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CLINICAL RESEARCH

Surgical anatomy of innervation of the gallbladder in humans and *Suncus murinus* with special reference to morphological understanding of gallstone formation after gastrectomy

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Abstract

AIM: To clarify the innervation of human gallbladder, with special reference to morphological understanding of gallstone formation after gastrectomy.

METHODS: The liver, gallbladder and surrounding structures were immersed in a 10 mg/L solution of alizarin red S in ethanol to stain the peripheral nerves in cadavers (n = 10). Innervation in the areas was completely dissected under a binocular microscope. Similarly, innervation in the same areas of 10 *Suncus murinus* (*S. murinus*) was examined employing whole mount immunohistochemistry.

RESULTS: Innervation of the gallbladder occurred predominantly through two routes. One was from the anterior hepatic plexus, the innervation occurred along the cystic arteries and duct. Invariably this route passed through the hepatoduodenal ligament. The other route was from the posterior hepatic plexus, the innervation occurred along the cystic duct ventrally. This route also passed through the hepatoduodenal ligament dorsally. Similar results were obtained in *S. murinus*.

CONCLUSION: The route from the anterior hepatic plexus via the cystic artery and/or duct is crucial for preserving gallbladder innervation. Lymph node dissection specifically in the hepatoduodenal ligament may affect the incidence of gallstones after gastrectomy. Furthermore, the route from the posterior hepatic plexus via the common bile duct and the cystic duct to the gallbladder should not be disregarded. Preservation of the plexus may attenuate the incidence of gallstone formation after gastrectomy.

Key words: Innervation; Gallstone; Hepatoduodenal ligament; Gastrectomy; Whole mount immunohistochemistry

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INTRODUCTION

Over the last five decades, many reports have shown an at least 10% higher incidence of gallstones in people who have undergone gastrectomy than in the general population[1-9]. With respect to lymph node dissection, the incidence of gallstone formation was 16.3% after D3 and 8.5% after D2-1[8]. More recently, Kobayashi *et al*[10] reported that the incidence of gallstone formation is 27.9% after total gastrectomy and 7.8% after partial gastrectomy, 28.2% *versus* 7.5% for dissection or nondissection in the hepatoduodenal ligament, and 25.1% *versus* 8.2% at 5 years for reconstruction after gastrectomy with duodenal exclusion or non-exclusion, indicating that lymph node dissection in the hepatoduodenal ligament, total gastrectomy and exclusion of the duodenum are risk factors for gallstones after gastrectomy. However, the morphological understanding of the risk factors evaluated remains poor.

Examination of silver-impregnated and methylene blue-stained material has shown that the extrahepatic portion of human biliary tree has a rich nerve supply[11].

Innervation of the gallbladder (GB) and biliary pathways have been studied in men and other species by means of immunohistochemistry and/or histochemical methods (mainly the indirect immunofluorescent technique) using whole mount preparations or sections of the GB. Most neurotransmitters such as vasoactive intestinal peptide (VIP), cholecystokinin (CCK), acetylcholine, serotonin, dopamine, nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), galanin, tyrosine hydoxylase (TH), neuropeptide Y (NPY), peptide YY (PYY), pancreatic polypeptide (PP), somatostatin, substance P (SP), and gastric inhibitory peptide (GIP), have been comprehensively discussed[12-27]. However, there has been no detailed description concerning clinico-anatomical and morphologic studies on the innervation, especially the extrinsic neural distribution and spread, of the GB and the biliary pathways.

Therefore, in the present study, the currently used gross anatomical dissection was not employed, but the effective method to label and dissect autonomic nerves of the viscera was employed, as in our previous studies[28-31]. We attempted to clarify the innervation of the GB in humans from a clinico-anatomical point of view, and to obtain a morphological understanding of gallstone formation after gastrectomy. Furthermore, an experimental animal, *Suncus murinus* (*S. murinus*), has been shown to exhibit general morphological characteristics more similar to those of humans than other currently used laboratory animals, such as mouse, rat, rabbit[28,30,31]. Hence, this animal was employed for a comparative study to confirm our morphological observations in humans in the present study.

