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Real-time imaging of the lymphatic channels and sentinel lymph nodes of the stomach using contrast-enhanced ultrasonography with Sonazoid in a porcine model.

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SUMMARY

Contrast-enhanced ultrasound (CEUS)-guided method in combination with Sonazoid has not been clinically or experimentally evaluated with regard to its utility for identifying sentinel lymph node (SLN) in the stomach. Therefore, we have attempted to evaluate the usefulness of CEUS-guided method with Sonazoid for imaging of the lymphatic channels and SLN of the stomach in a porcine model, by compared with the conventional Evans blue dye-guided method. Twenty-eight, 2 to 3-month-old, swine weighing 17-30kg were utilized for this experiment. Anesthesia was maintained with 2.0~3.0% isoflurane/O_2 inhalation. Sonazoid was injected into the intra- and sub-mucosal layers of the stomach. The intragastric or transcutaneous CEUS-guided method was used to identify the lymphatic channels and SLN of the stomach. Contrast imaging using the CEUS-guided method with Sonazoid enabled us to produce clear images of the afferent lymph vessel and SLN of the stomach until 2 hours after the injection of Sonazoid. In addition, intranodal flowing of the microbubbles agent could be clearly identified using tissue linear harmonic images of the SLN. The SLN detection rate was not significantly different between the CEUS- and dye-guided methods. However, the Evans blue dye was flowed out quickly (~15minutes after the injection) through the true SLN into next LN of stomach. In conclusion, the use of the CEUS-guided method with Sonazoid may be the most useful clinical procedure for producing real-time images of the SLN of the stomach, and the linear harmonic images are also useful for evaluating intranodal structure within the SLN.

INTRODUCTION

The sentinel lymph node (SLN) is the first lymph node that receives drainage from a primary tumor. Morton et al. (1) initially demonstrated the SLN concept in a feline model and later confirmed it in a clinical study of patients with breast cancer and melanoma. The clinical impact of the SLN concept has become one of the most important topics in surgical oncology (1-3). Recently, gastric cancer has also been identified as a target for SN navigational surgery (SNNS) (4-10).

The dye-guided or radioisotope (RI)-guided method, or a combination of both, is conventionally used for SLN mapping in gastric cancer (4-10). The dye-guided method is convenient and safe. However, it has been reported to be associated with a high false negative node ratio because the small dye particles can readily diffuse through the true SLN and transverse multiple nodes (4-10). The RI-guided method has several advantages over the dye-guided method for identifying SLN. However, lymph vessels cannot be visualized. The high radioactivity at the primary injection site may also interfere with the intraoperative detection of nearby lymph nodes (11-12).

Recently, contrast-enhanced ultrasonography in combination with Sonazoid was adopted to detect the SLN in patients of breast cancer (13). However, the clinical usefulness of CEUS-guided method in combination with Sonazoid is still controversial, because the superiority of CEUS-guided method in preclinical or clinical studies to compare with the conventional dye- or RI-guided method has

not been conducted in terms of SLN detection rate and accuracy. In addition, few or no study exists to confirm experimentally or clinically the usefulness of CEUS-guided method in combination with Sonazoid for imaging the SLN of stomach.

To address the possibility that the CEUS-guided method in combination with Sonazoid will develop the useful SLN detection method in patients with gastric cancer, we have attempted to evaluate firstly (1) the usefulness of the CEUS-guided method in combination with the intra- and sub-mucosal injection of Sonazoid for imaging the lymphatic channels and SLN of the stomach in a porcine animal model and then (2) to compare with quantitatively the SLN detection rate and accuracy obtained using the conventional Evans blue dye-guided method in the same animal model.

MATERIALS AND METHODS

In this experiment, we imaged the lymphatic channels and SLN of the stomach in a porcine model. The procedure was designed to identify the SLN and its afferent lymph vessels using lymphosonography in combination with Sonazoid and then the usefulness of the procedure was compared with the imagings of the lymphatic channels and SLN obtained with the conventional Evans blue dye-guided method in the animal model.

Animals, anesthesia, and monitoring

This experiment was approved by the Institutional Animal Care and Use Committee of Shinshu University. Twenty-eight, 2 to 3-month-old, crossbred [(Yorkshire × Landrace) × Duroc] swine (11 males and 17 females) weighing 17~30kg (23.0±0.8kg, n=28) were utilized in the animal experiments in a humane and ethical fashion. The animals were fasted overnight, and pre-anesthesia sedation was intramuscularly administered with 0.1~0.2mg/kg medetomidine hydrochloride (Orion Pharma, Finland) and 0.7~1.2mg/kg midazolam (Astellas Pharma, Japan). Anesthesia was maintained with 2.0~3.0% isoflurane (Dainippon Sumitomo Pharma, Japan)/O_2 inhalation, titrated to effect after endotracheal intubation. Ventilation was maintained at 10 to 15mL/kg/min for 10 to 15 breaths per minute. Electrocardiography (ECG) and heart rate were monitored (FCP-140, FUKUDA DENSI, Japan). Physiological saline solution (Otsuka Pharma, Japan) was administered at 10mL/kg/hr during the experiment. The animals were subsequently euthanized after the completion of the experiments.

Experimental procedure and protocols of CEUS-guided imaging

The swine were placed in a supine position on the operating table. The body

temperature of each animal was maintained at 36.5~37.5°C using a heating pad. The abdomen was then cut along the median line, and the stomach was gently dragged out to allow the pylorus and greater curvature of the stomach to be examined. In order to perform intragastric CEUS-guided imaging of the lymphatic channels and SLN of the stomach, a 5cm linear incision was made in the avascular region between the lesser and greater curvature of the stomach. The ultrasound probe was then inserted into the stomach through the incision and used to observe the lymphatic channels and SLN via the CEUS-guided method after the injection of Sonazoid (Daiichi-Sankyo Group, Japan). Thus, 0.01~0.3mL Sonazoid were injected into the intra- and sub-mucosal layers of the stomach at about 9cm above from the pylorus and about 2cm away from the greater curvature of the stomach, and then the injection site was gently massaged for 10s.

Sonazoid is a lipid-stabilized suspension of 2.4 to 3.5 micron perfluorobutane microbubbles that was originally developed as an ultrasound contrast agent. Several studies have been reported on the use of Sonazoid for CEUS-guided imaging of the lymphatic channels and SLN in animal models (14, 15). In these studies, the contrast agent was injected into the subject, and then transcutaneous, contrast-specific gray-scale or color-flow Doppler ultrasonography was performed. The agent can be clearly visualized on ultrasound as it passes through the lymphatic channels and SLN, but does not pass beyond the first-echelon lymph nodes (15). The safety of the intravenous administration of Sonazoid has been established in human studies evaluating its use for imaging of the liver and heart (16).

After the intragastric CEUS-guided imaging of the lymphatic channels and SLN of the

stomach, we sutured the abdominal wall and then closed the abdomen. Next, the lymphatic channels and SLN of the stomach were also re-evaluated in the same animal using the transcutaneous CEUS-guided method in combination with Sonazoid.

