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APPLICATION OF LOCOREGIONAL CHEMOTHERAPY USING LYMPH-DIRECTED SUBSTANCES IN A CANINE TRANSHIATAL ESOPHAGECTOMY MODEL

Y. Noguchi, M. Baba, M. Shimada, S. Nakano, S. Natsugoe, T. Aikou

The First Department of Surgery, Kagoshima University School of Medicine, Kagoshima City, Japan

ABSTRACT

We investigated whether lymph-directed substances injected into the mediastinal connective tissue of dogs reached the regional lymph nodes of the esophagus. In 46 dogs, 1.5 mL of cuttlefish particles or activated carbon particles containing 15 mg of bleomycin (CH-BLM) was injected at two sites: into the connective tissue between the trachea and the aorta via mediastinoscopy in 23 dogs (16 with cuttlefish particles and 7 with CH-BLM: mediastinal group), and into the crura of the diaphragm by means of laparotomy in 23 dogs (16 with cuttlefish particles and 7 with CH-BLM: crural group). Cuttlefish particles, distinguished by decolorization with melanin bleaching, showed selective affinity for lymphatics.

When cuttlefish particles were injected into mediastinal connective tissue, the rate of staining (# of black-stain positive nodes/# of examined nodes) was higher in the crural group than in the mediastinal group. In the crural group, bleomycin activity in lymph nodes was higher in the regions from the neck to the abdominal para-aortic region than at the injection site, excluding the peri-gastric region. If the topography of lymphatics and lymph flow kinetics in man are similar to that of the dog, then the crura of the diaphragm appears to be a potentially effective site for applying loco-regional chemotherapy for carcinoma of the esophagus in patients undergoing transhiatal esophagectomy.

Transhiatal esophagectomy combined with chemoradiotherapy for treatment of esophageal cancer is of current interest (1-4). In this operation, which is performed without thoracotomy, leaving lymph nodes behind in the mediastinum is problematic for cure. A greater possibility of tumor metastases may therefore exist in the mediastinum after transhiatal esophagectomy. At the same time, increased interest has recently been shown in targeting cancer chemotherapy using drug delivery systems. Hagiwara et al (5-8) have developed activated carbon particles (CH) as a drug carrier which has selective affinity for the lymphatics. However, mediastinal lymph drainage to the regional lymph nodes of the esophagus has not been fully elucidated, especially for patients with transhiatal esophagectomy. We, therefore, sought to clarify whether lymph-directed substances injected into the mediastinal connective tissue will reach the regional lymph nodes of the esophagus in a canine transhiatal esophagectomy model. If it does, what is an optimal site for loco-regional cancer chemotherapy targeting residual nodes after transhiatal esophagectomy? In the present study, we prepared cuttlefish particles, which have previously been reported as a lymph-directed substance (9) and CH adsorbed to bleomycin (CH-BLM).

