ORIGINAL

Direct lymphatic spreading route into the liver from the gallbladder : an animal experiment using pig

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Abstract : In the special occasion that the physiological lymphatic flow is obstructed, gallbladder carcinoma (GBC) may spread into the liver via lymphatic route. Therefore, this study was conducted to find out the direct lymphatic route draining into the liver from the gallbladder using pigs with ligated cystic ducts. After injecting the carbon particle suspension (CH40) or the contrast medium (Lipiodol) into the subserosal layer of the gallbladder, the lymphatic route into the liver was examined both macroscopically and histologically. In controls, CH40 or Lipiodol drained along the cystic duct toward the hepatoduodenal ligament. After occlusion of cystic duct, CH40 was interrupted at the ligated point, and then spread into the liver nearby the gallbladder bed, running off to the liver hilus, toward the hepatoduodenal ligament. This route was confirmed by the Lipiodol drainage into the right median lobe of the liver, equivalent to the segments V and IV a in humans. We presented for the first time the emergence of lymphatic drainage from the gallbladder into the liver after the occlusion of physiological lymphatic route using pigs. This implies that the direct spread into the segments V and IV a of liver should be considered in the surgical treatment of advanced GBC. J. Med. Invest. 51 : 210-217, August, 2004

Keywords **:** *gallbladder, lymphatic drainage, gallbladder carcinoma, heptic invasion, pig*

INTRODUCTION

The advanced gallbladder carcinoma (GBC) often happens to cause the metastasis to the liver, and this is a reason for a poor prognosis of GBC (1-3). Once carcinoma invades to the subserosal layer of the gallbladder, lymph node involvement and hepatic invasion or metastasis occur with very high rates (1-4). Therefore, it is very important to know the lymphatic drainage route from the gallbladder in the surgical treatments for GBC.

The anatomical analyses about the lymphatic systems of the gallbladder have been performed in the human cadavers or fetuses (5) and the experimental animals (6, 7), but these previous studies dealt with the route of lymphatic drainage under physiological conditions. On the lymphatic drainage routes between the gallbladder and the liver, it is a generally accepted idea that the lymphatic fluid flows physiologically in the direction from the liver parenchyma to the gallbladder, but never in the reverse direction (8, 9). Based on these anatomical data, surgical treatments were focused on the lymphatic drainage routes so far identified, and the resection was performed for the regions where the lymphatic fluid from the gallbladder flows through the cystic duct and the hepatoduodenal ligament toward the para-aortic lymph nodes (1-4, 10). But, there were several GBC cases, which had lymphatic metastasis into the liver by pathological findings (1).

The wall of the gallbladder has plentiful lymphatic vessels in subserosal layer and is rather thin on liver-facing side, without serous membrane (11). These anatomical characteristics are reminiscent of the lymphatic intercourse between the gallbladder and the liver (6, 8, 9). Lymph flow can be obstructed by cancer

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invasion. Therefore, in the special occasions that the physiological lymphatic flow is obstructed, it is possible that GBC may spread directly into the liver through the gallbladder.

The present study was undertaken to ascertain the presence or the emergence of the lymphatic drainage route from the gallbladder into the liver, with an animal model of the cystic duct occlusion using pigs. In this experiments, our hypothetical notion was examined by comparing the drainage routes between the control and the occluded animals after the injection of the carbon particle suspension (CH40) or the contrast medium (Lipiodol) into the subserosal layer of the gallbladder.

MATERIALS AND METHODS

Twenty male pigs weighing 18-24kg (AWAPORK, Japan) were used. The animals were kept on a 12/12 hr light/dark cycle and had food and water *ad libitum*.

We used the animals divided into two groups. One was the control group (n=8) without any surgical operation, and the other was the occluded group (n=12) with the ligated cystic duct before injecting the substances, CH40 or Lipiodol.

Seven days before injecting the substances, an animal model of cystic duct occlusion was made by ligating the cystic duct and the surrounding tissues of pig, as a case of GBC invading to the cystic duct.

Both the control and manipulated animals were anesthetized as described below, and 2 ml of the carbon particle suspension (CH40)(12), which was kindly provided by Dr. H.Yamagishi and Dr. A. Hagiwara (First Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan), was injected into the subserosal layer of the gallbladder through a 26-gauge needle under laparotomy. CH40 is the activated carbon particle that is taken up selectively into lymphatic system. After about 1-20 min, the lymphatic drainage route from the gallbladder into the liver was examined macroscopically and histologically, and compared with the non-occluded, control animals.