Ultrasound procedure

Conventional gray-scale ultrasound using a fundamental scanner (EUB-7500, Hitachi, Japan) and a 3.5MHz convex (EUP C715, Hitachi, Japan) or 13MHz flat linear array (EUP L74M, Hitachi, Japan) transducer was used prior to the injection of Sonazoid, with adjustment of the imaging parameters such as system and depth of field. After Sonazoid injection, gray-scale contrast harmonic or tissue linear harmonic imaging was used. This technique allows clear visualization of the contrast agent flowing through the lymph channels with good accuracy and spatial resolution. For example, using tissue linear harmonic imaging, the contrast agent can be clearly seen flowing into the lymph nodes. The mechanical index (MI) is a measure of the acoustic pressure generated within the ultrasound field (17). The MI values of the acoustic pressure employed in contrast or tissue harmonic imaging of Sonazoid ranging from 0.2 to 0.3 in order to reduce microbubble destruction. Flash replenishment imaging (FRI) was performed with the use of a higher MI (>1.0) to confirm the presence of Sonazoid as it caused the disappearance of the Doppler signal due to microbubble rupture. Micro-flow imaging (MFI) was also adopted to confirm the reflow of Sonazoid through the lymphatic channels and the SLN of the stomach. All precontrast and postcontrast scans were performed by the same sonographer.

Once the contrast agent had been identified, the injection site was gently massaged (10s) to expedite the flow of Sonazoid into the lymphatic channels. Subsequently,

lymphosonography was used to identify Sonazoid as it moved through the lymphatic channels to the SLN of the stomach. Once the SLN had been identified, the abdominal skin was marked with an indelible marker. In some cases, the contrast harmonic imaging was performed transcutaneously in the region where the skin had been marked after the abdominal skin had been stitched up.

After the ultrasound imaging recording had finished (n=23), dissection and isolation were carried out in the abdomen in order to identify the SLN or SL basins in the presence of the Evans blue dye. Thus, 0.1mL 1% Evans blue dye (Sigma, USA) containing 2% bovine serum albumin (BSA, Sigma Aldrich, St Louis, USA) were injected into the same intra- and sub-mucosal layers as the Sonazoid injected sites, and the morphologies of the excised lymphatic channels and SLN of the stomach were visualized.

Imaging analysis

All images were recorded as videos (10frames/s, 120s, EUB-7500, Hitachi, Japan). All data were analyzed frame by frame.

Experimental protocols of Evans blue dye-guided imaging

To compare with the SLN detection rate and accuracy between the CEUS-guided and the conventional dye-guided methods, we next investigated the lymphatic channels and SLN of the porcine stomach (n=5) using Evans blue dye-guided method. Thus, 0.1mL 1% Evans blue dye containing 2% BSA were injected into the same intra- and sub-mucosal layers of the stomach as the injection sites of Sonazoid, which is about 9cm above from the pylorus and about 2cm away from the greater curvature of the stomach. The flow patterns of Evans blue dye through the lymphatic channels, true SLN and the transverse lymph nodes were

observed intra-abdominally after the gently massage of the injection site for 10s. The flow patterns of Evans blue dye were photographed by a digital camera (RICOH, CX4, Japan) every 1min until 30min after the dye injection.

In some animals, to evaluate the corresponding SLN changes depending on the dye injected point, 0.1mL Evans blue dye containing 2% BSA were injected at about 9cm above from the pylorus and about 2cm away from the lesser curvature of the stomach, and then we investigated changes in the SLN position using the Evans blue dye-guided method.

Histological studies

To identify histologically the injected position of Sonazoid or Evans blue dye in the wall of porcine stomach, the histological analyses of the wall of the stomach pre-injected with 0.01mL Indian ink were conducted. For light microscopic observation, specimens including Indian ink were fixed with 10% formalin solution for 24hours. The specimens were dehydrated through graded series of ethanol and then embedded in paraffin in a routine manner. Sections of 3~4μm were processed by hematoxylin and eosin stain. The sections were examined with a light microscope (Leica, Wetzler, Germany) and photographed.

RESULTS

Figure 1 shows representative images of the operated stomach (Fig. 1A), photomicrographs of the macro- and micro-scopic sites of Sonazoid or Evans blue dye (Fig. 1B, C, D), and representative image produced by the intragastric CEUS-guided method using a contrast harmonic probe (Fig. 1E). As shown in the Fig. 1D, the injected Indian ink were clearly observed within the intra- and sub-mucosal layers of the stomach (Fig. 1D, arrow heads). In addition, the CEUS-guided contrast harmonic and tissue linear harmonic images were recorded in 23 pigs examined. In all cases (23 cases out of 23 pigs), the contrast agent, Sonazoid was easily identified as it flowed through the afferent lymph vessels and into the SLN of the stomach. Thus, the detection rate of the SLN of the stomach evaluated with the CEUS-guided method in combination with Sonazoid was 100.0% (n=23). The locations of the lymphatic channels and SLN of the stomach were confirmed using flash replenishment imaging (FRI) and micro flow imaging (MFI) in all cases.

Effects of the injected dose of Sonazoid on contrast harmonic imaging of the lymphatic channels and SLN of the stomach

Figure 2 demonstrates representative tracings of the effects of the injected dose of Sonazoid, which ranged from 0.01 to 0.1mL, on contrast imagings of the lymphatic channels and SLN of the stomach. In all cases (n=23), the afferent lymph vessels of the SLN were clearly identified within 30-seconds(s) of the intra- and sub-mucosal injection of more than 0.03mL Sonazoid. The SLN of the stomach were also identified using contrast harmonic imaging within 30-s of the Sonazoid injection in all cases (Fig. 2B, C). In contrast, the injection of Sonazoid at a dose of 0.01mL produced no contrast imaging of the afferent lymph

vessel or SLN of the stomach within 1 minute of its injection. However, around 5 minutes(m) after the injection of Sonazoid, a small SLN was dimly observed in the stomach (Fig. 2A).

Figure 3 shows representative tracings of the flash replenishment (FRI) and micro-flow images (MFI) of the afferent lymph vessels and SLN of the stomach produced using the CEUS-guided method at an MI of more than 1.0. The intra- and sub-mucosal injection of Sonazoid at a dose of 0.3mL allowed the rapid acquisition of clear contrast harmonic images of the afferent lymph vessels and SLN of the stomach (Fig. 3A). Thus, just after (less than 20s) the injection of 0.3mL Sonazoid, the afferent lymph vessels of the stomach were clearly identified (Fig. 3A, most-left panel). The contrast images of the afferent lymph vessels and SLN of the stomach became more and more clear in a time-dependent manner. This finding was confirmed in all cases (n=23). To confirm the flow of Sonazoid through the lymphatic channels and SLN of the stomach, we conducted an experiment in which we ruptured the perfluorobutane microbubbles, using the CEUS-guided method at a higher acoustic pressure (MI more than 1.0). In the same animal, we obtained FRI of the afferent lymph vessel and SLN of the stomach using ultrasound at an MI of 1.2, which resulted in a significant sharpening of the images of the afferent lymph vessels and SLN of the stomach (Fig. 3B, most-left panel). Around 2s after the FRI, no contrast images of the lymph vessel or SLN of the stomach were observed (Fig. 3B, 2s after the FRI). At more than 20s after the stimulation, the afferent lymph vessels and SLN of the stomach reappeared on the contrast harmonic images in a time-dependent manner (Fig. 3B, 20s after the FRI).

Next, to evaluate the time-dependent changes in the contrast harmonic images of the

lymphatic channels and SLN of the stomach, we investigated the changes in the images recorded every 10, 20, or 30m until 2 hours after the intra- and sub-mucosal injection of 0.3mL Sonazoid.