MATERIALS AND METHODS

Preparation of Cuttlefish Particles and CH-BLM

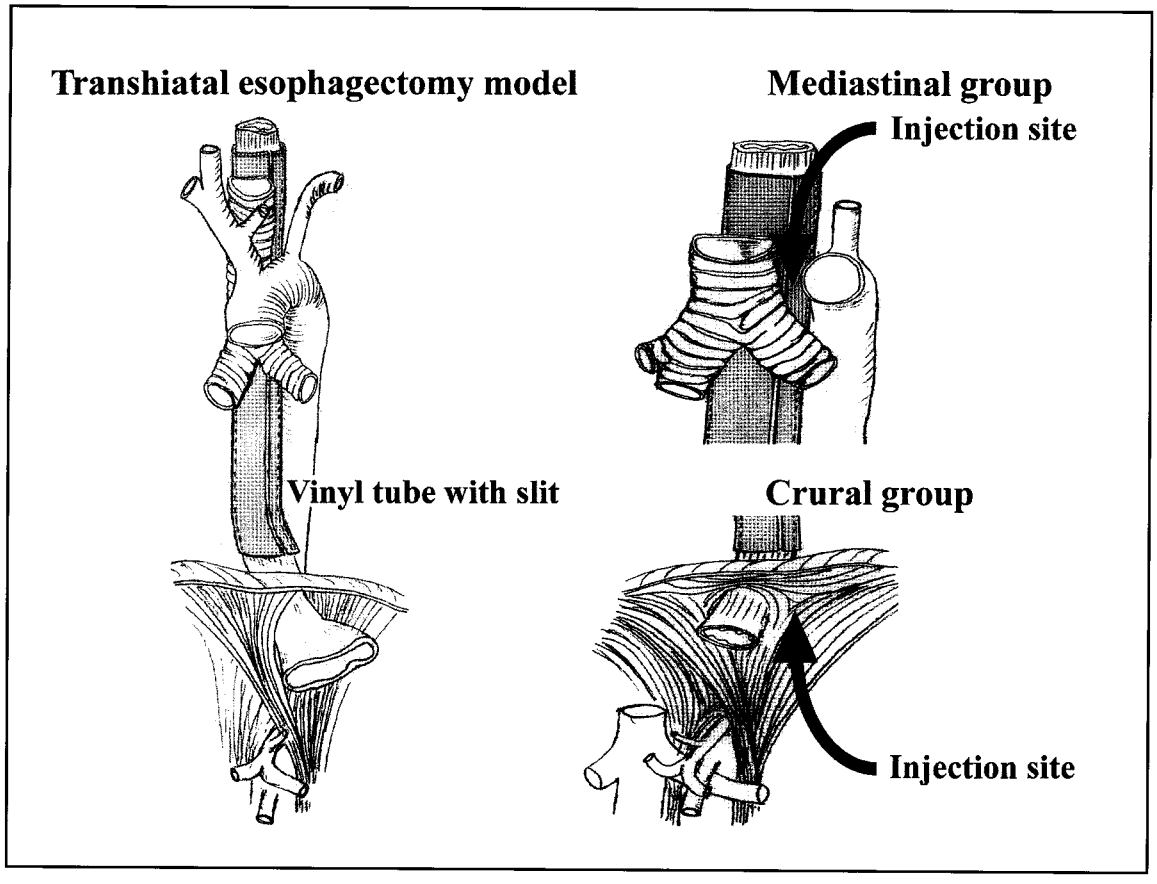

Fig. 1. Schematic diagram of transhiatal esophagectomy model and the sites where cuttlefish particles or CH-BLM were injected. Left: a vinyl tube with slit was inserted along the esophagus from the neck to the esophageal hiatus to "wrap" the thoracic esophagus. Right upper: arrow indicates the injection site (connective tissues between the trachea and the aorta) in the mediastinal group. Right lower: arrow indicates the injection site (the crura of the diaphragm) in the crural group.

An ink bag of the cuttlefish, weighing 10 g, was removed and incised. Cuttlefish ink was prepared by mixing it with 20 mL of saline and 5 mL of 0.1N NaOH and then filtering. The filtrate was centrifuged at 2500 rpm for 10 min. The sediment was resuspended in 20 mL of saline and adjusted to pH 4 by adding HCl. This mixture was again centrifuged at 2500 rpm for 10 min, the sediment resuspended in 20 mL of saline and surfactant, and then stirred by ultrasonic mixer. The cuttlefish particle preparation was heated at 56°C for 3 h and stored at 4°C.

CH-BLM was prepared by mixing 50 mg/mL of CH (#40 CH, Mitsubishi Chemical Industries Co., Ltd., Tokyo), 10 mg/mL of BLM (Nippon Kayaku Co., Ltd., Tokyo) and 20 mg/mL of polyvinylpyrrolidone K-30 (Nakarai Chemical Co., Ltd., Tokyo) in saline.

