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研究課題名（和文）超早期転移リンパ節に対するリンパ行性薬物送達法の創製

研究課題名（英文）Development of a lymphatic drug delivery system to treat very early metastatic lymph nodes

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：本研究では，以下の点を明らかにした．
(A) 臨床的N0リン パ節の四次元的形態解析：転移の初期段階にあるリンパ節では，腫瘍細胞が実質を腫瘍細胞に置き換え腫瘍巣を形成し，この形成過程で血管，リンパ洞が消失する．この消失により，超音波画像診断で観察される血流欠損が生じる．
(B) 臨床的N0リンパ節に対するLDDSの溶媒特性と抗腫瘍効果の検討：LDDSに最適な浸透圧(695- 2,780 kPa)，粘度(40mPas以下)を定めた．今後の製剤開発に指針になると，想定される．

研究成果の学術的意義や社会的意義
転移リンパ節に対する全身化学療法の奏効率は低い．この理由は何か？本研究では，リンパ節転移初期段階に形成される血流欠損が奏効率の低さの原因であることを示した．血流欠損は転移診断の指標になる．一方，転移リンパ節の治療に対してはLDDSが有効であり，LDDSの溶媒には最適な浸透圧と粘度が存在することを示した．LDDS製剤開発の指針を示した．

研究成果の概要（英文）：In this study, the following points were clarified.
(A) Four-dimensional morphological analysis of clinical N0 lymph nodes: In lymph nodes in the early stage of metastasis, tumor cells replace the parenchyma with tumor cells to form tumor nests, and blood vessels and lymph sinuses are lost during this process. This loss results in a blood flow defect observed on ultrasound imaging.
(B) Investigation of solvent characteristics and antitumor effects of LDDS on clinical N0 lymph nodes: The optimum osmotic pressure (695- 2,780 kPa) and viscosity (40 mPas or less) were determined for LDDS. It is assumed that this will be a guideline for the development of future formulations.

研究分野：医用システム

キーワード：リンパ節　転移　血流欠損　DDS

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１．研究開始当初の背景

Cancer patients have a 10-100% probability of lymph node (LN) metastasis, depending on the cancer type. We have shown that in the early stage of metastasis (clinical N0), tumor cells invasion and proliferation start from the marginal sinus of the LN (sentinel LN) via imported lymph vessels from the primary tumor and then penetrates the perforating branches of venous overlying LN.s; causes systemic metastasis to the whole body. This theory of metastasis has been proposed to explain the LN-mediated metastasis of tumor cells. Furthermore, this concept suggests local control of the N0 sentinel LN. Therefore, clinically can prevent distant metastasis. The lymphatic drug delivery system (LDDS) being developed by the Principal Investigators is a treatment method that controls N0 LNs. In this study, we aim to develop an LDDS with a high response rate for clinical N0 LNs regardless of cancer type.

２．研究の目的

This study addresses the following three issues to demonstrate that LDDS has a high response rate against clinical N0 LNs independent of cancer type.

[Subject A] Four-dimensional morphological analysis of clinical N0 LNs

[Subject B] Verification of solvent characteristics and antitumor effect of LDDS on clinical N0 LNs.

[Subject C] Verification of administration factors and antitumor effect of LDDS on clinical N0.

３．研究の方法

(1) **Cell lines.** Two types of non-keratinizing tumor cells were used to eliminate the effect of keratinization in the present study, namely: malignant fibrous histiocytoma-like (KM-Luc/GFP) cells, which express a fusion of the luciferase and enhanced green fluorescent protein genes and C3H/He mouse mammary carcinoma (FM3A-Luc) cells, which express the luciferase gene.

(2) **Mice.** Experiments were carried out under established guidelines and approved by the Institutional Animal Care and Use Committee of Tohoku University (2016BeA-019, 2016BeA-005, 2016BeLMo-003). MXH10/Mo-lpr/lpr (MXH10/Mo/lpr) mice (13–16 weeks of age) were used in all experiments.

(3) **Mouse model of LN metastasis.** 1.0×10^6 KM-Luc/GFP or 1.0×10^6 FM3A-Luc cells in 60 μL of vehicle (20 μL of PBS plus 40 μL of 400 mg/mL Matrigel) were injected into the right subiliac LN (SiLN) of mice, anesthetized with 2% isoflurane in oxygen, to induce metastasis in the right proper axillary LN (PALN) via lymphatic vessels. In controls, 60 μL of the vehicle was injected into the SiLN. Inoculation (24-gauge needle) was guided by a high-frequency ultrasound system (HF-US) VEVO770 (FUJIFILM VisualSonics, Tokyo, Japan) using a central frequency of 25-MHz transducer (RMV-710B, axial resolution 70 μm, FUJIFILM VisualSonics). The inoculation day was defined as day 0. Metastasis to the PALN was assessed using in vivo bioluminescence imaging

(IVIS; PerkinElmer, Waltham, MA, US) on days 0, 4, 7 and 10 for KM-Luc/GFP cells and days 0, 14, 21, 28, and 35 for FM3A-Luc cells. Metastasis was considered successful when PALN luciferase activity exceeded the background level in controls (~4×10^4 photons/s).