Figure 3C demonstrates representative tracings of the contrast images of the afferent lymph vessels and SLN of the stomach recorded every 10~30m. The SLN of the stomach was clearly identified at 10m after the intra- and sub-mucosal injection of 0.3mL Sonazoid (Fig. 3C, most-left panel at the upper tracing). Very similar contrast images of the lymph vessels and SLN stomach were produced at 20, 40, 60, 90, and 120m after the injection of 0.3mL Sonazoid (Fig. 3C, upper and lower tracings). In addition, we confirmed that the contrast agent did not pass beyond the first-echelon lymph node.

Tissue linear harmonic imaging of the lymphatic channels and SLN of the stomach

To evaluate the lymph flow within the SLN of the stomach in detail, we produced tissue linear harmonic images of the SLN of the stomach using a linear transducer (EUP L74M, 13MHz, Hitachi, Japan).

Figure 4A shows representative tracings of the distribution of Sonazoid within the SLN of the stomach. The microbubbles were clearly identified in the marginal and trabecular sinuses within the SLN of the stomach. Thus, the video composed of tissue linear harmonic images enabled us to visualize the Sonazoid flowing within the SLN of the stomach.

Confirmation of the SLN of the stomach using Evans blue dye-guided method

To evaluate whether the CEUS-guided images of the SLN of the stomach agreed with those produced after the same position injection of Evans blue dye as one of Sonazoid, we

compared the CEUS-guided images with the photomicrographs of the SLN of the stomach produced using Evans blue dye.

Figure 4B shows representative CEUS-guided images and a photomicrograph of the SLN of the stomach produced by the Evans blue dye injection. Parts of the SLN of the stomach were clearly stained by Evans blue dye (Fig. 4B-3). Very similar areas of the SLN of the stomach were identified by the contrast harmonic (Fig. 4B-1) and tissue linear harmonic images (Fig. 4B-2) of the SLN of the stomach.

Transcutaneous contrast-enhanced ultrasonography of the SLN of the stomach

Next, to evaluate the similarity of intragastric CEUS-guided images of the SLN of the stomach with those obtained by the transcutaneous CEUS-guided method, we investigated the SLN of the stomach using the transcutaneous CEUS-guided method in the same animal after we had identified the SLN of the stomach using the intragastric CEUS-guided method and then sutured the abdominal wall. Figure 5 demonstrates representative tracings of intragastric and transcutaneous CEUS-guided images (Fig. 5A, C) and a photomicrograph of the operated animal (Fig. 5B). The contrast harmonic images of the SLN of the stomach produced after the intra- and sub-mucosal injection of 0.3mL Sonazoid were clear (Fig. 5A). In the same animal, the abdominal wall was sutured (Fig. 5B), and then the SLN of the stomach was confirmed to be located in the same position using the transcutaneous CEUS-guided method (Fig. 5C). In addition, in the case of transcutaneous method, the contrast imaging of the SLN of the stomach was augmented by ultrasound stimulation at an MI of more than 1.2 (Fig. 5C, 170s). At 2s after the stimulation, the image of the SLN of the stomach had completely disappeared (Fig. 5C, 2s after FRI). The image reappeared 20s

after stimulation with FRI (Fig. 5C, 20s after FRI).

Imaging of the lymphatic channels and SLN of the stomach using the conventional dye-guided method

To compare the SLN detection rate and accuracy in the animal experiments between the CEUS-guided and the conventional dye-guided methods, we finally evaluated the lymphatic channels and SLN of the stomach by the intra- and sub-mucosal injection of 0.1mL 1% Evans blue dye of the stomach (n=5). Figure 6A shows representative photomicrographs of the Evans blue dye-guided images of the afferent lymph vessel, true SLN, the efferent lymph vessel, next lymph node, and its efferent lymph vessel of the stomach photographed every 1minute (m) until 15m after the injection. The true SLN, the next lymph node, and each efferent lymph vessel were clearly identified at 3m after the injection of Evans blue dye. In addition, the Evans blue dye within the SLN of the stomach was stained heterogeneously and then disappeared in a time-dependent manner. At 15m after the Evans blue injection, the dye within the SLN was diluted and then the dye within the next lymph node became gradually condensed. Thus, the phenomenon of overflowing out of Evans blue dye through true SLN, which may produce a high false negative node, was confirmed within 3m after the Evans blue dye injection. The detection rate of the SLN of the stomach evaluated by the dye-guided method at 3m after the injection of Evans blue was 100.0% (n=5). However, the accuracy of the Evans blue dye-guided method was confirmed in the present experiments to be significantly lower than the CEUS-guided method, because the dye-guided method was associated with a high false negative node ratio due to overflow out of the SLN.

To evaluate the SLN changes depending on the injected point, we conducted

preliminally another experiments for idetifing the SLN of the stomach using the 0.1mL 1% Evans blue dye intra- and sub-mucosal injection, which was about 9cm above from the pylorus and about 2cm away from the lesser curvature of the stomach. Figure 6B demonstrates representative photomicrographs of the Evans blue dye images of true SLN before (Figure 6B, 8m) and after (Figure 6B, 15m) the injection of Evans blue dye at the lesser curvature of the stomach. The additional injection of Evans blue dye at the lesser curvature of the stomach produced the new image of corresponding SLN of the stomach. Thus, the SLN of the stomach was clearly different from the SLN of the stomach identified by the injection of Evans blue dye at the greater curvature of the stomach.

DISCUSSION

Gastric SLN identification using the CEUS-guided method with Sonazoid

The identification of SLN; i.e., the LN that tumor cells reach first, is important for deciding whether axillary LN dissection should be performed in breast cancer patients. The current standard methods of SLN detection are the dye-guided and RI-guided methods. Although the dye is inexpensive, some skill is required to detect it. Moreover, since anaphylactic reactions to the dye, although rare, have been reported (18), care is necessary during use of the dye. On the other hand, the RI-guided method requires many hours to detect SLN after the radioactive colloid has been injected. Another disadvantage of this method is that it must be performed in a hospital that can handle radioactive materials. To overcome these problems, the CEUS-guided method of SLN detection has recently been developed.

Early gastric cancer is the most suitable target of SLN mapping in gastrointestinal (GI) cancer because individualized and minimally invasive surgery based on SLN biopsy might be applicable (19-21). However, in cases of gastric cancer, 5 ~10% of SLN are located in the second compartment that may be accounted for by aberrant drainage routes from the primary lesion. No suitable technique for identify lymphatic networks and SLN located in the second compartment is available. In addition, the utility of using the CEUS-guided method in combination with Sonazoid to identify the lymphatic channels and SLN of the stomach has not been evaluated in a clinical setting. Therefore, in the present experiments, we attempted to perform real-time imaging of the lymphatic channels and SLN of the stomach in a porcine model using the CEUS-guided method in combination with Sonazoid and evaluate