Experimental Protocol and Administration of Cuttlefish Particles and CH-BLM

Transhiatal esophagectomy was performed in 46 adult mongrel dogs weighing 12-15 kg. Under anesthesia with nembutal (0.5 mL/Kg), the esophagus was exposed at the neck, and a vinyl tube with a full-length slit was inserted from the neck to just above

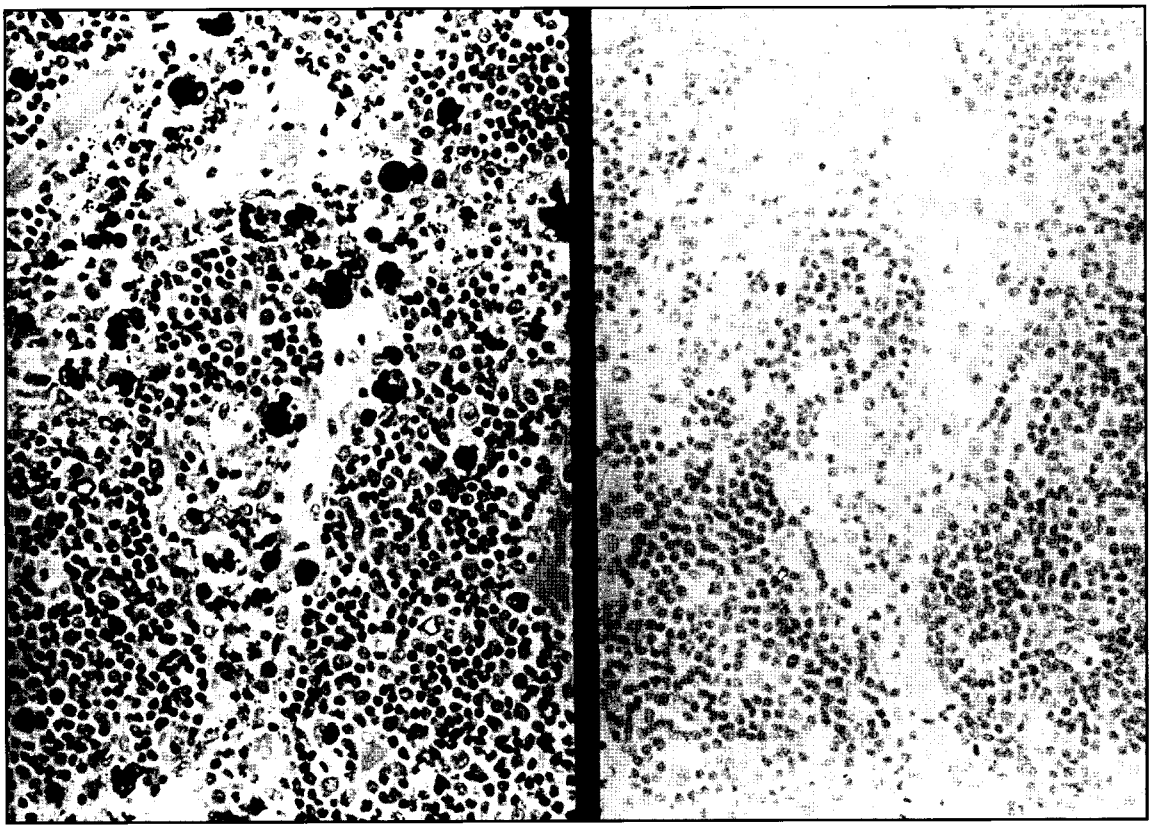

Fig. 2. Light microscopic view of a black-stain positive lymph node (x400). Left: hematoxylin and eosin stain. Right: eosin stain after melanin bleaching treatment; cuttlefish particles were decolorized thereby discriminating lymph nodes stained with anthracotic pigment from cuttlefish particles.

the diaphragm, wrapping the esophagus completely so that the esophagus was blocked from the surrounding connective tissue. One and a half mL of cuttlefish particles or CH-BLM was injected at two sites: the connective tissue between the trachea and the aorta via mediastinoscopy in 23 dogs (16 with cuttlefish particles and 7 with CH-BLM: mediastinal group), and the crura of the diaphragm by means of laparotomy in 23 dogs (16 with cuttlefish particles and 7 with CH-BLM: crural group) *(Fig. 1)*.