In the others of the control and manipiulated animals, the contrast medium (Lipiodol) was injected into the lymphatic ducts in the gallbladder subserosal layer through 24-gauge needle at a constant speed of 2 ml/h to confirm the direct lymphatic route into the liver. Lipiodol is the ethylester of iodinated poppy-seed oil fatty acid, which is used for clinical lymphangiography. The lymphatic drainage route from the gallbladder was checked by roentogenography up to 2 hr after the injection and also by histological examination.

In histological examination, the liver was fixed in a formalin fixative and paraffin sections were made. These sections were stained by hematoxylin and eosin in the animals of CH40 injection, and by Osmium or Sudan staining in those of Lipiodol injection.

Anesthesia was processed as follows. Anesthesia was induced with ketamine hydrochloride (10 mg/kg) and atropin sulfate (0.01～0.02 mg/kg) by intramuscular injection. The sedated pig was given bromo vecronium, a muscle relaxant, and thiamilal intravenously. After positioning of endotracheal tube, a ventilator was connected to the tube for delivering 35% oxygen. Anesthesia was maintained by intravenous infusion of bromo vecronium and sodium pentobarbital in a low dose.

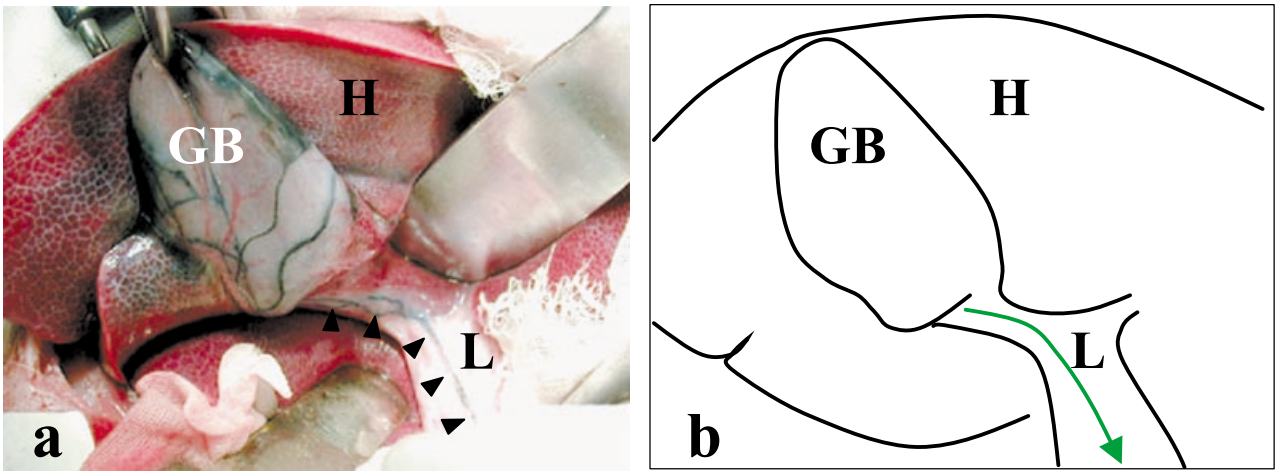

Figure 1. The injection of CH40 in the gallbladder subserosa in animals with non-occluded, intact cystic duct. a. The lymphatic drainage routes are shown as the black streams or stains from the gallbladder. In normals, lymphatic fluid drains down along the cystic duct and the hepatoduodenal ligamant (indicated by arrowheads). b. The lymphatic drainage route from the gallbladder is illustrated in the schematic diagram (green arrow). GB, gallbladder ; H, liver ; L, hepatoduodenal ligament.

