Experimental Study on Fluorescent Microspheres as a Tracer for Sentinel Node Detection

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Abstract. Background: Common tracers for sentinel node navigation surgery are blue dye and technetium-99m-labelled colloids. However, in most esophageal or lung cancer patients, it is impossible to detect the sentinel node among mediastinal nodes by blue dye because of the anthracotic pigmentation of mediastinal nodes. The use of technetium-99m-labelled colloids requires a special facility, while a large hot-spot at the injection site prevents detection of the sentinel node around the primary lesion. To overcome these problems, we investigated the use of fluorescent microspheres (0.1-20 μm in diameter) as tracers in animals and detected fluorescence-positive nodes by a simple ultraviolet light irradiation method.

Materials and Methods: Two milliliters of fluorescent microspheres 0.1-20 μm in diameter diluted to 2.5% weight per volume was injected via the tail vein of 30 rats; systemic side-effects were examined. One milliliter of 1.0 μm-diameter microspheres dilution was injected on the backs of 30 rats; local side-effects were examined. A microsphere dilution (0.2 ml, 1.0 μm-diameter microspheres) was injected into the footpad of 18 rats; the lymphatic pathway and drainage were examined. Five milliliters of 1.0 μm-diameter fluorescent microspheres was injected endoscopically into the submucosa of the esophagus, stomach and small and large bowels of 6 domestic pigs, and 5 ml was injected into the subadventitia of the esophagus or subserosa of the stomach, and small and large bowels. Fluorescence-positive lymph ducts or nodes were carefully observed under ultraviolet light irradiation. Results: No systemic side-effects were observed in rats. Only mild edema and a mild inflammatory reaction were observed on the backs of rats. Fluorescent microspheres 0.1, 0.5 and 1.0 μm in diameter were detected in lymph ducts or nodes of pigs within 1 hour after injection. Conclusion: Sentinel node navigation surgery with the use of fluorescent microspheres might be feasible and advantageous for patients with esophageal or lung cancer, especially in the mediastinum.

The sentinel node is the first draining lymph node on the direct lymphatic drainage pathway from the primary tumor site (1). Sentinel node biopsy is becoming a standard diagnostic procedure for evaluation of regional lymph node status in cases of malignant melanoma and breast cancer (2). The common tracers used to detect sentinel nodes are vital blue dyes (3, 4) and technetium-99m-labelled colloids (5), and the detection and accuracy rates are both over 95% (6, 7). However, the feasibility of sentinel node mapping in patients with esophageal cancer is still unclear. Recent studies of lymphatic mapping by means of technetium-99m-labelled tin colloids have been reported that support the validity of the sentinel node concept in esophageal cancer (8, 9), but it is impossible to detect the sentinel node by blue dye in esophageal and lung cancer patients because of the dark color of the regional lymph nodes produced by anthracosis in the mediastinum. Moreover, the particle size of the blue dye is considered too small to reflect the flow or dynamics of cancer cells. Technetium-99m-labelled colloids (tin, rhenium, phytate, etc.) are of comparatively large particle size, from 0.3 μm to more than 1 μm (10), but a special facility and technique are required; radioisotope colloids are so strictly regulated in Japan that their use is permitted only in the hospital’s restricted safety room, not in the operating room. To overcome these problems, we used fluorescent microsphere beads of 0.1 to 20 μm in diameter under ultraviolet light. The aim of this study was to examine, in animals, the safety and feasibility of using fluorescent microspheres as an alternative tracer in sentinel node mapping.

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Materials and Methods

After approval of the treatment protocol by the institutional animal review board of Hiroshima University, Japan, 2-week-old female rats were obtained (CLEA Japan, Inc., Tokyo, Japan) 3 days before the planned procedure. The rats were housed in groups of 3 in a temperature-controlled (21°C) environment under a 12-hour light/dark cycle with free access to food and water. Each rat was anesthetized by inhalation of diethyl-ether in an enclosed glass bottle, and additional intermittent inhalation was performed as needed when the animals were injected with microsphere dilutions or killed for dissection.

For histological examination, the dissected organs were fixed in formalin, embedded in paraffin, cut into 4-μm sections, and mounted onto glass slides for microscopic examination under hematoxylin and eosin staining.

Evaluation of side-effects of fluorescent microspheres in rats. Fluorescent microspheres diluted to 2.5% weight per volume (FluorescentTM Plain Yellow Green Microspheres, 0.1 μm-20 μm in diameter, Polysciences, Inc., Warrington, PA, USA) were injected through the tail vein of 30 rats for 3 week’s observation of systemic side-effects. The rats were separated into 5 groups of 6 each, and the rats in each group were injected with a 2-ml dilution of microsphere (2.5% w/v) 0.1, 0.5, 1.0, 10.0 or 20.0 μm in diameter. The rats were carefully observed for unusual behavior and killed 3 weeks after the injection for evaluation of changes in the liver, kidney and lung. The dissected organs were assessed histologically. For evaluation of effects at the injection site, 30 rats, separated into 5 groups of 6 each, were injected in the subdermal layer of their back skin with 1 ml of 1.0-μm-diameter microsphere dilution, and they were killed at 1 hour, 6 hours, 24 hours, 3 days, or 7 days after injection. The back skin of each rat was obtained by dissection and examined histologically.