MARERIALS AND METHODS

Cadavers

The study was performed on 10 cadavers (5 men and 5 women) with a mean age of 79.8 (range 50 to 94) years. All cadavers free from diseases of the abdominal viscera were selected from among bodies used for anatomy research and practice at the Kanazawa University School of Medicine in 1999-2000 (Table 1).

Table 1 Cadavers used in this study and GB innervation

Case	Sex	Age	Death	AHPlx[1]	PHPlx[2]	Phrenicus[3]
A	F	94	Pneumonia	O	O	R&L
B	F	81	Cerebral hemorrhage	O	O	R
C	F	83	Myocardial infarction	O	O	R
D	M	87	Myocardial infarction	O	O	-
E	M	86	Pneumonia	O	O	-
F	M	73	Cerebral hemorrhage	O	O	R
G	M	87	Cerebral hemorrhage	O	O	R
H	M	75	Myocardial infarction	O	O	R
I	F	82	Subarachnoid hemorrhage	O	O	R
J	F	50	Pneumonia	O	O	R

[1]Route arising from the anterior hepatic plexus (AHPlx); [2]Route arising from the posterior hepatic plexus (PHPlx); [3]There are offshoots of the right (R) or left (L) phrenic nerve to the hepatic portal vein.

Animals and tissue preparation

Adult laboratory house musk shrews, *S. murinus*, were obtained from a closed breeding colony bred and maintained in the Department of Anatomy and Neuroembryology, Kanazawa University, Japan. The animals were housed and handled in accordance with the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Briefly, all shrews were kept individually after weaning (20 d after birth) in plastic cages equipped with a wooden nestbox containing paper strips, in a conventionally conditioned animal room (23℃-27℃, no humidity control, and 14 L: 10 D light). Commercial trout pellets containing 45.0% protein, 3.5% fat, 3.0% fiber, 13.0% ash and 26.2% complex carbohydrate (Nippon Haigou Shiryou, Tokyo, Japan) and water were supplied *ad libitum*. The mother colony, JIc: CR, was maintained in the Central Institute for Experimental Animals, Kawasaki, Japan[28,30,31]. Adult animals (6 females and 4 males, weighing 45-80 g) were first anesthetized with ether and then given an intraperitoneal injection of a solution of urethrane (sodium ethyl carbamate, 900 mg/kg). After each *S. murinus* was completely narcotized, the abdominal cavity was opened, and a catheter was inserted retrogradely into the abdominal aorta at the level immediately above the bifurcation of this artery into the common iliac arteries. Perfusion was commenced with normal saline containing heparin (10 KU/L), and thereafter with 4% paraformaldehyde buffered with 0.01 M sodium phosphate (PFA, pH 7.4). After perfusion, the animals were injected with neoprene latex to label the blood vessels. Thereafter, the abdominal organs including the liver, GB, stomach, duodenum, common bile duct, and pancreas were extracted *en bloc* with the related nerves and vessels, and then postfixed with 4% PFA at 4℃ overnight for whole mount immunostaining.

Anatomical procedures under stereoscopic microscopy

Anatomical procedures for the cadavers were in accordance with our previous descriptions[29-31]. The viscera of the upper abdomen (including the liver, GB, pancreas, lower esophagus, stomach, and duodenum) were resected en masse with the abdominal aorta (the region including the celiac artery and superior mesenteric artery), portal system, and nerves (including the vagus nerve, celiac ganglion, and plexus). The resected specimens were immersed in a 10 mg/L solution of alizarin red S (Wako, Osaka, Japan) in ethanol to melt the fat tissue and to stain the peripheral nerves. The solution was changed three times, every 2 to 3 d in principle, but this process was longer depending on the degrees of fat elimination and staining. The area of each sample surrounded by the horizontal plane that passes through the portal region and the lower margin of the horizontal part of the duodenum, and the sagittal plane that passes through the descending part of the duodenum and hilum of the spleen was dissected to the depth of the celiac plexus with the aid of a stereoscopic microscope, keeping the sample completely immersed in 100% ethanol. On dissection, lymphatic vessels and lymph nodes were removed, with particular attention paid to the preservation