Measurement of Cuttlefish Particles and BLM Activity

Two days after injection, the dogs were killed. Lymph nodes were taken and embedded in paraffin for measurement of the incorporated cuttlefish particles. Two sections for each node were made; one was stained with hematoxylin and eosin and the other with eosin after melanin bleaching treatment. These were compared with each other under a light microscope, because unlike anthracotic pigment, cuttlefish particles are decolorized by the demelanization treatment. When more than one third of the section was stained black with cuttlefish particles, it was considered a black-stain positive mode *(Fig. 2)*.

As for BLM activity, lymph nodes, connective tissues near the lymph nodes and at the injection site, and 1 cm^3 each of liver and lung were taken 2 days after injection. They were weighed and trichloroacetic acid solution was added twice, then homogenized

TABLE 1
The % and Ratio (Parenthesis) of Black-Stained Positive Lymph Nodes (to all examined) According to the Injection Site of Cuttlefish Particles

	MEDIASTINUM	CRURAL
Neck	**54.5** (6/11)	**50.0** (6/12)
Upper mediastinum	**65.4** (51/78)*	**53.9** (41/76)
Right paratracheal LN	48.6 (18/37)	50.0 (18/36)
Left paratracheal LN	78.3 (18/23)	66.7 (16/24)
Pretracheal LN	83.3 (15/18)	43.8 (7/16)
Middle mediastinum	**42.6** (20/47)	**21.3** (10/47)
Right tracheobronchial LN	18.8 (3/16)	6.7 (1/15)
Left tracheobronchial LN	66.7 (10/15)	18.8 (3/16)
Infracarinal LN	43.8 (7/16)	37.5 (6/16)
Peri-gastric	**21.4** (3/14)	**26.7** (4/15)
Abdominal para-aortic	**28.8** (17/59)**	**79.5** (58/73)
Right side of aorta	24.1 (7/29)	82.9 (29/35)
Left side of aorta	33.3 (10/30)	76.3 (29/38)
Total	**46.4** (97/209)	**53.4** (119/223)

*p<.05; **p<.01; LN: lymph nodes

by a mixer. The supernatant was used as a sample for bioassay. BLM was calculated as μg BLM/g of tissue. Blood samples were taken at 30 min, 1 h, and 3 h after injection. BLM was measured by thin agar plate bioassay using bacillus subtilis PCL-219. The lower limit of BLM activity detected by this assay was 0.04 μg/g.

Division of Lymph Nodes and Connective Tissues Examined

The regions for lymph nodes and connective tissues were divided into five sections: neck, upper, and middle mediastinum, peri-gastric, and abdominal para-aorta. *Neck* is the region along the jugular vein and at the supraclavicular fossa of both sides. *Upper mediastinum* is the region around the trachea including the right and left paratracheal and pretracheal nodes. *Middle mediastinum* is the region around the tracheal bifurcation including the right and left tracheobronchial, and subcarinal nodes. The *peri-gastric region* includes the lesser curvature of the stomach and left gastric artery. The *abdominal para-aortic region* involves the left renal vein subdividing into the right and left side of the