the effectiveness of the CEUS-guided method in terms of the SLN detection rate and accuracy to compare with the conventional dye-guided method. Thus, this study is the first demonstration that using the CEUS-guided method in combination with the intra- and sub-mucosal injection of Sonazoid (0.05~0.3mL) enables the production of clear contrast harmonic images of the afferent lymph vessels and SLN around 20s after the injection. The images of the lymph vessels and SLN last 120min after the injection of 0.3mL Sonazoid. In addition, SLN mapping of the perigastric region can be performed using the transcutaneous CEUS-guided method. In conclusion, the use of the CEUS-guided method in combination with the intra- and sub-mucosal injection of Sonazoid could become the most useful clinical procedure for producing real-time images of the lymphatic channels and SLN of the stomach, and also for evaluating SLN located in the second compartment. Thus, the SLN concept, but not sentinel basins is applicable to gastric cancers when we use the real-time CEUS-guided method in combination with Sonazoid. In addition, the SLN detection rate was confirmed to be not significant different between the CEUS-guided and the conventional dye-guided methods. However, from the point of accuracy in the detection of the SLN of the stomach, the CEUS-guided method in combination with Sonazoid was confirmed to be more excellent than the dye-guided method. Therefore, SLN may be a good target for selective lymphadenectomy for early gastric cancer associated with a risk of micrometastasis. However, further investigations are necessary to evaluate the molecular and functional mechanisms of the flow of intra- and sub-mucosally injected Sonazoid into the initial lymphatics, but not the blood capillaries or venules. In addition, further studies will be needed in future to evaluate the corresponding SLN changes depending on the injected point

of Sonazoid using the CEUS-guided method.

Evaluation of intranodal structure within the SLN

Another important aspect of this study is that intranodal flowing of Sonazoid can be clearly identified on videos composed of tissue linear harmonic images within the SLN of the stomach. Thus, we concluded that the CEUS-guided method in combination with Sonazoid enabled us to visualize the intranodal structure and the real-time flowing of Sonazoid within the SLN of the stomach.

The SLN is the most common and earliest site of malignant tumor metastasis. Lymph nodes act as a mechanical barrier to prevent the passage of tumor cells through the node and also act as a biological barrier to inhibit tumor growth in the node (22-26). On the other hand, it is also known that primary tumors alter the tumor tissue microenvironment prior to the formation of metastases (27, 28). Recently, we (29) demonstrated that intracellular adhesion molecule-1 (ICAM-1) in the premetastatic regional lymph node is involved in producing a suitable environment for micro-metastasis within the lymph nodes.

According to the results of our present and previous studies, we would like to propose the new clinical possibility that microenvironmental changes within premetastatic or micrometastatic SLN could be identified on tissue linear harmonic images within the SLN of the stomach using Sonazoid associated with molecular markers such as ICAM-1. If such contrast agents are developed, changes in the intranodal structure depended upon flow patterns of Sonazoid or the distribution of ICAM-1 expression within the SLN may be identified clinically. Further investigation will be, in future, needed to evaluate the relationship between these changes within the SLN and the micrometastases of carcinoma

cells.

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COMPETING INTEREST STATEMENT

The authors declare that no competing financial interests exist.

REFERENCES

1. Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992; 127:392-399.
2. Veronesi U, Pagnelli G, Galimberti V, et al. Sentinel-node biopsy to avoid axillary dissection in breast cancer with clinically negative lymphnodes. Lancet 1997; 349:1864-1867.
3. Edwards MJ, Martin KD, McMasters KM. Lymphatic mapping and sentinel lymph node biopsy in the staging of melanoma. Surg Oncol 1998; 7:51-57.
4. Tajima Y, Yamazaki K, Masuda Y, et al. Sentinel node mapping guided by indocyanine green fluorescence imaging in gastric cancer. Ann Surg 2009; 249:58-62.
5. Hiratuka M, Miyashiro I, Ishikawa O, et al. Application of sentinel node biopsy to gastric cancer surgery. Surgery 2001; 129: 335-340.
6. Kitagawa Y, Fujii H, Mukai M, et al. Radio-guided sentinel node detection for gastric cancer. Br J Surg 2002; 89:604-608.
7. Ichikura T, Morita D, Uchida T, et al. Sentinel node concept in gastric carcinoma. World Surg 2002; 26:318-322.
8. Carlini M, Carboni F, Petric M, et al. Sentinel node in gastric cancer surgery. J Exp Clin Cancer Res 2002; 21:469-473.
9. Isozaki H, Kimura T, Tanaka N, et al. For the esophagus gastrointestinal surgical treatment study group. An assessment of the feasibility of sentinel lymph node-guided surgery for gastric cancer. Gastric Cancer 2004; 7:149-153.

10. Osaka H, Yashiro M, Sawada T, et al. Is a lymph node detected by the dye-guided method a true sentinel node in gastric cancer? Clin Cancer Res 2004; 10:6912-6918.
11. Kitagawa Y, Fujii H, Kumai K, et al. Recent advances in sentinel node navigation for gastric cancer: a paradigm shift of surgical management. J Surg Oncol 2005; 90:147-151.
12. Ichikura T, Chochi K, Sugasawa H, et al. Individualized surgery for early gastric cancer guided by sentinel node biopsy. Surgery 2006; 139:501-507.
13. Omoto K, Matsunaga H, Take N, et al. Sentinel node detection method using contrast-enhanced ultrasonography with Sonazoid in breast cancer: preliminary clinical study. Ultrasound Med Biol 2009; 35:1249-1256.
14. Goldberg BB, Merton DA, Liu JB, et al. Sentinel lymph node in a swine model with melanoma: contrast-enhanced lymphatic US. Radiology 2004; 230:727-734.
15. Goldberg BB, Merton DA, Liu JB, et al. Contrast-enhanced sonographic imaging of lymphatic channels and sentinel lymph node. J Ultraound Med 2005; 24:953-965.
16. Marwick TH, Brunken R, Meland N, et al. Accuracy and feasibility of contrast echocardiography for detection of perfusion defects in routine practice: comparison with wall motion and technetium-99m sestamibi single-photon emission computed tomography. The Nycomed NC100100 Investigators. J Am Coll Cardiol 1998; 32:1260-1269.

17. McCulloch M, Gresser C, Moss S, et al. Ultrasound contrast physics: A series on contrast echocardiography, article 3. J Am Soc Endocardiogr 2000; 13:959-967.
18. Rodier J, Janser J. Surgical technical details improving sentinel node identification in breast cancer. Oncol Rep 1997; 4:281-283.
19. Kitagawa Y, Saha S. Current status and future clinical applications of lymphatic mapping in gastrointestinal cancer. J Gastroenterol 2007; 42:927-931.
20. Kitagawa Y, Kitajima M. Gastrointestinal cancer and sentinel node navigation surgery. J Surg Oncol 2002; 79:188-193.
21. Kitagawa Y, Kitano S, Kubota T, et al. Minimally invasive surgery for gastric cancer – toward a confluence of two major streams: a review. Gastric Cancer 2005; 8:103-110.
22. Carr I, McGinty F. Lymphatic metastasis and its inhibition: an experimental model. J Pathol 1974; 113:85-95.
23. Cobb RA, Steer HW. Tumour cell trapping in rat mesenteric lymph nodes. Be J Exp Pathol 1987; 68:461-474.
24. Fisher B, Fisher ER. Barrier function of lymph node to tumor cells and erythrocytes, 1. Normal nodes. Cancer 1967; 20:1907-1913.
25. Hewitt HB, Blake E. Quantitative studies of translymphnodal passage of tumor cells naturally disseminated from a non immunogenic murine squamous carcinoma. Br J Cancer 1975; 31:25-35.

26. Nagata H, Arai T, Soejima Y, et al. Limited capability of regional lymph nodes to eradicate metastatic cancer cells. Cancer Res 2004; 64:8239-8248.

