vessels. Further studies with a variety of methods will have to determine whether the prelymphatic vessels directly continue to the lymphatic vessels. Other investigations should examine the nature of the fibroblast-like cells lining the prelymphatic vessels in relation to lymphatic vessels.

Fig. 11. A schematic representation of pathways of fluid and dendritic cells (Dc) extending from the sinusoids (S) through the space of Disse, channels in the limiting plate, and interstitial space of the portal tract to portal lymphatic vessels (L). Arrows indicate the presumable flow direction as deduced from the present study. C: collagen fiber, iA: interlobular artery, iV: interlobular vein, iB: interlobular bile duct, F: fibroblast, I: Ito cell (or stellate cell), K: Kupfer cell, N: nerve, PbP: peribiliary plexus, a: afferent vessel of PbP, e: efferent vessel of PbP.

phatic endothelial cells.

Fluid pathways from the space of Disse to the interstitial space of the liver capsule

Our transmission electron microscopic studies have demonstrated that the space of Disse around the hepatic sinusoids close to the portal tract communicates through the spaces containing collagen fibers between hepatocytes with the interstitial space of the hepatic capsule. Our tracer study has shown that HRP was located along collagen fibers running from the space of Disse through the space between limiting plate hepatocytes to the interstitial space of the hepatic capsule. Our previous studies (Ohtani, 1989, 1992b)

successfully demonstrated that the collagen fibers in the space of Disse are connected with those in the hepatic capsule (also see Poonkhum *et al.*, to be published elsewhere). These seem to indicate that fluids in the hepatic sinusoids located close to the hepatic capsule can flow at least in part to the interstitial space of the hepatic capsule, and finally enter the capsular lymphatic vessels.

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