(4) **Contrast-enhanced high-frequency ultrasound imaging with spatiotemporal analysis of pixel intensity variation.** Mice were placed in a lateral position on the heated stage after inhalation of anesthesia with 2% isoflurane in oxygen and rested on a heated stage at 38°C throughout the imaging session. A contrast-enhanced HF-US, with a central frequency of 40-MHz transducer (RMV-704B, axial resolution 40 μm), was used to acquire two-dimensional (2D) images of the microvasculature within the PALN on days 0, 4, 7 and 10 post-inoculation of KM-Luc/GFP cells (n = 6) or days 21, 28 and 35 post-inoculation of FM3A-Luc cells (n = 10). The transducer was fixed to a three-dimensional (3D) stage control system (Marsk-204-MS; Sigma Koki, Tokyo, Japan).

(5) **Intranodal lymphangiography of the PALN using a contrast-enhanced HF-US imaging system.** The solution I (n = 2) or II (n = 2) was injected into the SiLN at a constant rate of 20 μL/min (total volume, 400 μL) to deliver it to the PALN. The HF-US imaging system (VEVO770 with an RMV-710B transducer) was used to record two-dimensional and three-dimensional images of the PALN in contrast mode at 3-minute intervals for 30 minutes from the start of injection.

(6) **Contrast-enhanced ex vivo micro-CT imaging.** Ex vivo contrast-enhanced imaging was performed using a micro-CT scanner specifically developed to image small laboratory animals (scanXmate/E090, Comscantecno Co., Kanagawa, Japan), which did not permit us to monitor the blood vessel parameters of the same mouse over time. As previously described, a gelatin-based barium contrast agent (nontoxic due to its insolubility; 1.0 ± 0.3 μm in size) was prepared. Briefly, the heart's left ventricle was perfused first with PBS to remove the blood and then with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) to fix the tissues. Pre-heated gelatin-based barium contrast agent was injected better to visualize blood vessel branches, including small capillaries. Mice were refrigerated (4 °C) for at least 2 h after perfusion to solidify the gelatin-based contrast agent and prevent wash-out during fixation. PALNs were harvested and scanned at resolutions of 8–20 μm for plain angiography and a slice thickness of 100 μm for CT. Acquired slice data were rendered as 3D images (Amira, Maxnet Co., Ltd, Tokyo, Japan). Samples were obtained on day 4 (n = 5), day 7 (n = 6) and day 10 (n = 5) for the KM-Luc/GFP group and on day 0 (n = 6), day 14 (n = 5), day 21 (n = 6) and day 28 (n = 5) for the FM3A-Luc group. The PALNs resected on each day were then used for histological analyses.

(7) **Solvent characteristics.** The osmotic pressure of the injected solutions was set between 588 kPa and 3,000 kPa, and the viscosity was set between 1 mPa·s and 55 mPa·s. Five solutions (I, II, III, IV, and V) were prepared using glucose or polysorbate 80 to adjust the osmotic pressure and viscosity to the required values. Solutions I and II consisted of 50% glucose (Otsuka Pharmaceutical), distilled water, and an ultrasound contrast agent (Sonazoid; Daiichi-Sankyo) used intranodal lymphangiography of PALN

by an HF-US imaging system. Glucose was used to maintain the shape of the Sonazoid lipid membrane. Solutions III, IV, and V consisted of polysorbate 80 (NOF), saline solution (Otsuka Pharmaceutical), ethanol, distilled water, and indocyanine green (ICG, 100 μg/mL, Daiichi-Sankyo) with and without cisplatin (CDDP; Fujifilm Wako Pure Chemical) of 1 mg/kg. The osmotic pressure, Π, was calculated by adjusting the molar concentrations of the components according to Van't Hoff's law. The viscosity, μ, of each solution was measured at room temperature (25.6-25.8°C) using two tuning fork vibration viscometers (SV-1H and SV-1A; A & D).

