Therapeutic effect of cisplatin given with a lymphatic drug delivery system on false-negative metastatic lymph nodes

Asuka Tada,1,2,3 Sachiko Horie,1,2 Shiro Mori1,2,4 and Tetsuya Kodama1,2,3

1Laboratory of Biomedical Engineering for Cancer; 2Biomedical Engineering Cancer Research Center, Graduate School of Biomedical Engineering; 3Department of Electronic Engineering, Graduate School of Engineering, Tohoku University; 4Department of Oral and Maxillofacial Surgery, Tohoku University Hospital, Sendai, Japan

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Correspondence
Tetsuya Kodama, Laboratory of Biomedical Engineering for Cancer, Graduate School of Biomedical Engineering, Tohoku University, 4-1 Seiryo, Aoba, Sendai, Miyagi 980-8575, Japan.
Tel/Fax: +81-22-717-7583; E-mail: kodama@tohoku.ac.jp

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Lymph node metastasis occurs before distant metastasis in many cancers.1–4 Although LN dissection is effective for LN identified by imaging techniques as containing metastases,5 resection is invasive and often challenging.6 Systemic chemotherapy is also used but has limitations such as low selectivity, low drug retention in metastatic LN and serious adverse effects.7,8 As metastatic LN can be a source of systemic metastasis,9–11 effective therapies are needed for early-stage LN metastasis. We have established a colony of MXH10/Mo-lpr/lpr (MXH10/Mo/lpr) inbred mice, which develop systemic swelling of LN that reach up to 10 mm in diameter (similar in size to human LN).12 Furthermore, we have developed diagnostic13,14 and therapeutic9,15–18 methods for the early stage of LN metastasis. We define a LN during the early stage of metastasis as a false-negative LN, because its size at that stage of tumor development is not significantly different from that of a LN not containing tumor cells (i.e. a normal LN).16,19–21 Anatomical studies in MXH10/Mo/lpr mice have revealed that the PALN and AALN are located in the axillary area and that the PALN is downstream of both the AALN and SiLN.10 We have provided proof-of-concept for a LDDS that could be used to treat/prevent LN micrometastasis.9,18 The principle is that a chemotherapeutic drug is injected before surgical resection into an upstream LN (within the dissection area) so that it is delivered at high concentrations to a downstream metastatic LN (outside the dissection area). In the present study, we evaluated the anticancer effect of CDDP delivered to a false-negative LN using a LDDS. In initial experiments, the accumulation of ICG in the PALN (the “target” LN) was compared between injection into the AALN (an “upstream” LN) and systemic administration through the tail vein. Next, tumor cells were inoculated into the SiLN to induce metastasis to the PALN and CDDP was injected into the AALN to investigate its anticancer action in the metastasized PALN.

Materials and Methods

All in vivo studies were approved by the Institutional Animal Care and Use Committee of Tohoku University.

Mice.

MXH10/Mo/lpr mice12 were bred at the Institute for Animal Experimentation, Graduate School of Medicine, Tohoku University, Japan. Thirty-five male and female mice were used (weight, 30–42 g; age, 16–18 weeks).

Cell culture.

C3H/He mouse mammary carcinoma (FM3A-Luc) cells expressing the luciferase gene12 were maintained in RPMI-1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% (v/v) FBS (Hyclone, GE

Systemic administration of drugs into the blood circulation is standard treatment for prevention of metastasis. However, systemic delivery cannot maintain sufficiently high concentrations of anticancer drugs in lymph nodes (LN). Here, we show that giving cisplatin (CDDP) using a lymphatic drug delivery system (LDDS) has the potential to treat false-negative metastatic LN. We found that in MXH10/Mo-lpr/lpr mice, which develop systemic swelling of LN up to 10 mm in diameter, accumulation of indocyanine green (ICG), which has a similar molecular weight to CDDP, in a target LN was greater for lymphatic delivery of ICG than for systemic (i.v.) administration. Furthermore, CDDP administration with a LDDS inhibited tumor growth in false-negative metastatic LN and produced fewer adverse effects than systemically given CDDP. We anticipate that drug delivery using a LDDS will, in time, replace systemic chemotherapy for the treatment of false-negative metastatic LN.
Biodistribution of ICG after administration systemically or with a LDDS. One hundred and twenty microlitres of 100 μg/mL ICG (molecular weight, 775; excitation wavelength, 774 nm; emission wavelength, 805 nm; Daichi Sankyo, Osaka, Japan) was injected into the tail vein (IV group; n = 4) or AALN (LDDS group; n = 7) of mice anesthetized using 2% isoflurane (Abbott Japan, Chiba, Japan) in O2. Injection into the AALN was carried out using a 27-G butterfly needle under the guidance of a HFUS imaging system (VEVO770; VisualSonics, Toronto, Canada) using a 25-MHz transducer (RMV-710B; axial resolution, 70 μm; focal length, 15 mm; VisualSonics). Before and 0.5, 2, 6 and 24 h after ICG injection, fluorescence intensity was measured using a biofluorescence imaging system (in vivo imaging system; IVIS; Xenogen, Waltham, MA, USA). At 24 h after ICG injection, 1 mL blood was collected from the tail vein of mice anesthetized with 10–20 μL of 10% EDTA for 30 min at room temperature and centrifuged (4°C, × 13 000 g) for 10 s to obtain plasma. Fluorescence intensities of the plasma samples were measured using IVIS. After blood sample collection, heart, lungs, liver, bilateral kidneys, spleen, PALN and AALN were harvested and weighed. Fluorescence intensity of each organ was measured using IVIS and quantified according to organ weight.

