Microanatomical profiles on the lymphatic system in the human ampulla of Vater (immunohistochemistry and scanning electron microscopy)

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Abstract

Background Little information is available regarding microanatomy of lymphatic system in the ampulla of Vater, though it is of critical importance for an understanding of tumor progression via the lymphatics and determination of surgical strategy. The present study, therefore, aimed to demonstrate the distribution and microanatomical profiles on the lymphatic system in the ampulla.

Methods The fine distribution and structure of the lymphatic vessels were investigated in the ampulla and the stomach by immunohistochemistry for lymphatic- (D2-40) and blood vascular- (CD31) specific markers and scanning electron microscopy. The densities of lymphatic and blood vessels were also compared.

Results The duodenal papilla densely developed the lymphatics with distinct aspects of lymphatic capillaries, together with blood vessels. The density of lymphatic capillaries in the extramuscular layer in the ampulla was higher than those of both the other ampullary layers and the gastric extramuscular (subserosal) layer.

Conclusions The ampulla of Vater showed widespread lymphatic capillaries throughout the entire wall. The specific vascular system is suited to produce lymph everywhere and drain without via such a large vessel as lymphatic collector. This suggests that tumor cells invade the lymphatics and metastasize more easily in the ampulla than in the other gastrointestinal regions.

Keywords Ampulla of Vater · Immunohistochemistry · Lymphatic system · Lymphatic vessels · Scanning electron microscopy

Introduction

Carcinoma in the ampulla of Vater (CAV) is a rare disease throughout gastrointestinal tract disorders [1], but its malignant potential is thought to be worse than the other gastrointestinal cancers. Surgically localized resection of CAV is sometimes performed if the tumor is diagnosed as an early stage cancer [2], whereas most cases have undergone pancreaticoduodenectomy because of its high metastatic potential to lymph nodes. Since pancreaticoduodenectomy is highly invasive enough to invite various severe complications, it goes without mentioning that application of lower invasive surgery is better for keeping patients’ quality of life [3]. A new strategy governing lymph dynamic metastasis of CAV is desired to resolve the above problem. The elucidation of precise organization of its lymphatic vasculature is referred to be essential to develop such a surgical strategy leading to lower invasiveness as sentinel lymph node theory used in cases of breast cancer [4] and gastric cancer [5] for prediction of lymph node metastasis. However, even the microanatomical structure of the lymphatic system remains to be explained in the case of the ampulla of Vater. Therefore, the present study aimed to demonstrate precise organization of the lymphatic vasculature in the human ampulla of Vater.
**Materials and methods**

**Human tissue preparation**

The specimens of common bile duct, pancreatic head and duodenum containing the ampulla of Vater were collected from three cases of adult male cadavers with neither gastrointestinal diseases nor abdominal malformations under approval from the Human Research Ethics Committee of the Hirosaki University Graduate School of Medicine (Reference number 2015-227). The tissue pieces of the gastric corpus were also taken from the cadavers to compare the vascular densities between the duodenal papillary region and in the gastric wall. The tissues were immersed in 10% buffered formalin for at least 5 days, transitionally cut from the bile duct to the duodenal papilla at every 1 cm, and the tissue slices were embedded in paraffin. They were cut into several 5 μm thick sections for hematoxylin-eosin (HE) and immunohistochemical staining, and the remaining tissues were processed for scanning electron microscopy.

**Immunohistochemistry**

The deparaffinized tissue sections were incubated in a 0.01 M citrate buffer (pH 6.0) at 121°C for 15 min to retrieve antigenicity of the objective proteins before immunostaining. Some sections were immersed in 0.3% H2O2 in phosphate buffered saline (PBS; 1/15 M, pH 7.4) containing 0.1% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 20 min to block the endogenous peroxidase activity. They were incubated in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA), and then with a mixture of antibodies for podoplanin (D2-40; DAKO, Santa Clara, CA, USA) and CD31 (EP3095; Abcam, Tokyo, Japan) at 4°C overnight. Following a rinse in PBS, they were treated with alkaline phosphatase (AP)-conjugated anti-mouse IgG (Histofine Simple Stain MAX-AP; Nichirei Biosciences, Tokyo, Japan) and visualization by AP reaction (Vector blue substrate kit, Vector LaboratorieS). They were then treated with PO-bounded anti-rabbit IgG (Histofine Simple Stain MAX-PO) and visualized by AEC substrate chromogen (DAKO). In contrast, the tissue sections of human stomach were processed for similar staining procedure, but immunolocalization of D2-40 and CD31 were colored red and brown by AP reaction (Vector red substrate kit, Vector Laboratories) and DAB reaction, respectively. All the stained sections for light microscopy were examined with a BX-60 light microscope equipped with DP72 digital imaging system (Olympus, Tokyo, Japan). Control immunostaining were carried out using the same procedures, except for the use of a non-immunized serum instead of the corresponding antibodies. Completely negative results were observed.

