Effective Therapy Using a Liposomal siRNA that Targets the Tumor Vasculature in a Model Murine Breast Cancer with Lung Metastasis

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Although metastatic cancer is a major cause of death for cancer patients, no efficacious treatment for metastasis is available. We previously showed that the growth of a primary tumor could be inhibited by the administration of an anti-angiogenic small interfering RNA (siRNA) that is encapsulated in an RGD peptide-modified lipid nanoparticle (RGD-LNP). We herein report on the delivery of siRNA by an RGD-LNP to the vasculature is also effective for treating metastatic tumors. We compared the RGD-LNP with the polyethylene glycol (PEG)ylated LNP (PEG-LNP) in terms of accumulation in a lung-metastasized model. Despite malformed structure of vasculature in the metastasized lung, the accumulation of the PEG-LNP in the metastasized lung was lower than that for the RGD-LNP, which suggests that the delivery strategy based on vascular permeability is not completely effective for targeting metastasis tumors. The systemic injection of the RGD-LNP induced a significant silencing in the metastasized vasculature, but not in the normal lung. In addition, the continuous injection of the RGD-LNP encapsulating siRNA against a delta-like ligand 4 (DLL4) drastically prolonged the overall survival of metastasized model mice. Accordingly, our current findings suggest that vasculature targeting would be more effective than enhanced permeability and retention effect-based therapy for the treatment of metastatic cancer.

INTRODUCTION

Metastasis is a major cause of cancer-related death.1,2 The 5-year survival rate has not been improved in the period from 2005 to 2015, despite the tremendous advances in cancer treatment, while that for cancer patients without distant metastasis has been improved.3 Thus, an innovative therapy is needed for the treatment of advanced, metastatic cancer.

According to the previous report showing that tumor endothelial cells (TECs) in lymphatic metastasis tumors comprised a supportive phenotype as well as in primary tumors,4 we focused on the use of TECs as a target for the treatment of metastatic tumors. To regulate a gene of interest, we previously developed a TEC-targeting small interfering RNA (siRNA)-loaded lipid nanoparticle (LNP) (RGD-LNP), which was composed of a fusogenic cationic lipid (YSK05) and a cyclic RGD peptide that recognized αVβ3 integrin, which is highly expressed in TECs.5,6 Systemically injected RGD-LNP caused the efficient knockdown in TECs, with an ED50 of approximately 0.75 mg/kg in a human renal cell carcinoma model.7 Angiogenesis-related gene vascular endothelial growth factor receptor 2 (VEGFR2), which is inhibited by RGD-LNP, resulted in a delay in the growth of the primary tumor.

In previous studies, cationic nanoparticles have frequently been used to target the vasculature of a metastasis site, because cationic liposomes were reported to readily accumulate in angiogenic vessels in tumors and sites of chronic inflammation.8 For example, Santel et al.9 reported that siRNA lipoplex AtuPlex, which contained the cationic lipid AtuFect, inhibited tumor growth in a pulmonary metastasis model. Dahlman et al.10 demonstrated that an original cationic polymer 7C1/siRNA complex successfully suppressed Lewis lung carcinoma metastasis. However, such cationic nanoparticle also non-specifically silenced off-target organs.10,11 In an attempt to eliminate such non-specific silencing, we show that an RGD-LNP had a high selectivity for the metastasis site.

Additionally, although it is well known that the properties of vessels in experimental primary tumors (subcutaneous implantation) are angiogenic, malformed, and leaky in an experimental lung metastasis tumor model (intravenous [i.v.] implantation), specifically delivering nanoparticles to such tissue is unclear. Although recent studies have revealed that cancer cells hijack pre-existing vessels in metastatic lesions, a process known as vessel co-option, the properties of such vessels remain unclear despite their clinical significance.12,13 Although it has been clearly demonstrated that macromolecules with a prolonged circulation time exhibited a significant therapeutic effect, there is no clear, direct evidence to show that the enhanced permeability of the vasculature in the metastatic lung could be attributed to such a therapeutic effect.14,15

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A recent report revealed that doxorubicin-loading liposomes were not significantly more effective than free doxorubicin in clinical studies.\textsuperscript{17} It should therefore be important to confirm whether delivering nanoparticles via leaky tumor vessels (enhanced permeability and retention effect) is effective or not at the metastasis site, since clinical patients suffer, not only from the effects of a primary tumor but also from a metastatic tumor in many cases. In this study, we report the characteristics of vessels in a lung metastasis model, that RGD-LNP would be a useful nanoparticle for the treatment of metastatic cancer, and that nanoparticles with a prolonged circulation time would not tend to accumulate in a metastasized lung.