(8) **Administration of CDDP with the LDDS.** CDDP (or control solution) was administered with the LDDS on day 7 after tumor cell inoculation (defined as day 0T). Solution III, IV, or V with or without CDDP (Table 1) was injected into the right SiLN at a constant rate of 10 μL/min (total volume, 200 μL) using a syringe pump (Legato100; KD Scientific). Solution III with CDDP was administered into the tail vein (bolus injection) as a control experiment. Luciferase activity (used as an indicator of tumor growth) was measured on days 0T (before and after drug injection), 3T, 6T, and 9T using a bioluminescence imaging system (IVIS Lumina LT Series III; PerkinElmer). The SiLN and PALN were imaged on days −7T, 0T, 3T, 6T, and 9T using the HF-US imaging system, and their volume was calculated as described previously. Mouse body weight was measured on days −7T, 0T, 3T, 6T, and 9T. No statistically significant toxicity or blood biochemical parameter changes (T-Bil, A.L.T., AST, Cre, or BUN) in our previous study using CDDP at 5 mg/kg.

(9) **Histological analysis.** LNs were fixed overnight in 10% formalin at 4 °C, dehydrated, embedded in paraffin, serially sectioned (3–5 μm), and either stained with hematoxylin and eosin (HE) or immunostained for CD31-positive cells. Histological images were captured using a digital camera (DP72; Olympus, Tokyo, Japan).

(10) **Statistical analysis.** Data are presented as the mean ± SEM Differences between groups were determined by one-way or two-way ANOVA followed by Tukey's posthoc test. $P < 0.05$ was considered to be a statistically significant finding.

４．研究成果

(1) Perfusion defects

Perfusion defects in LNs were observed with each imaging modality, and the LNs were subjected to pathological analyses to determine whether they were cN0 or N1. The present finding was that tumor cells were replaced in microvascular areas to form a perfusion defect that is not pathologically necrotic tissue in the early metastasis stage. During perfusion defect formation, replacement of parenchyma with tumor supplied by only a few microvessels occurs, intranodal pressure increased, pO_2 remained constant, and tumor neovascularization was not induced. These phenomena have also been observed in nonenlarged LNs. The characteristics of perfusion defect formation in LNs may explain why metastatic LNs show an inadequate response to hematologic systemic chemotherapy using small-molecule and macromolecular anticancer agents. Issue B. This study found that the optimal osmotic pressure range of LDDS solution for the treatment of metastatic

LNs is 695-2,780 kPa and for viscosity less than 40 mPa·s. Up to this viscosity value, the macroscopic flow dynamics of the drug did not significantly differ.

(2) Optimal physicochemical parameters of solvent for LDDS.

LDDS is assumed to be capable of treating and preventing clinical N0 and downstream LNs by filling the inside of clinical N0 LNs with drugs and delivering the drug to downstream LNs. In this year's study, the antitumor effect of LDDS on N0 metastatic LNs

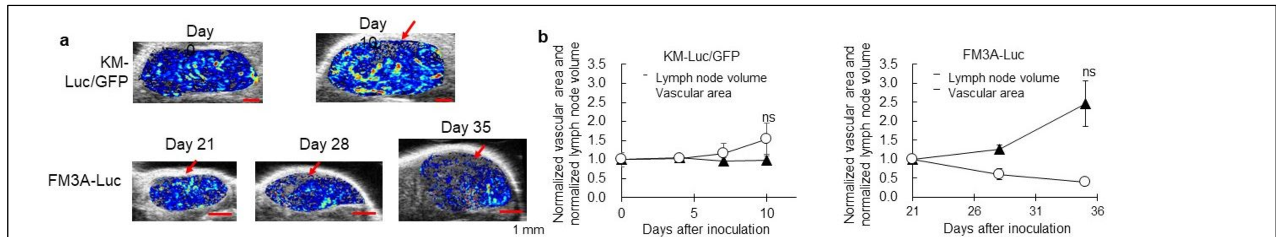

Figure 1. Contrast-enhanced high-frequency ultrasound imaging. Representative heatmaps of the PALN on days 0 and 10 after inoculation of KM-Luc/GFP cells into the SiLN and on days 21, 28, and 35 after inoculation of FM3A-Luc cells. The heatmaps were generated from contrast-enhanced high-frequency ultrasound images using UCAD software. Arrows: perfusion defect. b Changes in normalized vascular area ratio and normalized volume of the PALN. KM-Luc/GFP or FM3A-Luc cells were inoculated into the SiLN to induce metastasis to the PALN. KM-Luc/GFP cells: day 4 (n = 6), day 7 (n = 6) and day 10 (n = 6). FM3A-Luc cells: day 21 (n = 10), day 28 (n = 9) and day 35 (n = 10). No significant changes in normalized LN volume or normalized vascular area ratio in the metastatic PALN (one-way ANOVA and Tukey's post-hoc test) were observed. Data are shown as the mean ± SEM ns not significant. Spatial resolution: > 30 µm. Scale bar: 1 mm