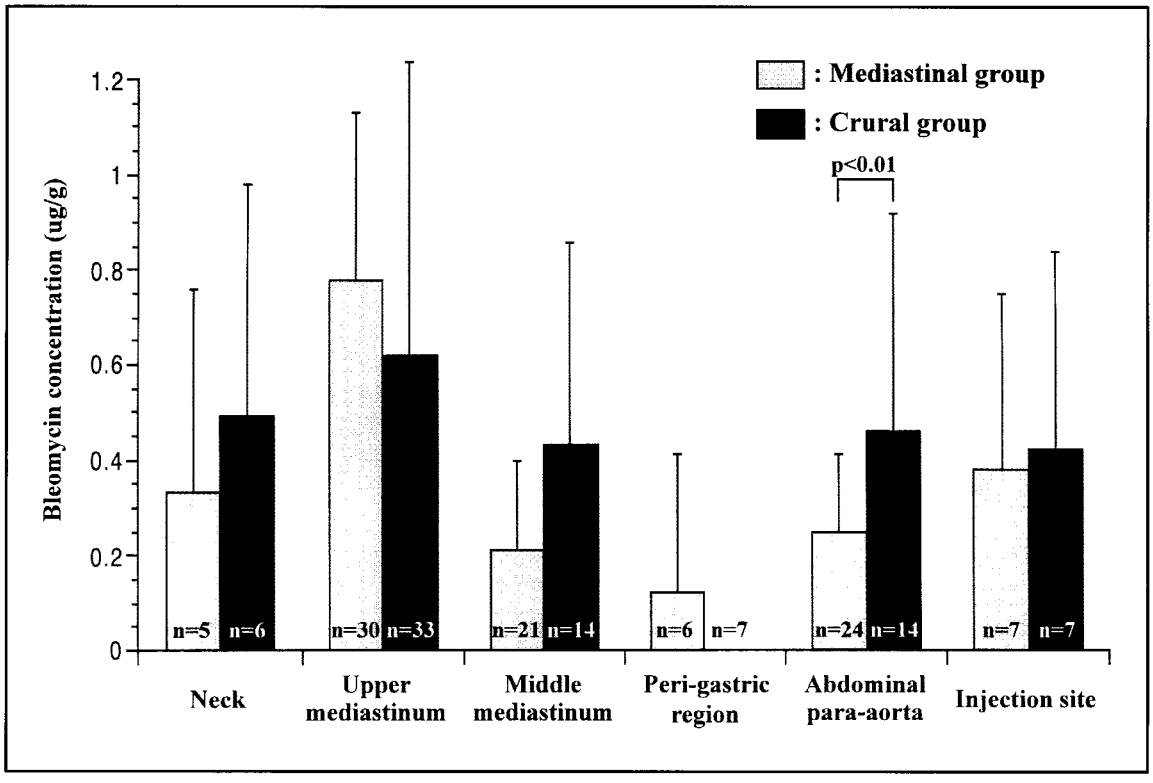

Fig. 3. Bleomycin (BLM) distribution in lymph nodes according to injection site. Numbers in the bar chart indicate the number of lymph nodes examined. BLM concentrations in the crural group were higher in all regions except the peri-gastric site where BLM activity in seven nodes were below detection.

aorta. The lower mediastinum along the lower third of the esophagus was excluded from this study, since lymph nodes and connective tissues obtained at this site were not adequate for examination.

Statistics

Data are presented as the median value ± standard deviation (SD). The chi-square test with Yates correction was used for categorical variables and the Mann-Whitney U test for nonparametric data. P values <.05 were considered significant.

RESULTS

Black-Stain Positive Lymph Nodes

A total of 432 nodes obtained from 32 dogs were examined. The rate of black staining (# of black-stain positive nodes/# of examined nodes) was 53.4% (119/223) for the crural group and 46.4% (97/209) for the mediastinal group *(Table 1)*. The rate of black staining was significantly high in the upper mediastinum for the mediastinal group and in the abdominal para-aorta for the crural group. In the crural group, more than half of examined nodes were black-stain positive in the neck, upper mediastinum, and abdominal para-aortic regions.