27. Hiratsuka S, Watanabe A, Aburatani H, et al. Tumor-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. Nat Cell Biol 2006; 8:1369-1375.

28. Johnson JP. Cell adhesion molecules of the immunoglobulin supergene family and their role in malignant transformation and progression to metastatic disease. Cancer Metastasis Rev 1991; 10:11-22.

29. Kawai Y, Kaidoh M, Ohhashi T. MDA-MB-231 produces ATP-mediated ICAM-1-dependent facilitation of the attachment of carcinoma cells to human lymphatic endothelial cells. Am J Physiol Cell Physiol 2008; 295:C1123-C1132.

FIGURE LEGENDS

Figure 1

A: Representative images of the operated stomach in a porcine model.

B: Representative photomicrograph of the Sonazoid injection point (low magnification).

C: Representative photomicrograph of the Sonazoid injection point (high magnification).

D: Representative histological photomicrograph of the gastric wall pre-injected with Indian ink. The Indian ink were confirmed at the intra- and sub-mucosal layers (arrow heads) of the stomach. The marker is 100μm.

E: Representative photomicrograph produced using the intragastric CEUS-guided method during the identification of the lymphatic channels and SLN of the stomach using a contrast harmonic probe.

Figure 2

Representative tracings of the effects of Sonazoid injected at doses ranging from 0.01 to 0.1mL on contrast imaging of the lymphatic channels and SLN of the stomach in a porcine model.

White arrowhead: the SLN of the stomach

Dotted arrowhead: the afferent lymph vessel

s: seconds m: minutes IVC: inferior vena cava PV: portal vein

Figure 3

A: Representative tracings of contrast harmonic images of the afferent lymph vessels and

SLN of the stomach obtained using the intragastric CEUS-guided method in combination with the intramucosal injection of 0.3mL Sonazoid.

B: Representative tracings of flash replenishment images (FRI) and contrast harmonic images obtained at 2, 10, and 20s after FRI stimulation in the same animal.

White arrowhead: the SLN of the stomach

Dotted arrowhead: the afferent lymph vessel

s, m, IVC, and PV represent the same parameters as in Fig. 2.

C: Representative tracings of contrast harmonic images of the SLN of the stomach obtained using the intragastric CEUS-guided method at 10, 20, 40, 60, 90, and 120m after the intramucosal injection of 0.3mL Sonazoid.

White arrowhead, s, m, IVC, and PV represent the same parameters as in Fig. 2.

Figure 4

A: Representative tracings of tissue linear harmonic images of the afferent lymph vessels and SLN of the stomach obtained with the intragastric CEUS-guided method at 500, 510, 520, 530, and 540s after the intramucosal injection of 0.3mL Sonazoid.

White and dotted arrowheads, s, and PV represent the same parameters as in Fig. 2.

B: Representative tracings of contrast harmonic (1) and tissue linear harmonic (2) images of the SLN of the stomach in the same animal.

B (3): Representative photomicrograph of the SLN of the stomach identified by injecting Evans blue dye into the same location as the Sonazoid.

White arrowhead, m, and PV represent the same parameters as in Fig. 2.

Figure 5

A: Representative tracing of contrast imaging of the SLN of the stomach obtained using the intragastric CEUS-guided method in combination with the intramucosal injection of 0.3mL Sonazoid.

B: Representative photomicrograph of the transcutaneous CEUS-guided method in the same animal.

C: Representative tracings of the contrast harmonic and flash replenishment images (FRI) of the SLN of the stomach obtained using the transcutaneous CEUS-guided method after the intramucosal injection of 0.3mL Sonazoid in the same animal.

White arrowhead and s represent the same parameters as shown in Fig. 2.

Figure 6

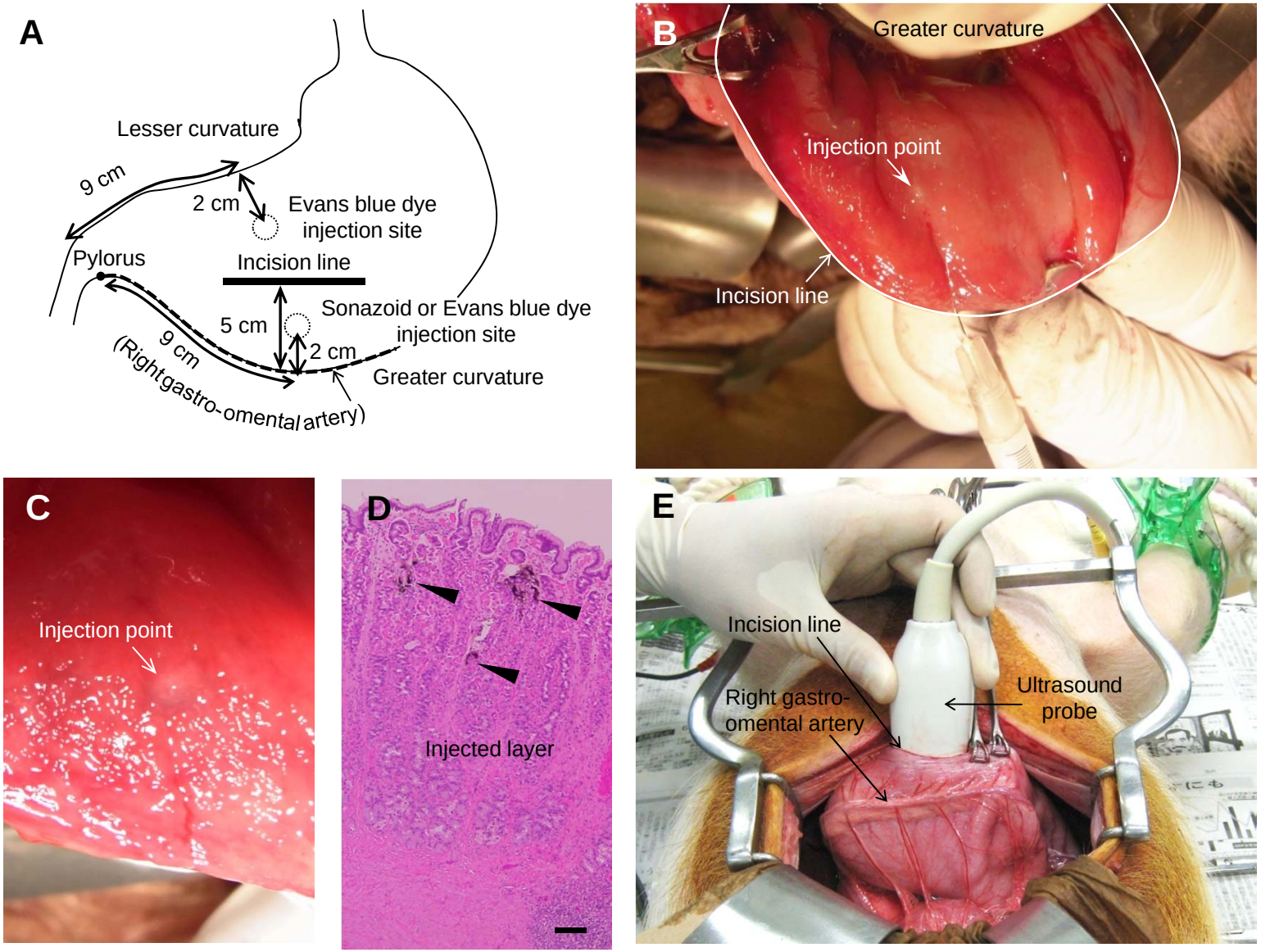
A: Representative photomicrographs of the afferent lymph vessel the SLN, the efferent lymph vessel, the next lymph node, and its efferent lymph vessel obtained using the conventional Evans blue dye-guided method at 3, 4, 8, and 15minutes (m) after the intra- and sub-mucosal injection of the dye.