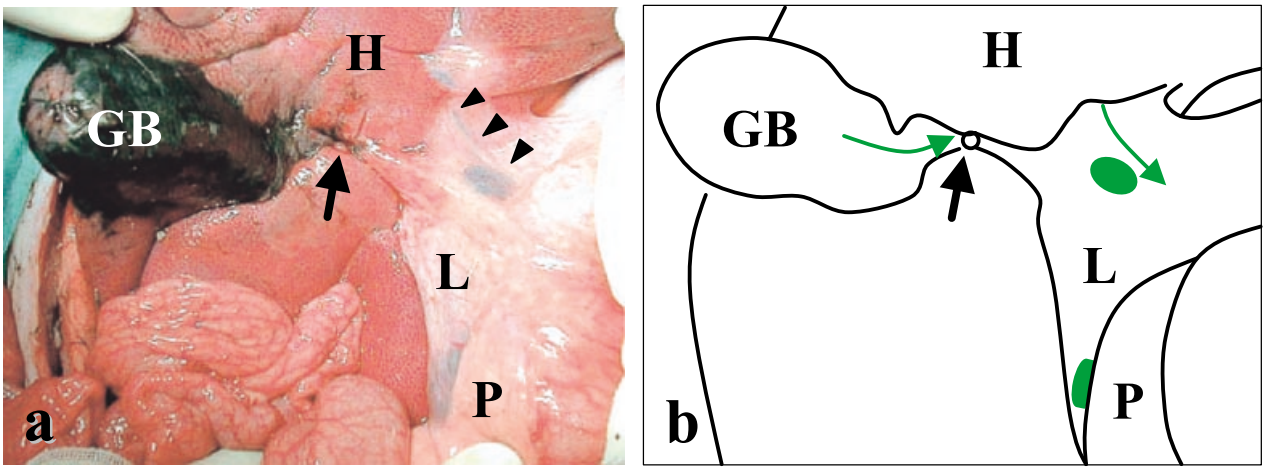

Figure 2. The injection of CH40 in the gallbladder subserosa in pigs with occluded cystic duct and the surrounding tissues. Arrows indicate the ligated portion of the cystic duct. a. The lymphatic drainage is interrupted at the ligated portion of the cystic duct. The black streams (arrowheads) appear from the liver hilus, draining into the hepatoduodenal ligament. No stream of CH40 is found between the ligated portion and the hepatoduodenal ligament. In the later period of 20 min, the stain of CH40 appeared in the retro-pancreatic lymph nodes. b. The lymphatic drainage route (green arrows) and lymph nodes (green ellipses) is illustrated in the schematic diagram. GB, gallbladder ; H, liver ; L, hepatoduodenal ligament ; P, pancreas.

All experimental procedures used in this study were approved by the Animal Care and Use Committee of The University of Tokushima School of Medicine.

RESULTS

Injection of the carbon particle suspension (CH40)

The spreading route of CH40 through the lymphatic vessels appeared as the black streams or stains on the surface of digestive organs and their surroundings. In non-occluded, controls, the injected CH40 drained from the bottom of the gallbladder to its neck, and then to the hepatoduodenal ligament along the cystic duct (Fig. 1 a, b). In animals with the occluded cystic duct, however, the drainage route of CH40 was changed. The flow of CH40 toward the cystic duct was interrupted at the ligated portion, and CH40 emerged from the liver hilus and its stream continued to the hepatoduodenal ligament, without any stains in the regions between the ligated points of the cystic duct and the hepatoduodenal ligament. In the later period of 20 min, the stain of CH40 appeared in the retro-pancreatic lymph nodes, but any stream of CH40 was not detected between the ligated portions of the cystic duct and the hepatoduodenal ligament, although the whole surface of the gallbladder was totally stained with CH40 (Fig. 2 a, b).

The histological examination of the liver showed that in non-occluded, controls, any trace of CH40 was not seen (Fig. 3 a, b), but in occluded animals, lymphatic vessels in interlobular connective tissues were dilated and filled with particles of CH40 (Fig. 3 c, d).

Injection of the contrast medium (Lipiodol)

In non-occluded, controls, the Lipiodol drained, through the cystic duct, into the hepatoduodenal ligament toward the para-aortic lymph nodes. A single stream of Lipiodol was traced from the gallbladder toward the para-aortic lymph nodes. No stream of Lipiodol was seen within the liver (Fig. 4 a, b).

After the ligation of the cystic duct, the lymphatic vessels on the surface of the gallbladder were so dilated that the puncture was easy to perform (Fig. 4 c). In these animals, the injected Lipiodol drained to the hepatoduodenal ligament, but stagnated at the ligated portion although some streams were traced toward the hepatoduodenal ligament. However, the ligation of the cystic duct induced the new appearance of lymphatic drainage route, leading into the liver parenchyma nearby the gallbladder bed, through the liver hilus, toward the hepatoduodenal ligament (Fig. 4 d). The right median lobe, where the new alternative lymphatic routes flew into the liver of pigs, corresponds to the right inferior anterior segment (segment V) and the lower part of the left medial segment (segment IVa) in humans.

It may take some time before these new alternative lymphatic routes are constructed because immediately after ligating the cystic duct, there was no lymphatic drainage of Lipiodol into the liver while the interruption of the lymphatic flow was seen at the ligated portion (Fig. 4 e).