BLM Activity in Lymph Nodes

A total of 174 nodes obtained from 14 dogs were examined. Comparing BLM

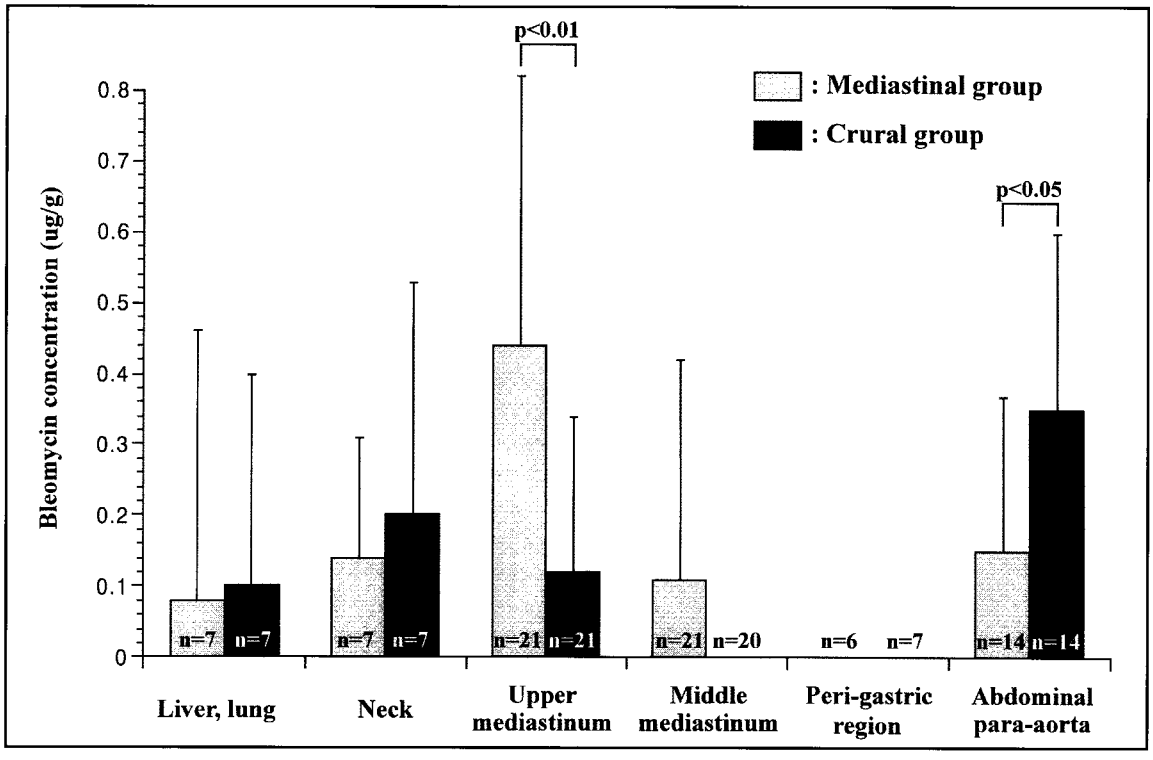

Fig. 4. Bleomycin (BLM) distribution in connective tissues according to injection site. Numbers in the bar chart indicate the number of connective tissues examined. BLM activity in connective tissues at the peri-gastric region were below the detection limit.

activity in the mediastinal and crural groups, it was significantly higher in the abdominal para-aorta in the crural group *(Fig. 3)*. In the crural group, BLM activity was higher in the regions from the neck to the abdominal para-aorta than at the injection site (BLM level of 0.42 ± 0.29), excluding the peri-gastric area. In the mediastinal group, BLM activity in all regions except the upper mediastinum was less than 0.33 ± 0.43.

Bleomycin Activity in Connective Tissues and Blood Samples

BLM activity was highest in the upper mediastinum with a level of 0.44 ± 0.38 for the mediastinal group and in the abdominal para-aorta with a level of 0.35 ± 0.25 for the crural group *(Fig. 4)*. BLM activity in the regions not in the vicinity of the injection site was less than 0.20 ± 0.33. BLM activity in the liver or lung was less than 0.10 ± 0.31 for both groups, and activity in the blood was 0.12 ± 0.31 at 30 min, 0.06 ± 0.12 at 1 h, and below the detection limit at 3 h after injection.