B left panel (8m): Representative photomicrograph of the afferent lymph vessel, the SLN, and the efferent lymph vessel obtained using the Evans blue dye-guided method at 8m after the Evans blue dye injected at 2cm away from the greater curvature of the stomach.

B right panel (15m): Representative photomicrograph of another SLN of the stomach obtained at just 2m after the additional injection of Evans blue dye into the 2cm away from

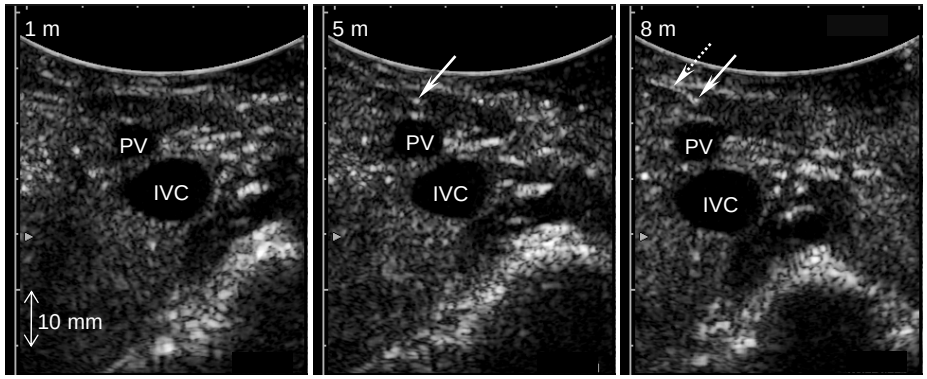
the lesser curvature of the stomach.

New Fig. 1

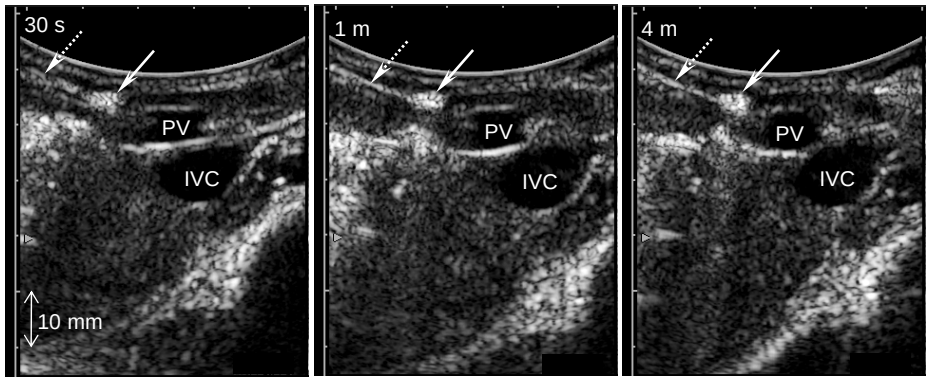

A
Lesser curvature
9 cm
2 cm
Evans blue dye injection site
Pylorus
Incision line
5 cm
Sonazoid or Evans blue dye injection site
2 cm
9 cm
(Rightgastro-omental artery)
Greater curvature
B
Greater curvature
Injection point
Incision line
C
Injection point
D
Injected layer
E
Incision line
Right gastro-omental artery
Ultrasound probe