Induction of PALN metastasis by injection of tumor cells into the SI LN. Each mouse was anesthetized with 2% isoflurane in O2, and the unilateral SI LN was injected (under HFUS guidance) with FM3A-Luc cells (3.3 × 105 cells/mL) suspended in 10 μL PBS plus 20 μL of 400 mg/mL Matrigel (Collaborative Biomedical Products, Bedford, MA, USA). PALN tumor growth was assessed every 3 days post-inoculation through measurements of luciferase activity: 10 min after i.p. injection of luciferin (150 mg/kg; Promega, Madison, WI, USA) under anesthesia (2% isoflurane in O2), the fluorescence signal from the PALN was measured over a 1-min period using IVIS. PALN metastasis was considered to have occurred when the luciferase activity exceeded the background level in controls (1 × 106 photons/s). The day on which PALN metastasis was confirmed was defined as day −1T.

Treatment of metastatic PALN with CDDP. Mice with confirmed PALN metastasis were randomly divided into LDDS and IV groups. The LDDS group was further divided into three subgroups (n = 5): 0 μg/g CDDP (saline without CDDP), 0.5 μg/g CDDP and 5 μg/g CDDP. For the LDDS subgroups, CDDP (molecular weight, 300; Wako Pure Chemical Industries) in 120 μL saline was injected into the AALN using a 27-G butterfly needle under HFUS guidance. For the IV group, a bolus injection of CDDP (5 μg/g; n = 5) was given into the tail vein. Luciferase activity was measured with IVIS at days 3T, 6T and 9T after administration. SI LN, PALN, AALN and lungs were harvested at day 9T, and luciferase activities of the samples (in six-well plates) were measured by IVIS (0.3 mg/mL luciferin).

Measurement of PALN and AALN volumes. Proper axillary lymph node and AALN volumes were measured using a HFUS imaging system (VEVO770) with a 25-MHz transducer (RMV-710B) on the inoculation day and days 0T and 9T.

Blood biochemistry to assess renal/hepatic toxicity. Sixteen mice were assigned to the IV group (5 μg/g CDDP, n = 3) and three LDDS groups: 0 μg/g CDDP (n = 4), 0.5 μg/g CDDP (n = 4) and 5 μg/g CDDP (n = 5), respectively. Blood was drawn from the left ventricle at day 9T (under general anesthesia) and plasma obtained by centrifugation at 13 000 g for 10 s. Hepatic and renal functions were evaluated from plasma measurements of T-Bil, ALT, AST, Cre and BUN (Oriental Yeast, Tokyo, Japan).

Histological analysis. Tissues (PALN, AALN and kidney) were excised at day 9T, fixed overnight in 10% formalin at 4°C, dehydrated, embedded in paraffin, serially sectioned (3–4 μm), and either stained with H&E or immunostained for LYVE-1-positive and CD31-positive cells (Discovery XT Automated Staining Processor; Ventana Medical Systems, Tucson, AZ, USA).

Statistical analysis. Data are presented as mean ± standard deviation (SD). Differences between groups were determined by non-parametric tests (Mann–Whitney U-test, Wilcoxon signed rank test or Kruskal–Wallis test with post-hoc test) using GraphPad Prism 6.1 (GraphPad). P < 0.05 was considered statistically significant.

Results
ICG biodistribution. These experiments evaluated drug retention in a LN after administration by a LDDS or i.v. injection. In the LDDS group, the highest fluorescence signal was detected in the axillary area at 2 h post-injection of ICG into the AALN (Fig. 1A), and the accumulated ICG was retained in the axillary area at 24 h post-injection. In the IV group, no fluorescence signal was detected in the axillary area, whereas a notable fluorescence signal was detected in the liver for up to 6 h. Next, ex vivo ICG fluorescence intensity was measured in various excised organs and plasma samples 24 h after post-injection of ICG (Fig. 1B). Notable fluorescence signals were detected in the kidneys, liver, PALN and AALN in the LDDS group, but only in the kidneys and liver in the IV group (Fig. 1Ba). Weight-normalized fluorescence intensity (Fig. 1Bb) differed significantly between the LDDS and IV groups for the liver (P < 0.05), PALN (P < 0.05) and AALN (P < 0.01), whereas the plasma fluorescence intensity did not differ between groups (Fig. 1Bc).