DISCUSSION

Lymph drainage from the esophagus to the regional lymph nodes has been well described (10-12). Injecting sky-blue dye directly into the esophageal wall, Haagensen found that the main lymph drainage from the upper two thirds of the esophagus is toward the lymph nodes in the neck and upper mediastinum, and from the lower third is toward the lymph nodes around the celiac axis and abdominal aorta (10). However,

lymph drainage from the mediastinum to the regional lymph nodes of the esophagus has not been detailed. Elucidation of such lymphatic drainage may be critical for applying loco-regional cancer chemotherapy to patients who are scheduled to undergo transhiatal esophagectomy for carcinoma of the esophagus because lymph nodes in the mediastinum are not well dissected and removed by this procedure; moreover, esophageal cancer frequently is associated with lymph node involvement in the mediastinum (13-16). We have, therefore, prepared a canine transhiatal esophagectomy model and examined injection sites for loco-regional cancer chemotherapy which can be reached without thoracotomy.

We have repeatedly experienced difficulties in delineating mediastinal lymph drainage after injecting a vital staining dye, such as carbon particles, because many lymph nodes have coexistent anthracosis. Thus, we took advantage of the properties of cuttlefish particles, which are decolorized by demelanization treatment; these particles can be discriminated from anthracosis and have selective affinity for lymphatics (5-6). In this study, the rate of black-stain positive nodes was high after injecting cuttlefish particles at the diaphragmatic crura, compared with the connective tissue between the trachea and aorta. In addition, when injecting cuttlefish particles at the diaphragmatic crura, more than half of the lymph nodes examined were black-stain positive in the neck, upper mediastinum, and abdominal para-aortic regions. We suggest that lymph-directed substances such as cuttlefish or carbon particles are taken up by lymphatics in the connective tissue which is densely connected to the extramural lymphatics in the esophagus; these are then transported to the regional lymph nodes, particularly after injection at the diaphragmatic crura, first toward the abdominal para-aortic region, second toward the upper mediastinum (chiefly with the assistance of negative pressure in the thoracic cavity), and finally toward the neck, in part via the thoracic duct.

Activated carbon particles adsorbing bleomycin, which release a fixed concentration of anticancer drug over a long period, have also been prepared as a lymph-directed substance, since cuttlefish particles are glycoprotein which may cause anaphylaxis after repeated injection. We have previously reported that after injection of bleomycin adsorbed to activated carbon or silica particles into the esophageal wall by endoscopy, bleomycin activity was found in both regional lymph nodes and connective tissues, not only in the mediastinal region but also in the cervical and abdominal regions in patients with esophageal cancer (17-19). Similarly, in this study, injecting bleomycin adsorbed to activated carbon particles into the crura of the diaphragm resulted in bleomycin activity in the lymph nodes which was higher in the regions from the neck to the abdominal para-aorta than at the injection site, with the exception of the perigastric region which can be easily cleared by abdominal surgery.

Unfortunately, in this study, the bleomycin activity in the connective tissue was very low in regions other than the vicinity of the injection site, although bleomycin did accumulate in the regional lymph nodes. Because more than 40% of esophageal cancer patients who undergo three-field lymphadenectomy and esophagectomy have extension of carcinoma into perinodal connective tissues (20,21), the crura of the diaphragm appear to be the most effective site for applying loco-regional chemotherapy for carcinoma of the esophagus, especially for patients undergoing transhiatal esophagectomy.

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Yasuhiko Noguchi, M.D.
The First Department of Surgery
Kagoshima University School of Medicine
8-31-1 Sakuragaoka
Kagoshima City, Japan 890
Telephone: 099 275 5359
Fax: 099 265 7426