Osmium or Sudan staining was performed in the liver sections to ascertain the presence of Lipiodol in the lymphatic vessels (Fig. 5 a, b). In the animals with ligated cystic ducts, the lymphatic vessels in the interlobular connective tissues were so dilated that black-

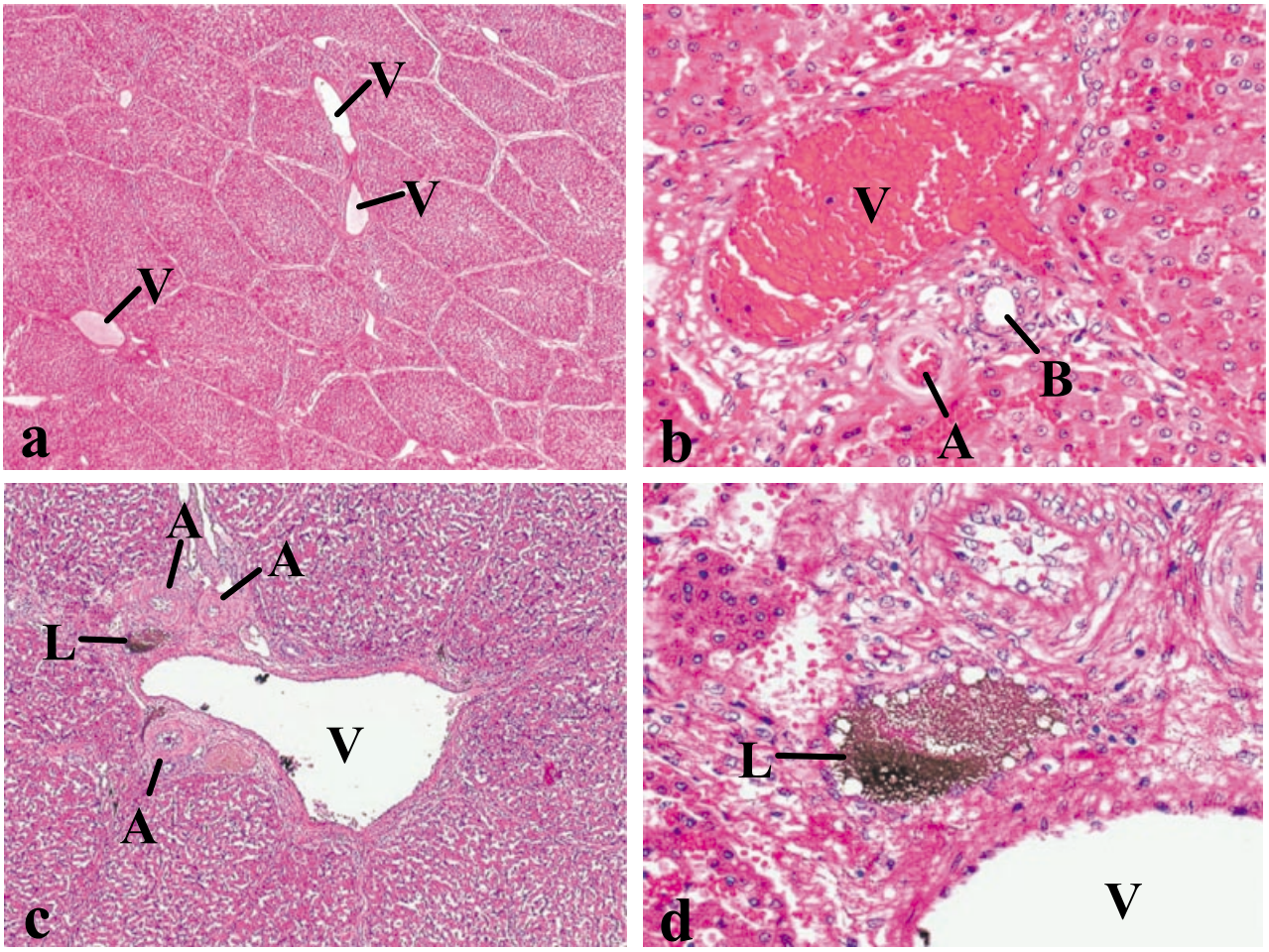

Figure 3. Histology of the liver after the injection of CH40 in animals with non-occluded (a, b) and occluded (c, d) cystic duct. Hematoxylin and eosin staining. a, b. No presence of CH40 is found in those with non-occluded cystic duct. In interlobular connective tissues, lymphatic vessels are not dilated and no particles of CH40 are seen (a). Only red blood cells are found in the interlobular arterial (A) and venous (V) vessel (b). c, d. The particles of CH40 are found in the interlobular connective tissues, and these dark particles are filled in the dilated lymph vessels (L) but not in interlobular arterial (A) and venous (V) blood vessels. A part of c is magnified in d. B, interlobular bile duct. a. ×10, b. ×150, c. ×30, d. ×150.

stained contrast medium were contained, while the apparent morphological change was not seen in the arterial and venous vessels and the biliary ducts and any stained materials were contained.