**Scanning electron microscopy**

The deparaffinized tissues were processed for SEM as described previously [6]. Briefly, they were immersed in 1% tannic acid solution and 1% osmium tetroxide solution for 1 h each, dehydrated in a graded ethanol series, freeze-dried by t-butylalcohol, and observed under a JEOL JSM-6510 (JEOL, Tokyo, Japan). Captured images were processed with imaging and photo editing software, Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA).

**Morphological assessment and statistical analyses**

The distribution of lymphatics and blood vessels were calculated in the immunostained tissue sections. In particular, numbers of the two vessels were obtained in 10 fields of view, which were set in the area of 500 μm² and randomly chosen in each region. The vessels with obvious lumens were preferentially calculated. The calculated numbers of the two vessels were compared among the mucosal, intramuscular and extramuscular layers. The densities of lymphatic vessels in the ampulla of Vater and the stomach were compared in each region of the mucosal, intramuscular and extramuscular layers. Statistical analyses were performed as follows. Wilcoxon signed-rank test was carried out for comparison with the number of the two vessels. Mann–Whitney U-test was carried out for comparison with the number of lymphatic vessels in the ampulla of Vater and the stomach. Tukey’s honestly significant difference test and Steel–Dwass test were carried out for comparison with three groups. These statistical analyses were tested by Statistical package for social sciences (SPSS) version 21.0 (IBM, Armonk, NY, USA) and Statcel 3 software (OMS Publishing Inc., Saitama, Japan), and the threshold for significance was $P < 0.05$.

**Schematic illustration of the lymphatic system in the ampulla of Vater**

The distribution of lymphatic vessels was illustrated as a model that was reconstructed from the obtained data of immunohistochemistry, SEM and the above morphological analysis. The schematic illustration was generated using an illustration software, Adobe Illustrator CC (Adobe Systems).
Results

Under a light microscope, HE staining classified the three layers, mucosal (m) layer, intramuscular (im) layer equal to the sphincter of Oddi and extramuscular (em) layer tentatively in this study (Fig. 1a). The immunohistochemistry for podoplanin and CD31 demonstrated lymphatic and blood vessels with significant immunoreactivity on the tissue sections of both the ampulla of Vater and the stomach to allow a clear distinction between lymphatic and blood vessels (Figs 1b–f,2c–h). SEM examination of the tissue opposite to the section immunostained for podoplanin permitted a detection of lymphatic vessels and observation of their microanatomical structures in the ampulla of Vater (Fig. 3).

Structure of lymphatic system in the ampulla of Vater

The podoplanin-immunopositive lymphatic vessels were thoroughly distributed with dense supply of the CD31-positive blood vessels in the mucosal, intramuscular and extramuscular layer and appeared to be isolated from the duodenal lymphatic network (Figs 1,2). The lymphatics were irregular in shape, approximately 80 μm in diameter and consisted only of thin endothelial cells. They laid flat and smooth-surfaced endothelial cells with irregular contours, the luminal surfaces and contained some biological substances within the lumens (Fig. 3). Therefore, the lymphatic meshwork in the ampulla of Vater was predominantly composed of lymphatic capillaries. The lymphatic connections were seen between the above three layers (m, im and em) (Fig. 4).

Statistical analysis

While the density of blood vessels was significantly higher than lymphatic vessels in the mucosal, intramuscular and extramuscular layers (P < 0.01, P < 0.01 and P = 0.036), the density of lymphatic vessels in extramuscular layer was significantly higher than in intramuscular layer (P < 0.01). The blood vessels were significantly greater in number than the lymphatic vessels in all layers of the ampulla of Vater, and the density of blood vessels in the mucosa of the ampulla of Vater was significantly higher than that in the other layers (Fig. 2a). In contrast, the extramuscular layer in the ampulla of Vater showed a
higher lymphatic vascular density than those in both the other papillary layers and in the gastric extramucosal (subserosal) layer (Fig. 2b).