New Fig. 2

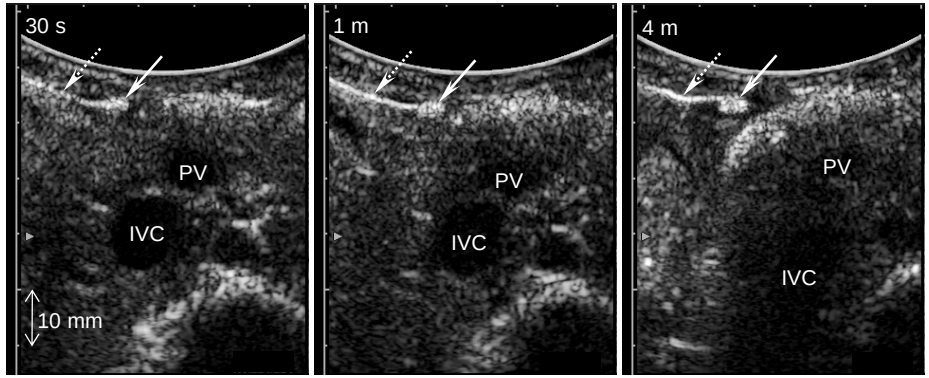
A injection volume : 0.01mL



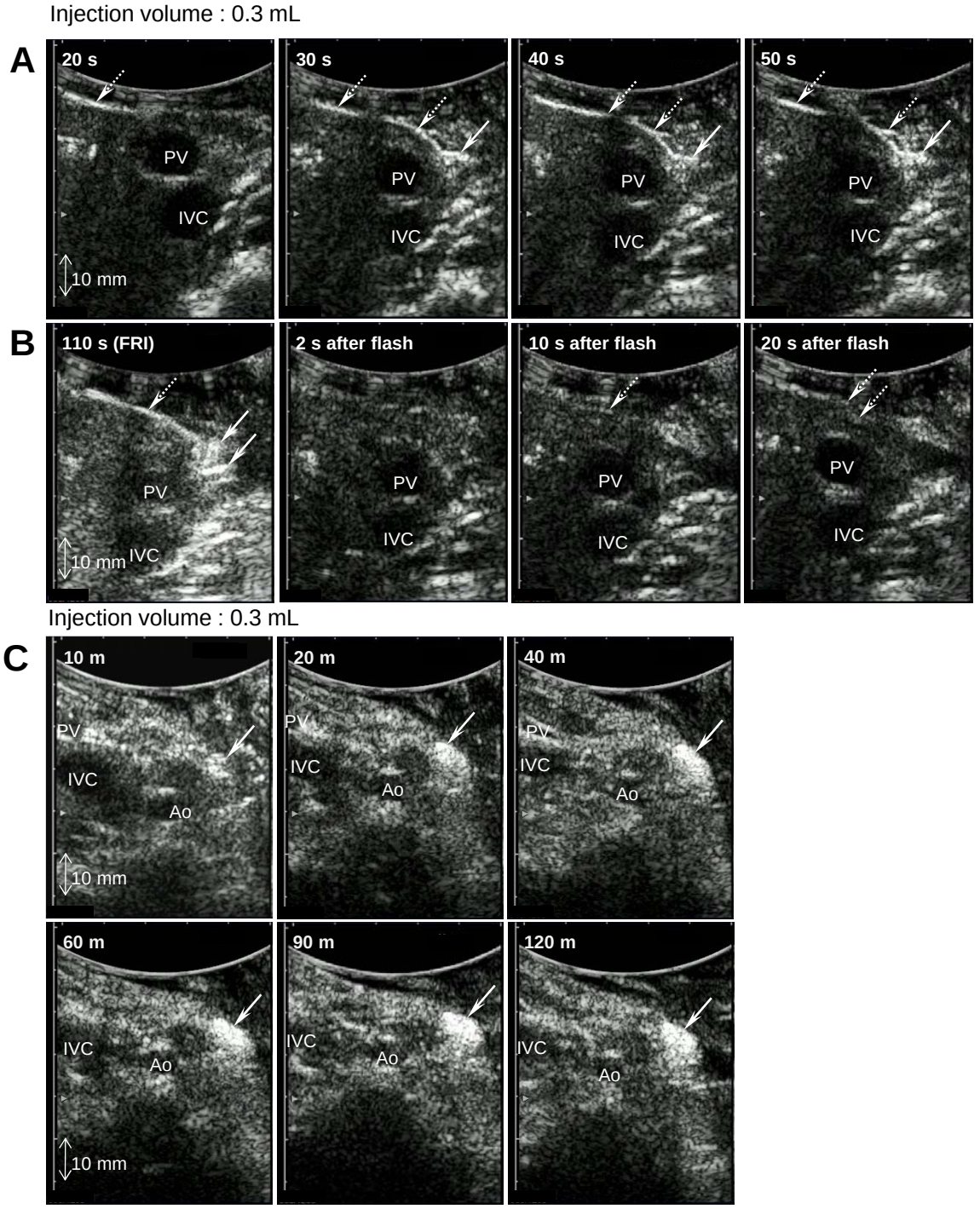
B injection volume : 0.03mL



C injection volume : 0.1mL



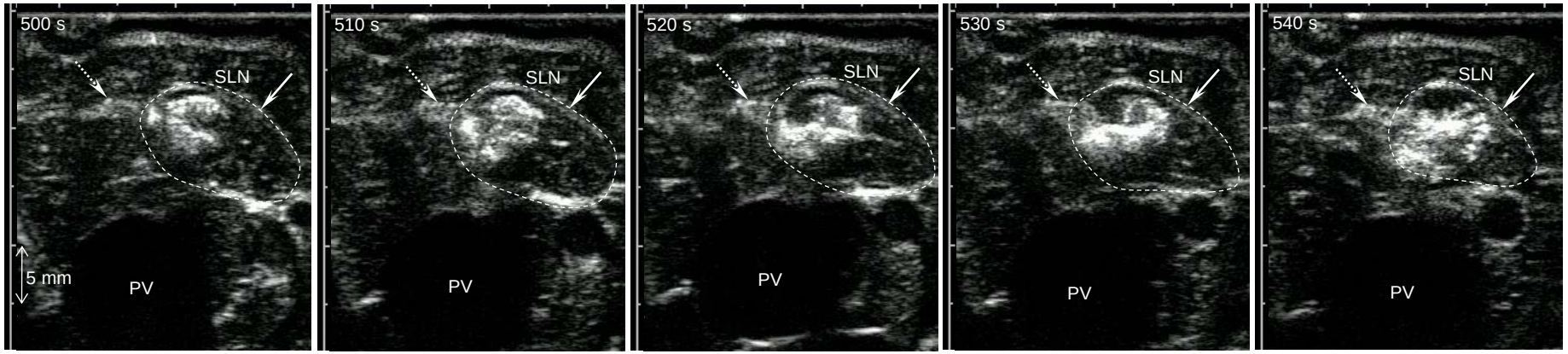
New Fig. 3


Injection volume : 0.3 mL
A
20 s
30 s
40 s
50 s
PV
IVC
10 mm
B
110 s (FRI)
2 s after flash
10 s after flash
20 s after flash
Injection volume : 0.3 mL
C
10 m
20 m
40 m
60 m
90 m
120 m
Ao

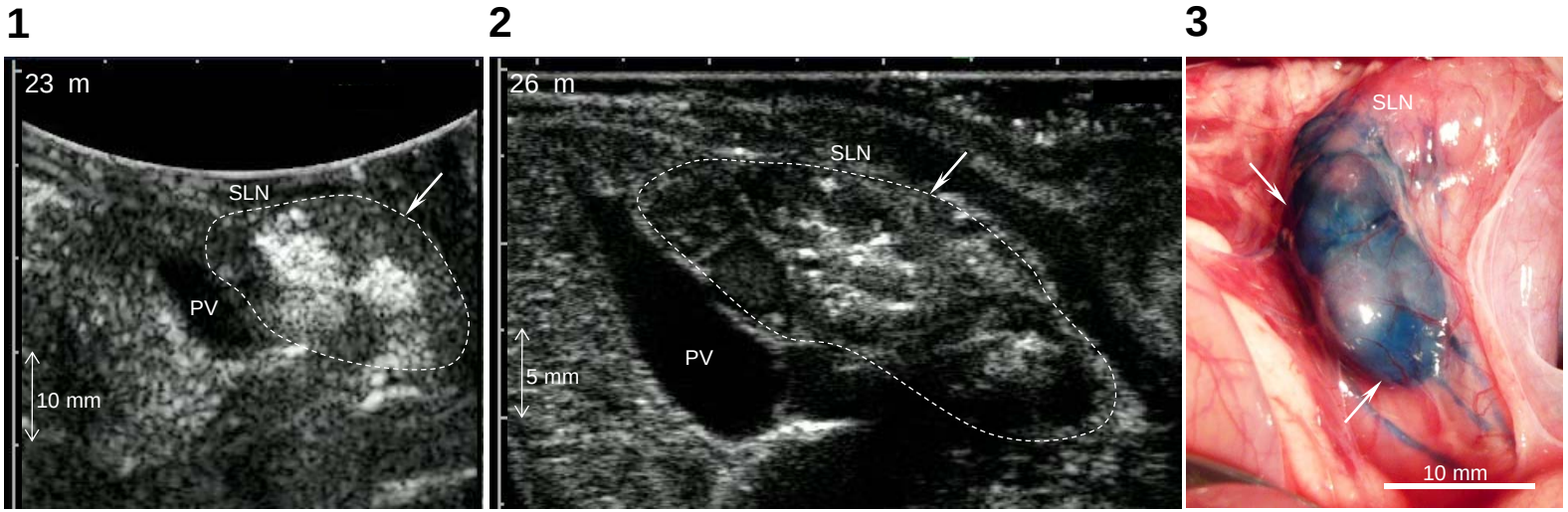
New Fig. 4

A

Injection volume : 0.3 mL



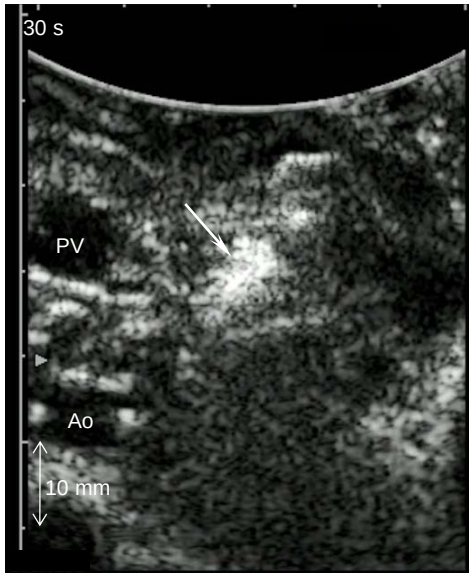
B Injection volume : Sonazoid, 0.3 mL
Injection volume : Evans blue, 0.3 mL