Schematic diagram of the lymphatic drainage route from the gallbladder

The lymphatic drainage route from the gallbladder is illustrated in the schematic diagram (Fig. 6 a, b). This lymphatic drainage pathway is composed of the two routes. One is the ordinary route, which drains lymphatic fluid from the gallbladder along the cystic duct and the hepatoduodenal ligament toward the para-aortic lymph nodes (Fig. 6 a). This route functions in normal conditions. The other is the new alternative route, which emerges when the ordinary route is stagnant (Fig. 6 b). This route runs from the gallbladder bed, draining directly into the liver parenchyma nearby the gallbladder bed, through the liver hilus, toward the hepatoduodenal ligament and the para-aortic lymph nodes.

DISCUSSION

The present study revealed the presence of the direct lymphatic drainage route emerging from the gallbladder into the liver in the case of the physiological lymphatic flow is obstructed, using living pigs. In physiological conditions, the lymphatic fluid from the gallbladder drains along the cystic duct into the hepatoduodenal ligament, and it rarely drains from the gallbladder bed directly into the liver (6-9). The occlusion of the lymphatic flow along the cystic duct, however, induced the appearance of the lymphatic drainage route that derived from the gallbladder bed : this route ran directly into the liver nearby the gallbladder bed, bypassing the cystic duct, through the liver hilus, toward the hepatoduodenal ligament, and it merged finally to the ordinary lymphatic drainage route toward the para-aortic lymph nodes.

Two distinct substances were utilized to trace the lymphatic drainage route from the gallbladder in both