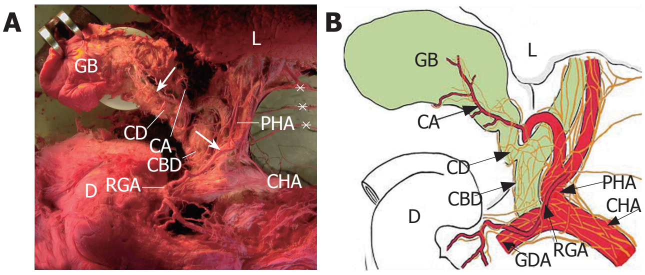

Figure 1 Innervation of the gallbladder (GB) from the ventral aspect (**A**) in a cadaver and a schematic representation of it (**B**). The branches innervating the GB originate from the anterior hepatic plexus, and run along the cystic duct (CD) and the cystic artery (CA). The hepatic divisions (*) of the vagus join in the anterior hepatic plexus in the proper hepatic artery (PHA). Arrows indicate nerve branches. CBD: common bile duct; CHA: common hepatic artery; D: duodenum; GDA: gastroduodenal artery; L: liver; RGA: right gastric artery.

not only of the nerves but also of the arteries and veins in the areas of the hepatic portal and hepatoduodenal ligament.

Whole mount immunohistochemistry

Whole mount immunostaining procedures for the *S. murinus* were performed as previously described[28,30,31]. Briefly, after rinsing in PBS, the fixed specimens were treated with 10 g/L periodic acid for 20 min to prevent any intrinsic peroxidase reaction. They were then incubated in freshly prepared 5 g/L Papain (Sigma) in 0.025 mol/L Tris-HCl buffer (pH 7.6) for 1 h, and then 25 g/L, 50 g/L, and 100 g/L sucrose in PBS for 30 min, respectively, followed by freezing and thawing three times. The specimens were incubated with the primary antibody (NFP-Ab) in PBS containing 2 g/L bovine serum albumin (BSA), 3 g/L Triton X-100, and 1 g/L sodium azide for 3 d at 4℃. After a thorough wash in PBS, the specimens were incubated with the secondary antibody labeled with peroxidase-conjugated affinity-purified sheep anti-mouse IgG (HRP) in PBS containing 2 g/L BSA and 3 g/L Triton X-100 for 3 d at 4℃. After a thorough wash in PBS, coloration was performed in 0.05 mol/L Tris-HCl buffer containing 20 mg/L 3, 3'-diaminobenzidine (DAB) and 0.1 mL/L H_2O_2 for 1-3 d at 4℃. The stained preparations were then stored in glycerin to obtain transparency. The primary antibody was the anti-neurofilament protein (NFP) antibody, a monoclonal mouse anti-all neurofilament consisting of three subunit proteins: NF-H (200 kDa), NF-M (160 kDa), and NF-L (70 kDa) (M0762, lot 089, clone: 2F11, Dako).

RESULTS

In humans

The innervation of the GB in humans involved three routes: *via* the anterior and posterior hepatic plexus, respectively, and the phrenic nerves.

Via the anterior hepatic plexus

The hepatic division of the vagus, arising from the anterior vagal trunk, ran through the hepatogastric ligament near the edge of the liver (caudal liver), and joined the anterior hepatic plexus in the hepatoduodenal ligament. The plexus, containing parasympathetic and sympathetic fibers, arose from the celiac plexus and wound around the common hepatic artery, i.e., the proper hepatic artery[30], and then sent some branches to the GB via the deep and superficial branches of the cystic artery, which were distributed in the peritoneal aspect of the GB and the site of attachment of this organ in the bed between the GB and the liver (Figure 1). This route was seen in all ten specimens.