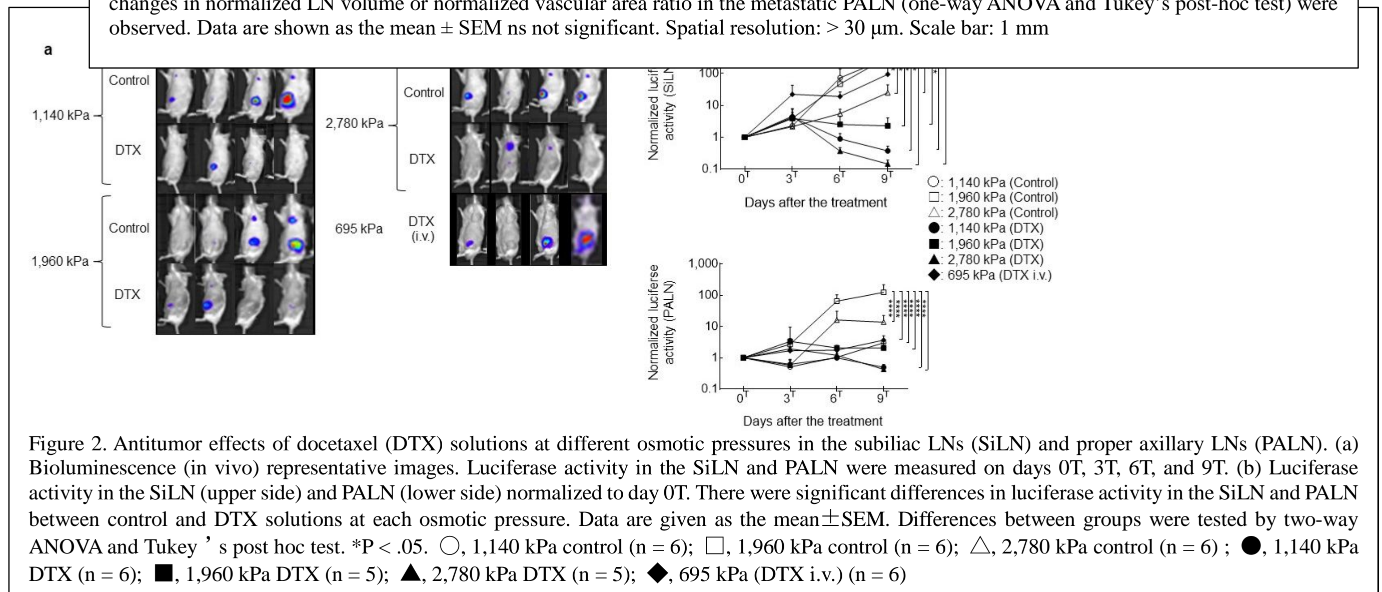

Figure 2. Antitumor effects of docetaxel (DTX) solutions at different osmotic pressures in the subiliac LNs (SiLN) and proper axillary LNs (PALN). (a) Bioluminescence (in vivo) representative images. Luciferase activity in the SiLN and PALN were measured on days 0T, 3T, 6T, and 9T. (b) Luciferase activity in the SiLN (upper side) and PALN (lower side) normalized to day 0T. There were significant differences in luciferase activity in the SiLN and PALN between control and DTX solutions at each osmotic pressure. Data are given as the mean±SEM. Differences between groups were tested by two-way ANOVA and Tukey's post hoc test. *P < .05. ○, 1,140 kPa control (n = 6); □, 1,960 kPa control (n = 6); △, 2,780 kPa control (n = 6) ; ●, 1,140 kPa DTX (n = 6); ■, 1,960 kPa DTX (n = 5); ▲, 2,780 kPa DTX (n = 5); ◆, 695 kPa (DTX i.v.) (n = 6)

was examined using the anticancer drug docetaxel dissolved in a solvent of appropriate osmolarity and viscosity obtained in the previous year, and MXH10/Mo/lpr mice were used. Tumor cells were transplanted into the SiLN and induced metastasis in the PALN; SiLN was defined as the N0 LN and PALN as a metastatic downstream LN. Docetaxel at adjusted osmolality (700-3,000 kPa) and viscosity (< 40 mPas) was administered to SiLNs; tumor growth in SiLNs and PALNs was suppressed. We anticipate these optimal ranges to be a starting point for developing more effective drug regimens to treat metastatic LN with the LDDS.

5．主な発表論文等

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10.1111/cas.14867	有
オープンアクセス	国際共著
オープンアクセスとしている（また、その予定である）	－

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3．雑誌名	6．最初と最後の頁
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掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
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オープンアクセスとしている（また、その予定である）	－

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10.1111/cas.15283	有
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オープンアクセスとしている（また、その予定である）	－

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関