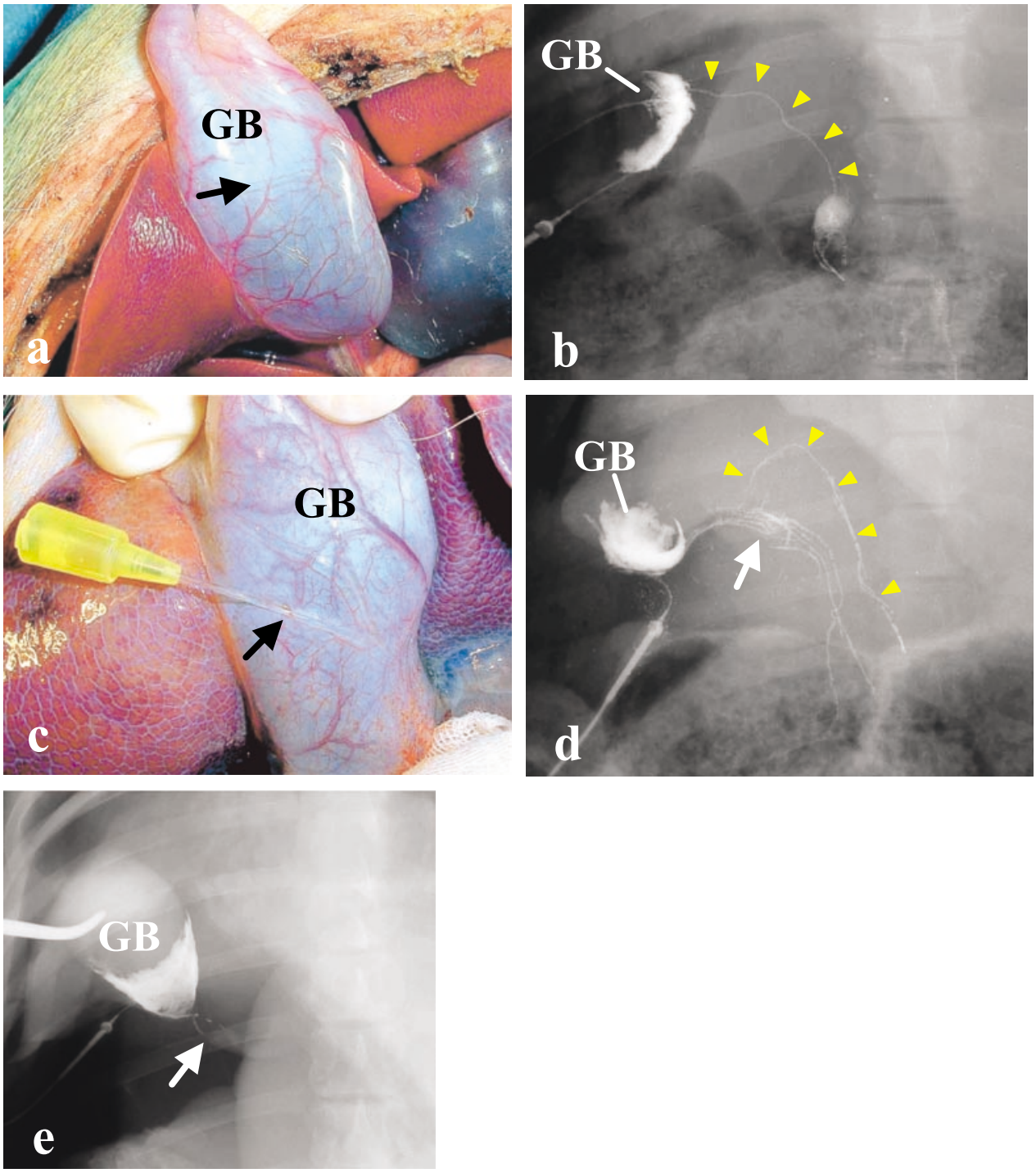

Figure 4. a. The injection of Lipiodol in the lymphatic duct (black arrow) of the gallbladder in animals with non-occluded cystic duct. b. Roentogenography after the injection of Lipiodol shows that Lipiodol drained from the gallbladder along the cystic duct and the hepatoduodenal ligament toward the retro-pancreatic lymph nodes. The lymphatic drainge route is indicated by arrowheads. c. The injection of Lipiodol in the lymphatic duct (black arrow) of the gallbladder in animals with occluded cystic duct. The lymphatic ducts are so dilated as to be punctured. d. Seven days after the ligation of the cystic duct, the alternative drainage route (arrowheads) of Lipiodol emeged from the gallbladder into the liver, and then toward the hepatoduodenal ligament. The conventional lymphatic drainage route stagnated at the ligated portion (white arrow), although some of the injected Lipodol escaped down from ligated portion. e. Immediately after ligating the cystic duct, the injected Lipiodol stagnated at the ligated portion (white arrow), but did not drain into the liver. GB, gallbladder ; HR, hilar region of the liver ; CD, cystic duct ; L, hepatoduodenal ligament.

controls and manipulated animals with ligated cystic duct and the surrounding tissues. Using CH40, the draining routes were traced by black streams or stains on the surface of the digestive organs. Therefore the tracing by CH40 was easy to detect the route on the surface or nearby regions, but the detection was difficult in the deep region of the liver. Hence, using CH40, it did not seem enough to see the whole lymphatic drain-