Treatment of false-negative metastatic LN using CDDP given by a LDDS. As PALN retention of ICG was higher for the LDDS group than for the IV group (Fig. 1), a similar effect was anticipated for CDDP. Tumor cells were inoculated into the SI LN (day 0) to induce PALN metastasis (Fig. 2Aa–2Ab). Luciferase activity increased with time after inoculation in both the SI LN and PALN (Fig. 2Ab) but was higher in the SI LN (injection site). PALN metastasis was detected at 21 days post-inoculation (Fig. 2Aa).

CDDP was given to metastatic PALN by LDDS or i.v. injection on day 0T (the day after detection of metastasis). Figure 2Ba and 2Bb shows the treatment effects in the various LDDS and IV groups. Figure 2Bb shows normalized in vivo luciferase activity versus days after treatment. Although a significant difference was not observed between each group, among the LDDS groups, PALN luciferase activity was lower in the 5 μg/g CDDP group than in the 0 μg/g CDDP or 0.5 μg/g CDDP groups (P < 0.05). The IV group (5 μg/g CDDP) showed no decrease in PALN luciferase activity versus the LDDS 0 μg/g CDDP group. Luciferase activity in the LDDS 5 μg/g CDDP group was highest at day 3T and then decreased at days 6T and 9T. Figure 2Ca shows ex vivo bioluminescence images of SI LN,
PALN, AALN and lung samples, obtained at day 9T. PALN luciferase activity was detected in the LDDS 0\(\mu g/g\) CDDP, LDDS 0.5\(\mu g/g\) CDDP and IV (5\(\mu g/g\) CDDP) groups but not in the LDDS 5\(\mu g/g\) CDDP group. Luciferase signals in the AALN or lung were not detected in any of the groups. Figure 2Cb shows ex vivo fluorescence images at 24 h post-injection of ICG. (a) Ex vivo fluorescence images of each organ. For the LDDS group (n = 7), ICG fluorescence was detected in the proper axillary lymph node (PALN) and AALN. However, for the IV group (n = 4), fluorescence levels in the PALN and AALN were below the limits of detection. (b) Ex vivo fluorescence intensity of each organ normalized to its weight. Statistically significant differences between the LDDS and IV groups were found for the liver, PALN and AALN (Mann-Whitney U-test: *P < 0.05, liver; *P < 0.05, PALN; **P < 0.01, AALN). (c) In vitro fluorescence intensity of plasma. There was no significant difference between the IV (n = 4) and LDDS (n = 4) groups (Mann-Whitney U-test).

Blood biochemistry and animal weight. To determine whether use of LDDS was associated with toxicity, blood biochemical parameters at day 9T and changes in animal weight during the study were assessed. There were no statistically significant differences between the four groups (Table 1).

Histological analysis. Figure 4 shows representative sections stained at day 9T with H&E, anti-CD31 antibody or anti-LYVE-1 antibody. In all groups, lymphatic vessels were expanded by tumor growth (Fig. 4A3, 4B3, 4C3, 4D3). In the LDDS 0\(\mu g/g\) CDDP, LDDS 0.5\(\mu g/g\) CDDP and IV groups, tumor cells were widely distributed in the PALN (Fig. 4A1 – 4A3, 4B1 – 4B3, 4C1 – 4C3, 4D1 – 4D3). In the LDDS 5\(\mu g/g\) CDDP group (Fig. 4C1 – 4C3), tumor cells were observed only within lymphatic vessels and not outside them. In sections of the kidneys (Fig. 4A4, 4B4, 4C4, 4D4), proximal tubular atrophy was observed in the IV (5\(\mu g/g\) CDDP) group but not in any of the LDDS groups.