On the other hand, the anterior hepatic plexus sent some branches directly to the GB along the cystic duct, i.e., forward to the neck, body, fundus of the GB. However, the route concentrated mainly in the cystic duct and the neck of the GB compared with the body and fundus of the GB (Figure 1). However, it was not observed that the branches arising from the hepatic division of the vagus were sent directly to the GB.

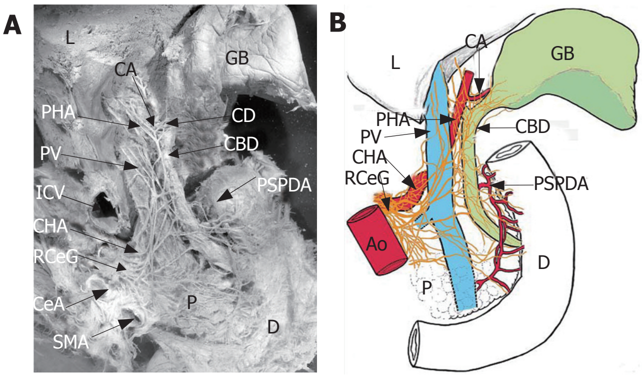

Figure 2 Innervation of the gallbladder (GB) from the dorsal aspect (**A**) in a cadaver and a schematic representation of it (**B**). The branches innervating the GB arise from the posterior hepatic plexus, and run along the cystic duct (CD). Ao: aorta; CA: cystic artery; CBD: common bile duct; CeA: celiac artery; CHA: common hepatic artery; D: duodenum; IVC: inferior vena cave; L: liver; PHA: proper hepatic artery; PSPDA: posterior superior pancreatoduodenal artery; PV: portal vein; RCeG: right celiac ganglion; SMA: superior mesenteric artery.

Via the posterior hepatic plexus

The posterior hepatic plexus or the dorsal hepatic plexus[30], arising from the celiac plexus on its right side and running along the dorsal side to the portal vein, was composed of 4-5 nerve fascicles, divided into two groups of nerve bundles, and the thickest branches, about 80% of the nerve fibers, extended along the upper part of the common bile duct and portal vein, joined the liver and the GB, or descended, sending branches to the proximal side of the descending part of the duodenum and the lower common bile duct[30] (Figure 2). Abundant communicating rami behind the common bile duct and portal vein between the ascending and descending plexuses were observed, showing the existence of direct bidirectional neural connections between the duodenal papilla and the biliary tract containing the GB (Figure 2).

As in the case of the anterior hepatic plexus, the branches sent to the GB were distributed mainly in the cystic duct and the neck of the GB (Figure 2). This route was also seen in all ten specimens.

Moreover, abundant communicating rami were seen between the anterior and posterior hepatic plexuses around the cystic duct.

Via the phrenic nerves

In addition, the phrenic nerves sent offshoots forward to the hepatic portal, which ran along the sagittal sulcus of the liver (data not shown). The offshoots were observed in eight out of ten cases, originating predominantly from the right phrenic nerve in seven and from both the right and left phrenic nerves in one (Table 1).

In S. murinus

In *S. murinus*, the GB was supplied by the cystic artery, a branch of the hepatic artery, corresponded to that in humans. However, there were no anterior and posterior hepatic plexuses in *S. murinus*.

Abundant plexus was found in the common bile duct of *S. murinus*, which arose from the celiac plexus via the common hepatic artery. The plexus ran along the common bile duct, descending to the lower common bile duct and the duodenal papilla, and ascending to the liver and common bile duct (Figure 3). Moreover, innervation of the GB occurred mainly in the cystic duct and the neck of the GB. Namely, it showed the existence of direct bidirectional neural connections between the duodenal papilla and the biliary tract including the GB as in humans (Figure 3).

Similarly, innervation of the GB along the cystic artery was also observed. However, it was much poorer than that in the route starting from the common bile duct, cystic duct (Figure 3).