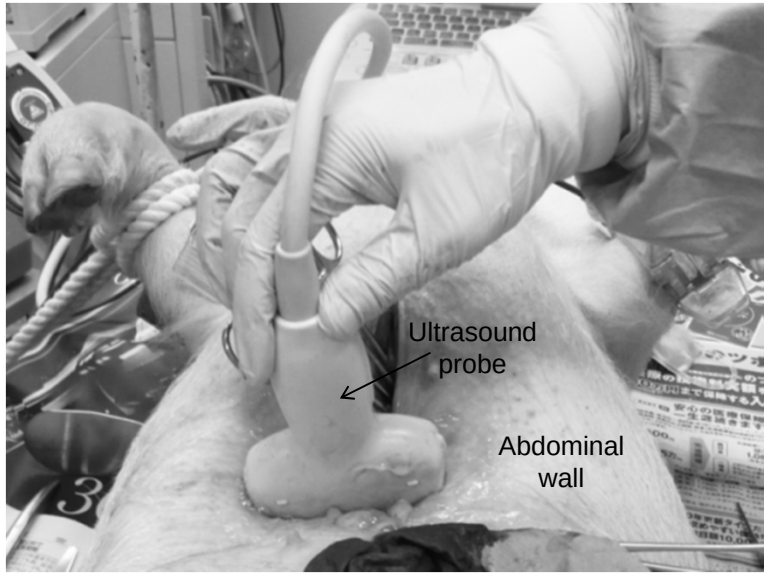
New Fig. 5

Injection volume : 0.3 mL

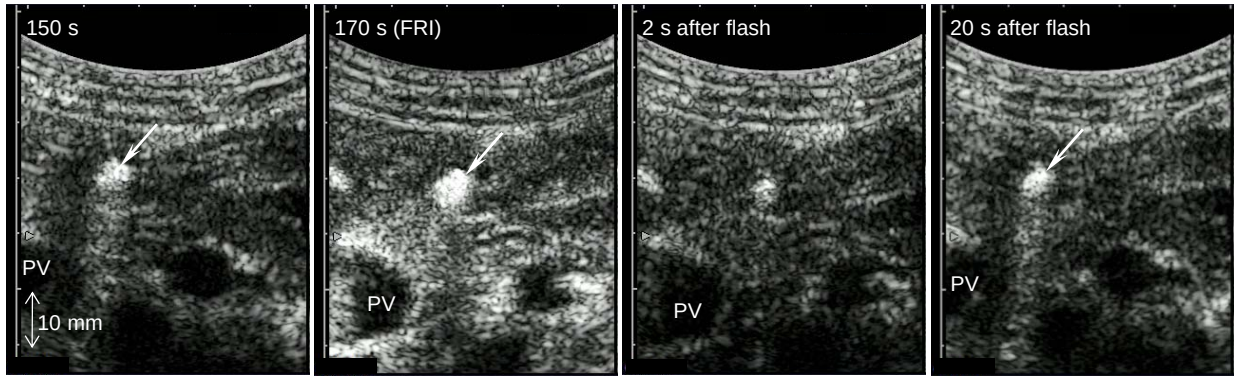
A intragastric contrast imaging



B transcutaneous abdominal ultrasonography



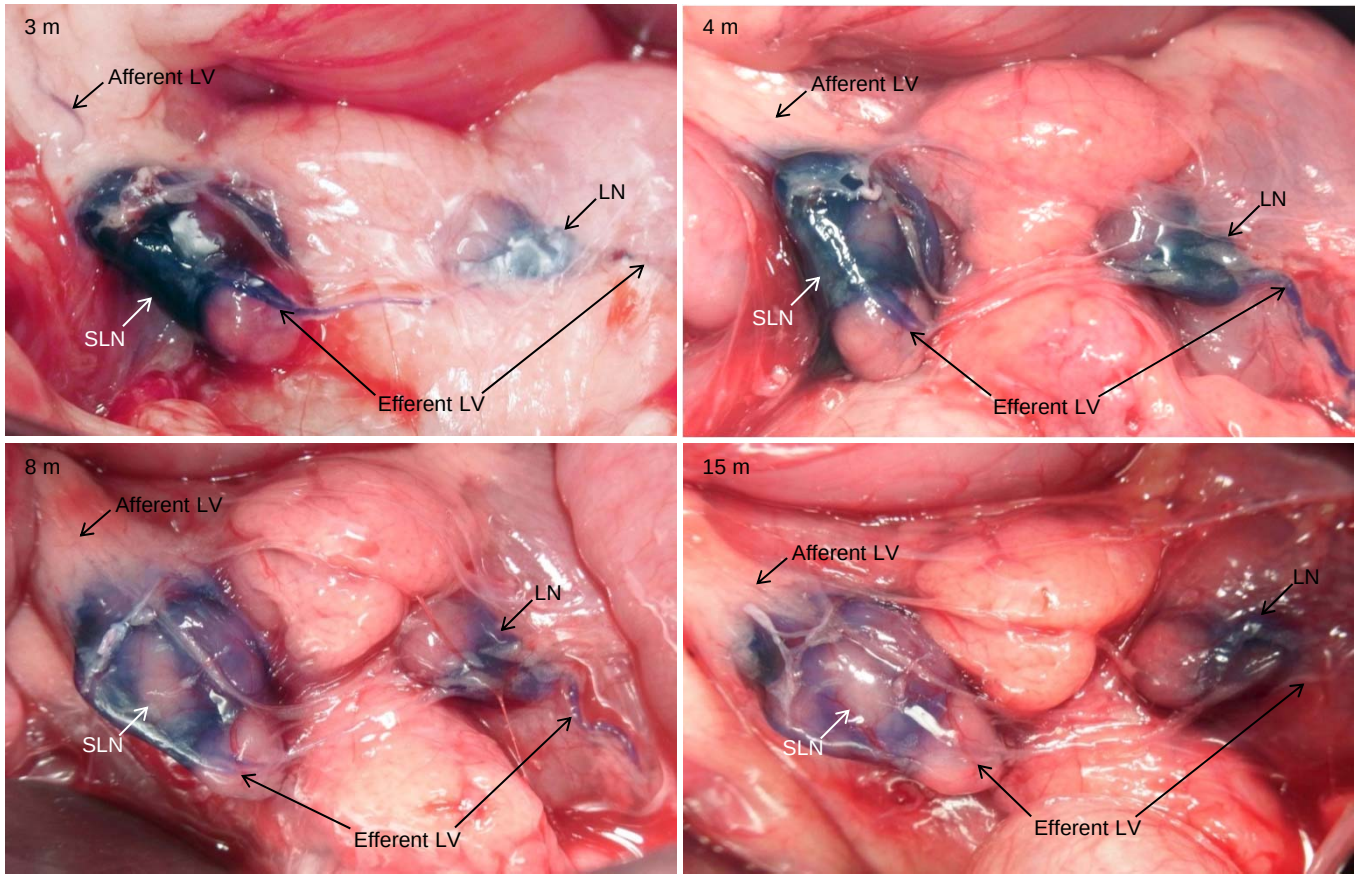
C transcutaneous contrast imaging



New Fig.6

Injection volume : 0.1 mL

A Evans blue dye-guided images (the greater curvature site injection)



Injection volume : 0.1 mL

B Evans blue dye-guided images (the greater and/or lesser curvature site injection)