Discussion

The present study has demonstrated the distribution and microanatomical profiles of the lymphatic system in the human ampulla of Vater by immunohistochemistry and SEM. The ampulla of Vater developed a precise network of lymphatic capillaries independent of the duodenal lymphatic system. The microanatomical organization of the lymphatic system of the ampulla of Vater as demonstrated by the present study is schematically presented in Figure 5.

The CAV is of low malignant potential [2], but the overall 5-year survival rate of CAV after resection is lower than other gastrointestinal cancers such as gastric and colorectal cancers [7, 8]. The pancreaticoduodenectomy has undergone CAV as a standard surgical treatment, whereas such local resection as surgical or endoscopic procedures are conducted in some cases of cancers in early stage [9, 10] instead of pancreaticoduodenectomy involving high morbidity and mortality. However, no distinct criteria have been indicated between local resection and pancreaticoduodenectomy.

Lymph node metastasis and lymphatic invasion on pathological findings have been reported to be poor prognostic factors of CAV after curative resection [11, 12]. Such complete resection as pancreaticoduodenectomy is required to improve these prognostic factors, but cause
high frequency of complication. In order to resolve this problem, such lymph node dissection procedures meeting curative resection and preservation of quality of life as sentinel lymph node theory [4, 5] for the other cancers should be required, and thus, the present study provides microanatomical information of the lymphatic system of the duodenal papilla. Carcinoma in situ in the duodenal papilla clinically caused no lymph node metastasis, but carcinoma limited to the mucosal layer and/or invaded the sphincter of Oddi showed a higher rate of lymph node metastasis in the ampulla of Vater [13, 14]. Yoon et al. [14] have reported that lymph node metastasis less frequently occurred in 9% of patients with early ampullary cancer (pT1 or pTis) (according to the 6th edition of AJCC cancer staging [15]) as compared with 50.8% of pT2, 38.1% of pT3, and 62.5% of pT4 cancer. Furthermore, Winter et al. [16] have reported that the rate of lymph node metastasis significantly increased with T stage (pT1, 28.0%; pT2, 50.9%; pT3, 71.7%; pT4, 77.3%). Therefore, the risk of lymph node metastasis increased with the depth of tumor invasion on pathological findings in CAV. The lymphatic vessels throughout the entire wall of the duodenal papilla were regarded as lymphatic capillaries by light and electron microscopic examinations (Figs 3, 5). The lymphatic capillaries play a pivotal role of fluid absorption and transport of immune cells and tumor cells. This suggested that carcinoma in every mucosal, intramuscular and extramuscular layers might invade to the lymphatic vessels and migrate to the lymph nodes easily. In addition, abundant distribution of blood vessels throughout the three layers (Fig. 2a) may contribute to be favorable situation for tumor metastasis.

In our investigation into the density of lymphatic vessels between the ampulla of Vater and the gastric wall, the extramuscular layer of duodenal papilla showed a high lymphatic vascular density as compared to the subserosal layer in the stomach (Fig. 2b). While the previous studies [6, 17] described that the mucosal lymphatic capillaries gradually converged to form such large channels as lymphatic pre-collectors in the submucosal and subserosal layers in the mammalian gastrointestinal tract, the extramuscular layer in the duodenal papilla demonstrated numerous lymphatic capillaries. These microanatomical profiles suggested that the cancer limited to the mucosal layer could be resected by local resection, while pancreatoduodenectomy should be selected against the cancers invaded into the intramuscular layer for response to its high metastatic potential. Furthermore, the distance from the mucosal epithelium to the intramuscular layer of the sphincter of Oddi was shorter than those of the stomach (200–500 μm vs. 1,000–2,000 μm). This also supported our hypothesis.

Several limitations of the present study should be acknowledged. First, the anatomy of pancreaticobiliary junction is known to be complex and tends to have several anomalies [18]. The present results are applied in cases showing standard anatomical condition of the ampulla of Vater. Second, although the lymphatic vessels observed in the present study ran from the common pancreaticobiliary channel to the common bile duct, it is obscure as to where they drain. We are now trying to...
demonstrate the lymphatic system also in the common bile duct and the main pancreatic duct.

In conclusion, the present study is first to provide the lymphatic system in the human ampulla of Vater systematically and implicated those intimate associations with lymphatic metastasis of the carcinoma.

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Conflict of interest None declared.

References