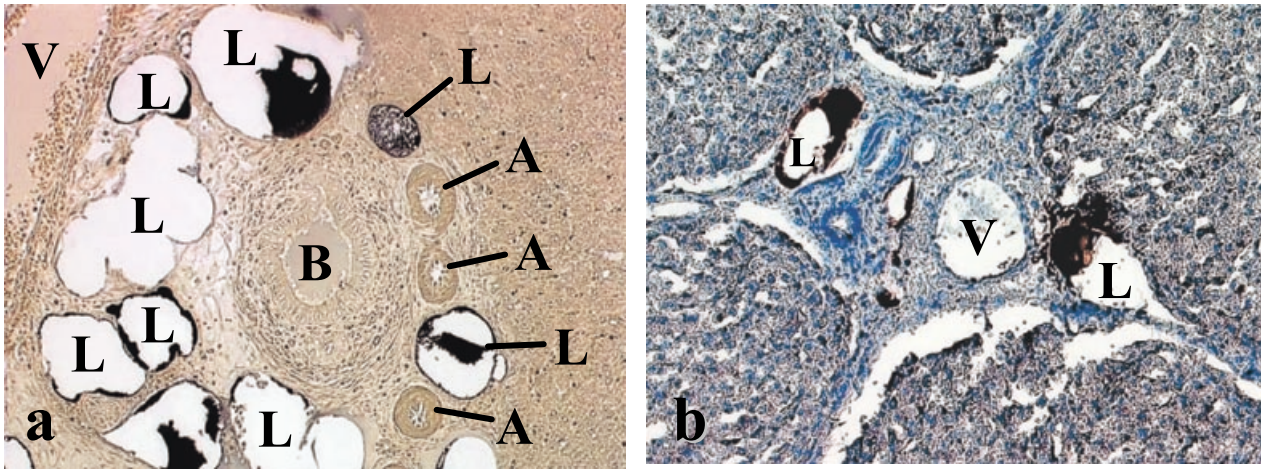

Figure 5. Histology of the liver after the injection of Lipiodol in animals with ligated cystic duct. a. Osmium staining. Lipiodol is stained black and filled in the dilated lymphatic vessels (L), but not in the arterial (A) and venous (V) blood vessels and the bile duct (B) in the interlobular connective tissues. ×40. b. Sudan staining. Lipiodol is also stained red and filled in the lymphatic vessels (L), but not in the venous (V) blood vessels in the interlobular connective tissues. ×40.

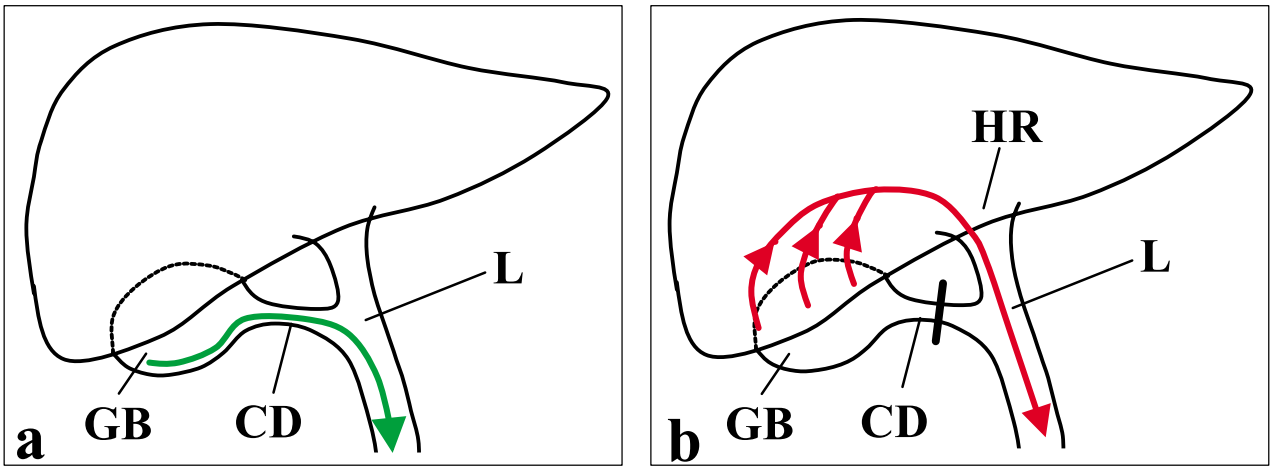

Figure 6. Schematic diagram showing the lymphatic drainage routes from the gallbladder. a. Under physiological conditions, the conventional lymphatic drainage route runs from the gallbladder along the cystic duct and the hepatoduodenal ligament toward the retro-pancreatic lymph nodes, and no drainge route is found into the liver. b. After the obstruction (bold line) of lymphatic route along cystic duct, the alternative bypassing lymphatic drainage route emerges from the gallbladder bed into the liver, through the liver hilus, and then toward the hepatoduodenal ligament. GB, gallbladder;HR, hilar region of the liver;CD, cystic duct;L, hepatoduodenal ligament.

age route bypassing from the gallbladder bed through the hilar region of the liver toward the hepatoduodenal ligament. To overcome these shortcomings of CH40, another substance, Lipiodol, was used to make sure the bypassing lymphatic route that emerges into the liver. The use of Lipiodol has some advantages as follows : 1. It is possible to get the real-time tracing of the lymphatic drainage route from the gallbladder in the living animals. 2. Several bypassing lymphatic routes, if any, can be traced simultaneously. 3. Even if the lymphatic drainage route may be deep in the liver, it is so easy to trace the route running within the liver mesenchyme by roentogenography. By Lipiodol, we made it clear that after the occlusion of the cystic duct, the alternative lymphatic drainage route merges from the gallbladder and drains into the liver parenchyma through the right median lobe of the liver toward the hepatoduodenal ligament and finally merges to the ordinary lymphatic drainage route toward the para-aortic lymph nodes. Use of Lipiodol indicated that there remained some lymphatic drainage route distal to the ligated portion of the cystic duct even if the tight ligation was done, and that from the gallbladder, there appeared several lymphatic drainage routes, which gathered together to a single stream.