Observation of microsphere drainage in lymphatics in rats. The left foot pad of 18 rats was injected with 0.2 ml of the microsphere (1.0 μm in diameter) dilution (2.5%, w/v) for observation of the lymphatic pathway to regional draining lymph nodes under ultraviolet light (365 nm) irradiation with a UV lamp (UVLMS, UVP, Upland, CA, USA). Inguinal lymph nodes with bright fluorescence were obtained by dissection and examined histologically.

Detection of the first draining lymph node of the alimentary tract in pigs. To investigate the feasibility of first draining lymph node detection by fluorescent microspheres, 6 domestic pigs were anesthetized via intravenous injection of 5 mg/kg thiopental sodium and 0.1 mg/kg vecuronium bromide. These two agents were supplemented with appropriate concentrations of sevoflurane and nitrogen oxide administered during one-lung ventilation. Under careful observation and monitoring of anesthesia by a veterinarian, a 5-ml dilution of microspheres 0.1, 0.5, 1.0, 10.0, or 20.0 μm in diameter was injected endoscopically into the submucosal layer of the esophagus and stomach. Thoracotomy and laparotomy were then performed, and microspheres were injected via the adventitia for the esophagus or via the serosa for the stomach and small and large bowels for investigation of the first draining lymph duct and node by UV light irradiation. In cases without obvious bright lymph duct or nodes, delamination of the membranes (pleura, omentum, mesentery) was performed to examine faint luminescence. The dissected nodes were cut into slices for macroscopic observation of obvious luminescence.

Results

No abnormal behavior or activity was observed during the 3-week observation period in any of the 30 rats with microspheres injected via the tail vein. Microscopic examination of the liver, kidney and lung showed no particular changes such as fibrosis, emboli, necrosis, or edema.

There was no macroscopic evidence of local edema or other changes around the injection site on the back skin in any of the 30 rats from 1 hour to 7 days after injection. Furthermore, microscopic examination revealed no particular pathological changes except very mild edema and some inflammatory reaction around the injection site. These changes were observed to almost the same degree in all rats, except at 1 hour after injection, there was little or no evidence of inflammation or edema in 6 rats.

With careful dissection from the footpad to the area of inguinal nodes in 18 rats, one bright inguinal lymph node was shown under ultraviolet irradiation in 2, 4 and 4 rats after 6 hours, 24 hours and 3 days, respectively (Figure 1A). However, linear fluorescence through the draining lymph duct in the lower limb, connecting the fluorescence-positive node and the injection site, was not evident.

The first draining lymph node and/or lymph duct in the alimentary tract of pigs was detected with microspheres 0.1, 0.5 and 1.0 μm in diameter. Despite careful observation for 6 hours, no bright lymph duct or node was seen with 10.0- or 20.0-μm microspheres in any alimentary tract. In relation to the esophagus, the first draining node was detected in 3 pigs without delamination of the mediastinal portion of the parietal pleura. Two of these 3 pigs showed only a bright duct directed to a particular node; no obvious bright node
was observed (Table I). In 2 pigs, careful delamination of the pleura revealed the first draining lymph node in front of the bright duct. After dissection of the lymph nodes, weak fluorescence was observed on the sliced surface of the nodes (Figure 1B). The fluorescence was focal and the brightness continued for the 6 hours in all cases. In relation to the stomach, a bright peri-gastric lymph node was detected in only 1 pig (Table I). In relation to the small intestine, a distinct bright lymph duct, dynamic flow and the draining lymph node were observed in 5 out of 6 pigs (Table I, Figure 1C). The fluorescent microspheres of 0.1, 0.5 and 1.0 μm showed obvious dynamic flow about 20 minutes after injection, and the brightness did not decrease. For the large bowel, it was difficult to follow the natural lymphatic flow in the mesocolon due to its anatomical spiral shape (Table I). Although it was difficult to detect the first draining node without a directional pilot, one bright lymph node located near the cecum was detected by careful delamination of the mesocolon membrane only in 1 pig. Thus, for all the organs examined in the 6 pigs, only one first draining lymph node and/or duct was detected for each injection site.

Discussion

The sentinel node concept is accepted in relation to surgeries for malignant melanoma and breast cancer (1, 4, 6, 7). However, in relation to malignant tumors of the alimentary tract, the concept is controversial. If the sentinel node concept is established in such cases, sentinel node navigation surgery will lead to precise staging, regional lymph node control and omission or reduction of extended lymph node dissection. Many Japanese surgeons have performed esophagectomy with extended three-field lymph node dissection under thoracotomy, laparotomy and neck surgery as standard surgical therapy for esophageal cancer with invasion below the submucosal layer (11). However, this stressful operation is associated with an increase in post-operative complications and, therefore, should be avoided if possible (11, 12). Detecting the first draining lymph node by sentinel node navigation surgery may be advantageous, the procedure being convenient, economical and reliable.