In addition, communicating rami arising from diaphragm, which were observed running along the ligament between the xiphoid process of the sternum and the GB with an approach to the fundus of the GB, innervated the GB (data not shown).

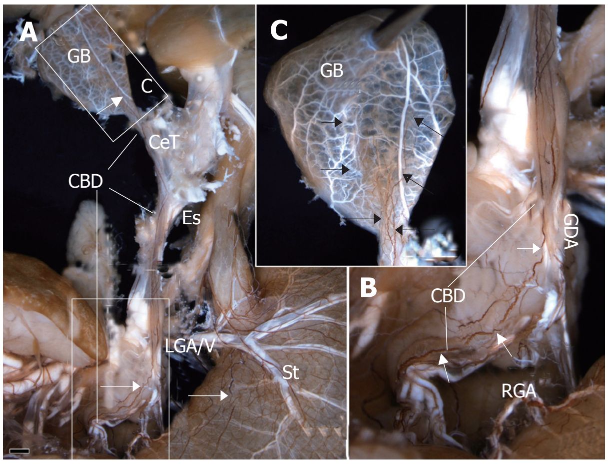

Figure 3 Innervation of the gallbladder (GB) in S. murinus revealed on whole-mount immunostaining (**A**) and high magnification of the boxed areas (**B** and **C**). Arrows indicate the stained/labeled nerve branches or bundles. CBD: common bile duct; CeT: celiac trunk; Es: esophagus; GDA: gastroduodenal artery; LGA/V: left gastric artery/vein; RGA: right gastric artery; St: stomach. Scale bar = 2 mm in **A**.

DISCUSSION

The present paper concerns detailed observation of the innervation of the GB and biliary pathways, with the aim of providing an anatomical basis for understanding the surgical clinical phenomenon of gallstone formation after gastrectomy.

Functionally, innervation of the GB in humans involves both sympathetic postganglionic nerve fibers and parasympathetic fibers. Morphologically, innervation of the GB occurred predominantly through two routes. One was from the anterior hepatic plexus containing the branches arising from the hepatic division of vagal nerves and the celiac plexus, the innervation occurred along the cystic arteries and the cystic duct. Invariably this route passed through the hepatoduodenal ligament. The other route was from the posterior hepatic plexus, containing the branches originating from the celiac branches of the posterior vagal trunk and the celiac plexus, the innervation occurred along the cystic duct ventrally. This route also passed through the hepatoduodenal ligament dorsally. Furthermore, nerve offshoots arising from the phrenic nerves were observed, and communicating twigs between the GB and the duodenal papilla were identified. Although there were no anterior and posterior hepatic plexuses, similar results were obtained for a laboratory animal, *S. murinus*.

The incidence of gallstone formation is higher in patients after gastrectomy than in the general population. Kobayashi *et al*[10] identified lymph node dissection specifically in the hepatoduodenal ligament as a significant risk factor for gallstone formation, suggesting that dissection of nerve fibres near the GB might be important. This viewpoint is consistent with our morphological observations, i.e., innervation of the GB, either *via* the anterior or posterior hepatic plexus, occurred through the hepatoduodenal ligament. However, innervation of the GB arising from the anterior hepatic plexus surrounded the common hepatic artery, i.e., the proper hepatic artery to the cystic artery. If skeletonization of the hepatoduodenal ligament, especially the common and proper hepatic arteries, can be avoided in dissection, preservation of innervation of the GB originating from the hepatoduodenal ligament is possible. Virtually, the entire idea of radical resections for gastric cancer remains somewhat controversial, with different results obtained in Asia *vs* the US. It is not necessary to perform skeletonization of the hepatoduodenal ligament for all cancer resections of stomach, although it is general maneuver to excise lymph nodes in the hepatoduodenal ligament. Both potential risk of metastasis and quality of life after operation should be taken into consideration. It is important to have a suitable excision of lymph nodes and to avoid skeletonization of the hepatoduodenal ligament for quality of life and the formation of cholelithiasis after gastrectomy.