Discussion
This is the first study to quantify treatment efficacy of a chemotherapy agent given by a LDDS to a false-negative metastatic LN. PALN metastasis, induced by tumor cell
First, we gave ICG into the AALN with the LDDS and evaluated ICG accumulation in the PALN (Fig. 1). It was assumed that CDDP would show similar flux/accumulation properties to ICG, as their molecular weights are relatively similar (i.e. CDDP: molecular weight, 300; ICG: molecular weight, 775). Signals in the axillary area were detected 24 h after ICG injection with the LDDS. Ex vivo measurements made at 24 h after ICG administration showed that fluorescence intensities in PALN and AALN were higher in the LDDS group than in the IV group (Fig. 1Bb). However, the LDDS group showed less hepatic accumulation of ICG than the IV group (Fig. 1Bb). Second, we investigated the anticancer effects of CDDP on a false-negative metastatic LN. In vivo bioluminescence measurements revealed that 5 μg/g CDDP inhibited
tumor growth when given by LDDS but not when given by i.v. injection (Fig. 2B,C). In addition, renal proximal tubular atrophy, a typical adverse effect of CDDP, was detected after i.v. administration but not after administration through LDDS. Thus, LDDS may be a superior technique for drug delivery and retention in target LN, enhancing the response rate compared to systemic chemotherapy. When LDDS was used to deliver CDDP, volume of the drug injection site (LN

Table 1. Evaluation of toxicity 9 days after treatment

|                  | LDDS       | IV         | Statistical significance |
|------------------|------------|------------|--------------------------|
|                  | 0 μg/g (n = 4) | 0.5 μg/g (n = 4) | 0.5 μg/g (n = 5) | 0.5 μg/g (n = 3) |
| CRE (mg/dL)      | 0.10 ± 0.01 | 0.14 ± 0.02 | 0.13 ± 0.01 | 0.10 ± 0.01 | NS           |
| BUN (mg/dL)      | 27.98 ± 1.06 | 28.8 ± 0.89 | 35.66 ± 4.39 | 33.1 ± 4.88 | NS           |
| T-BIL (mg/dL)    | 0.05 ± 0.02 | 0.04 ± 0.02 | 0.072 ± 0.01 | 0.06 ± 0.02 | NS           |
| ALT (mg/dL)      | 52.25 ± 13.29 | 53 ± 18.53 | 59.4 ± 13.98 | 39.67 ± 1.20 | NS           |
| AST (mg/dL)      | 150.75 ± 31.38 | 120.5 ± 18.77 | 142.2 ± 24.70 | 146 ± 23.10 | NS           |
| Weight change (g)| 1.60 ± 0.50 | 0.80 ± 0.90 | -1.00 ± 0.50 | 0.00 ± 1.10 | NS           |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cre, creatinine; IV, intravenous group; LDDS, lymphatic drug delivery system NS, not significant; T-Bil, total bilirubin. Values are means ± SEM.

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5 µg/g CDDP using LDDS significantly inhibited PALN tumor growth, but tumor cells were not completely eliminated (Fig. 4). Proper axillary lymph node volume increased after IV administration of CDDP (Fig. 3Ab), and this treatment only slightly inhibited PALN tumor growth (Fig. 2Bb). Interestingly, the volume of the AALN (located upstream of the PALN) was greatly reduced by 5 µg/g CDDP, which was probably because of bone marrow suppression by CDDP. The reduction rate was almost the same for direct injection of CDDP into the AALN by LDDS. That is, LDDS has the potential to treat targeted LN effectively compared to systemic chemotherapy, with side-effects such as bone marrow suppression. The present study has demonstrated an antitumor effect of CDDP given with a LDDS on a false-negative metastatic LN and compared this effect with systemic i.v. administration of CDDP. Use of LDDS showed a notable advantage over i.v. administration in terms of molecular retention of drug in the target LN. In addition, CDDP administration with LDDS produced inhibition of tumor growth in LN without the serious adverse effects normally produced after i.v. injection of CDDP. LDDS shows great potential as an effective treatment for false-negative metastatic LN. In the present study, we supposed that CDDP would show similar flux/accumulation properties to ICG, as their molecular weights are similar. However, parameters such as size, surface charge, molecular weight, hydrophobicity and composition determine molecular retention in lymph nodes by LDDS. In addition, intranodal veins anastomose the vein running over the lymph node.24 Thus, part of the drug injected into the lymph node flows into the vein. In LDDS, drug retention in lymph nodes and biodistribution in the whole body must be investigated. In addition, in future study, we intend to determine maximum tolerated dose, dose-limiting toxicity and to construct a dose-response curve to establish the most effective CDDP dose for completely treating tumor cells in metastatic LN.

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Disclosure Statement

Authors declare no conflicts of interest for this article.

Abbreviations

AALN accessory axillary lymph node
ALT alanine aminotransferase
AST aspartate aminotransferase
BUN blood urea nitrogen
CDDP cisplatin
Cre creatinine
HFUS high-frequency ultrasound
ICG indocyanine green
LDDS lymphatic drug delivery system
LN lymph node
PALN proper axillary lymph node
SiLN subiliac lymph node
T-Bil total bilirubin
VEGF vascular endothelial growth factor

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