Using both the injection of CH40 and Lipiodol, we confirmed the evidence that in controls with non-occluded cystic duct, the lymphatic drainage route from the gallbladder runs along the cystic duct and the hepatoduodenal ligament toward the para-aortic lymph nodes, and it never drains directly into the liver. This drainage route of the lymphatic fluid from the gallbladder was reported in earlier reports using human cadavers (5) and the patients with GBC or the cholangiocaricoma

(3, 4, 13). For the surgical curative treatment of GBC, the area of surgical resection has therefore been focused on the lymphatic drainage route along the cystic duct and the hepatoduodenal ligament down to the para-arotic lymph nodes (1, 2). However, the present results presented the new finding that the alternative lymphatic drainage route emerges directly into liver from the gallbladder, after the ordinary lymphatic route was obstructed, and that this bypassing lymphatic pathway appears to meet the ordinary lymphatic drainage route. The area in the liver where the alternative lymphatic drainage route emerged, was confirmed in the present study. Hence, in advanced GBC, which invades to the subserosal layer, this area in the liver should be taken into account for the resection, in addition to the lymph nodes around the hepatoduodenal ligament and the para-aortic region (1, 2, 14). It may be possible that GBC in the depth of invasion, which is recognized subserosal layer or deeper, has already spread into the liver through the alternative lymphatic drainage route found in the present study even if any signs of direct invasion into the liver are not seen (15).

The lymphatic drainage route downstream to the gallbladder has been studied (5, 13). The lymphatic drainage route in the biliary system comprises mainly the two pathways : the right and the left route. The right route runs down along the cystic duct and the common bile duct, and to the lymph nodes nearby pancreas head, and leads to the lymph nodes around the superior mesenteric artery and the abdominal aorta. The left route runs through the cystic duct and then along the proper and common hepatic artery, and leads to the lymph nodes around the celiac trunk. On the communication of the lymphatic fluid between the gallbladder and the liver, several studies presented the existence of lymphatic communication but demonstrated only the direction of the lymphatic drainage from the liver to the gallbladder (6-9). No existence of the lymphatic fluid draining from the gallbladder directly into the liver was pointed out in physiological conditions. The alternative lymphatic drainage route, which emerged in the liver nearby the gallbladder bed after the physiological lymphatic route was obstructed, met mainly with the right route. The gallbladder bed was situated in the right median lobe of the pig liver, which is divided into five lobes : left lateral, left median, right median, right lateral and caudate (16), and this area appeared to correspond to the segments V and IV a in humans. The histology of hepatic lobule in pigs is principally the same as in humans, but in humans the whole surface of hepatic lobule is covered with the interlobular connective tissues less tightly than in pigs. Though the species difference in the structure of the liver may be considered between pigs and humans, it is therefore imaginable that the lymphatic spreading of GBC goes fast, once the carcinoma invades into the lymphatic vessels in the liver.

The metastasis of GBC within the liver is found at high rates in surgery (about 50-90%) and in the examination of autopsies (about 90-100%)(2). The metastasis of GBC into the liver occurs by the following three routes : the direct invasion, the vascular spreading and the lymphatic drainage : the first two have been thought to be the major metastatic routes. This conviction was based mainly on the anatomical report (17) that the venous flow from the human gallbladder runs into the portal vessels in the area of segment V and IV a in the liver, via two pathways : one is through the Calot's triangle and the other is through the gallbladder bed. The last lymphatic drainage, which has ever been overlooked, is proved here to be the alternative metastatic route from the gallbladder into the liver.

In conclusion, we demonstrated the new evidence that the direct lymphatic drainage route from the gallbladder bed into the liver emerged after the ligation of the cystic duct and the surrounding tissues in pigs. This new bypassing lymphatic route ran into the liver nearby the gallbladder bed, which corresponds to the area of segments V and IV a in humans, toward the hepatoduodenal ligament and the para-aortic lymph nodes. The present findings provide the new data on the resection area in the surgical curative treatment of GBC. It may therefore be necessary that the direct lymphatic spread into the segments V and IV a of liver should be taken into consideration for the surgical treatment of advanced GBC, which invades the cystic duct and surrounding tissues.

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