Although the degree of lymph node dissection in D2 affects the incidence of gallstone formation as it has been shown that there is an at least% higher incidence of gallstone formation in people who have undergone gastrectomy than in the general population[10], we cannot agree with the idea that prophylactic cholecystectomy should be performed during radical gastrectomy, even if the GB is normal, in order to prevent the complications of acute cholecystitis and cholelithiasis[7]. Kobayashi *et al*[10] reported that most patients with gallstone formation after gastrectomy are asymptomatic and less than 0.5% of them require cholecystectomy, suggesting that preventive cholecystectomy appears to be unnecessary.

However, besides complete abolition of the innervation of the GB, gallstone development after gastrectomy also depends on the extent of gastrectomy (total or partial gastrectomy), on reconstruction after gastrectomy and exclusion or non-exclusion of the duodenum[10].

Furthermore, studies on other species have shown the existence of direct bidirectional neural connections between the GB and duodenum containing the sphincter of Oddi, indicating that local reflux between the GB and the sphincter of Oddi might be important in regulation of the pressure within the bile ducts and the flow across the sphincter[14,16,24,27,32,33], demonstrating the presence of this intrinsic neural pathway from the duodenum to the GB in these species. Our data also showed the presence of extrinsic neural connections between the duodenum and the GB in humans and *S. murinus*, and supported that local reflux between the GB and the duodenum, the sphincter of Oddi, might be important in the regulation of the flow in the GB and duodenum[31]. The integrity of these extrinsic neural connections may play a role both in maintaining the GB motor activity and in preventing gallstone formation after distal gastrectomy[34].

Whether the cystic duct plays any regulatory role in the process of GB filling and emptying remains unknown. The cystic duct clearly possesses contractile muscle and is the narrowest part of the extrahepatic biliary tree. Poiseuille's law dictates that only minor changes in the caliber of the duct could greatly influence the resistance to the flow of bile[35]. In dogs, the innervation density parallels the thickness of the musculature, which is the greatest toward the neck of GB[36]. Our data supported this viewpoint. In our study, abundant neuron fibers were distributed in the neck of GB and the cystic duct both in humans and *S. murinus*. This may be important for the emptying of GB and the cystic duct.

In addition, horseradish peroxidase is utilized to study the distribution of afferent fibers from the GB in cats. Afferent cell bodies have been found in the nodose ganglion and the T4 to L1 dorsal root ganglia[37,38]. Iwahashi *et al*[39] also reported that the GB of cats was innervated by bilateral phrenic nerves, the nodose ganglia and dorsal root ganglia T2-L3. The phrenic nerves were concerned in about 33% of the subjects, the right side predominating over the left. In our study, the human subjects with some twigs to the GB from the phrenic nerves accounted for 80% (eight of 10 cases). In one case both the right and left phrenic nerves were the origin of the twigs, the right phrenic nerve was predominant in the other seven cases. Iwahashi *et al*[39] and Inomata[38] showed that some afferent fibers from the GB traveling via the phrenic nerves, particularly on the right side, and entering the cervical segments may be a supplementary mechanism as to the generation of the referred pain in GB disease.

In conclusion, we revealed the details of innervation of the GB from morphologic and clinico-anatomical perspectives in this study. The whole mount immunostaining with a peripheral neuron marker for *S. murinus*, and the alizarin red S staining technique for humans are effective for peripheral nerve labeling. There are three routes for GB innervation in humans, of which the route from the anterior hepatic plexus via the cystic artery and/or duct is crucial for preserving GB innervation. Lymph node dissection specifically in the hepatoduodenal ligament may affect the incidence of gallstone formation after gastrectomy. However, the route from the posterior hepatic plexus via the common bile duct and the cystic duct to the GB should not be disregarded. Preservation of the plexus may attenuate the incidence of gallstone formation after gastrectomy. However, in the present study, we did not examine the intrinsic neurons system in the biliary duct (containing GB) or the upper gastrointestinal tract. Both extrinsic nerves and intrinsic neurons containing abundant neurotransmitters, participate in the regulation of GB motion.

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