Kitagawa et al. recently applied radio-guided sentinel node detection to esophageal cancer using technetium-99m-radiolabelled tin colloid solution, but this requires a special facility, special equipment and a complicated procedure. Handling of radioactive agents is so strictly regulated in Japan that we cannot inject them in the operating room; they are restricted to a safety room. Another problem is that the esophageal injection site presents a shine-through phenomenon; i.e., the large radioactive hot-spot at the injection site disturbs detection of the nearby radioactive lymph node by hand-held gamma probe and also in lymphoscintigraphy.
Whereas vital blue dye such as Isosulfan blue is convenient and economical for sentinel node detection (3, 4, 6), particles are less than 10 nm in diameter. Thus, the problem remains of how truly the vital blue dye mimics and reflects the dynamics of metastatic carcinoma cells in lymphatic systems, the cells being some 10 μm or more in diameter.

We used fluorescent microspheres to overcome the problem of discriminating the tracers from dark mediastinal lymph nodes, of using the tracers without legal restrictions and of prohibitive costs. Fluorescent microspheres present yellow-green luminescence under ultraviolet light, so detection of the first draining lymph node is not disturbed by the dark color of anthracotic mediastinal nodes. Other advantages of the present method are that we are able to use the microsphere tracer anywhere without strict regulation, the costs of the microsphere tracer and ultraviolet light lamp are acceptable, and detection of the first draining node is not confounded by plural bright regional lymph nodes.

Another important factor is that no systemic and few local side-effects were observed in our rats, supporting the safety of the microsphere used as a drug. The lack of side-effects is because the microspheres are made up of polystyrene, a material used for surgical sutures.

In pigs, the first draining lymph node was detected at 20 minutes after injection only with microspheres less than 1 μm in diameter. Microspheres with diameters of 10 μm or more were considered too large for lymphatic mapping, because the gap between adjacent endothelial cells on the microcapillary wall is a few micrometers (13). In our study, the detection rates for the fluorescent node related to the stomach and large bowel were low (1/6). By examination of the stomach, it was clear that almost all the injected microspheres were trapped in the vast submucosal space; tissue pressure was not high enough. Increasing the injection volume of the microsphere dilution or injecting adequate physiological saline after injection of the tracer may solve this problem. We used microspheres of 0.1-20.0 μm in diameter, much larger than those of blue dyes, so they might need much more than 6 hours to flow in the lymphatic system. In other words, each organ may have its own appropriate observation time for the present method. A much longer observation period, i.e., much earlier injection, might solve the problem of the low detection rate.

The low detection rate in the large bowel was also because the long large bowel of the pig is folded into a spiral, and it was impossible to follow the direction of the draining lymph duct in the mesocolon.

The staining with fluorescent microspheres was focal and localized under the capsule of the lymph node; this differs from the staining pattern of blue dyes. The luminescence did not decrease over the 6-hour observation period. The blue dyes are so small in diameter that they pass out of the lymph nodes through efferent ducts within approximately 10 minutes. Our results suggest that the microsphere dynamics might be similar to that of cancer cells because of the large diameter of the microspheres compared to that of blue dyes.

Concerning the invasion of tumor cells into lymph vessels, Deutsch et al. reported that melanoma cells penetrated the subendothelial space as single cells, fused with the endothelial cytoplasmic membrane, and subsequently destroyed the endothelial wall (14). It is impossible to mimic completely the action of tumor cells with any tracers used in lymphatic mapping, because tumor cells have active transport or metamorphic potential for entering into the lymphatic system. Despite the fact that no tracer, including fluorescent microspheres, can completely mimic the physiological dynamics of each metastasizing cancer cell in the lymphatic system, a tracer with a high rate of accuracy and low false-negative rate would be clinically useful for sentinel node detection.

The problem identified in the pigs is that the fat tissue, artery walls and nerves have their own "auto-fluorescence," making it difficult to distinguish the auto-fluorescence from the luminescence of the fluorescent microspheres. In a future study, a special sunglasses-like filter should be used to block the natural auto-fluorescence. Another problem is that if the sentinel node exists outside the mediastinum, it is impossible to detect by our method. In esophageal cancer patients, it is possible for the sentinel node to lie in the supraclavicular or abdominal area because the esophageal lymphatic drainage system is very complicated (15). Radio-guided sentinel node detection with lymphoscintigraphy can depict the hot node on the monitor if the node is in the supraclavicular or abdominal area. Another advantage of the radio-guided method is that the hot node can be imaged, thus localized, by surgeons pre-operatively. This is not true with the dye mapping or present method.

In conclusion, a fluorescent microspheres-traced sentinel node detection system might prove to be a clinically useful and simple method, especially for malignant esophageal tumor-related mediastinal nodes that show anthracotic pigmentation. However, further investigations are needed into the appropriate injection and observation times, into how to avoid confusion with auto-fluorescence, and into more sensitive ultra-violet spectrophotometric detection techniques.

References

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