RESULTS AND DISCUSSION

Permeable Vasculature of Metastatic Breast Cancer

Since the properties of tumor vasculature at a metastasis site and the consequent nanoparticle accumulation have not yet been unveiled,\textsuperscript{12,18} we first characterized the tumor vasculature at a metastasis site, namely, breast cancer that had metastasized to the lung. After systemically administering 4T1 cells to mice via the tail vein, the lung was observed by confocal laser-scanning microscopy (CLSM). The numbers of proliferating endothelial cells increased significantly with passing time (Figures 1A and 1B). The cancer cells first became clogged in the vessels and then formed a metastatic region (Figure S1). The endothelial cell adhesion protein vascular endothelial cadherin (VEcad) and type IV collagen (COLIV) containing the basement membrane of the vasculature were significantly reduced in the metastasis lung (Figures 1C and 1D). The alpha smooth muscle actin (\(\alpha\)SMA)-positive cells that line the vasculature (pericytes) are considered to be a marker of mature vessels.\textsuperscript{19–21} In the metastasis lung, \(\alpha\)SMA levels were also significantly decreased (Figure 1E). These results suggest that the vascular permeability in the metastasis lung would be increased and, therefore, had the same characteristic structure as that in the primary tumor. In other words, nanoparticles with a prolonged circulation time could accumulate in tumor tissue via the enhanced permeability and retention (EPR) effect, based on conventional understanding. Further studies directed at the structure
of vessels in a metastasis site with other metastasis models, such as spontaneous and other cancer types, will be needed if progress is to be made regarding nanoparticle-based therapy.

Comparison of PEGylated LNP and RGD-LNP in the Metastasis Lung

The accumulation of polyethylene glycol (PEG)ylated LNP (PEG-LNP) and RGD-LNP in the lung was compared by CLSM 1, at 3 and 7 days after implantation. PEG-LNP levels in the lung tissue were negligible. On the other hand, a larger amount of the RGD-LNP was observed in the metastasis lung (Figure 3C). These results suggest that tumor vasculature targeting (RGD-LNP) was a more potent strategy for therapeutic delivery than PEG-LNP. However, the accumulation of RGD-LNP at the metastatic site appeared to be lower than that in the primary tumor subcutaneous model. After a mouse was inoculated both subcutaneously (primary tumor) and systemically (lung metastasis) at the same time that the PEG-LNP was intravenously administered, an enormous amount of PEG-LNP was detected in the primary tumor (Figures 3A and 3B). On the other hand, only small amounts of PEG-LNP were observed in the metastasis lung (Figure 3C). These results suggest that tumor vasculature targeting (RGD-LNP) was a more potent strategy for therapeutic delivery than PEG-LNP. However, the accumulation of PEG-LNP at the metastatic site appeared to be lower than that in the primary tumor.

In previous studies, it was reported that the protein corona absorbed on the surface of a nanoparticle disturbed the capacity for targeting. For more efficient targeting, the properties of nanoparticles should be strictly optimized in terms of size, type of ligand used, and related issues. In addition, it also appears that EPR effect-based delivery in lung metastasis is not as effective as that in subcutaneous xenografts. This might provide an explanation for the critical difference in the therapeutic effect of doxorubicin-loaded nanoparticles between clinical and non-clinical conditions.
immunostained 24 hr after the final injection. VEGFR2 expression was significantly reduced in whole-lung tissue (Figures 4A and 4B). Since VEGFR2 expression was detected both in cancer cells and TECs, its expression in TECs, shown as yellow pixels in Figure 4, was evaluated. Since previous reports showed that VEGFR2 was expressed not only in TECs but also in cancer cells themselves for autocrine proliferative effects, the localization of VEGFR2 was consistent with other general results.26,27 Further, the cancerous regions and the non-cancerous regions were clearly separated from the whole collected lung tissue. The RGD-LNP injection led to a significant Vegfr2 mRNA knockdown only in the cancerous region and not in the non-cancer part (Figure 4C). The purity of the cancerous and non-cancerous regions was confirmed by tdTomato mRNA expression. The separated tissues were subjected to nested PCR with the tdTomato-specific primer, which is only expressed in tdTomato/luc2-4T1 cells. The PCR amplicon was detected exclusively in the cancerous region (Figure S4). Considering the above findings, it is clear that the RGD-LNP exerted highly selective gene silencing at the metastasis site.

**Therapeutic Effect by RGD-LNP**

Lastly, we assessed the therapeutic effect of RGD-LNP against lung metastasis. In this experiment, taking into account the fact that metastatic tumors in clinical patients were already resistant to chemotherapy, we used doxorubicin (DOX)-resistant tdTomato/luc2-4T1 cells, whose sensitivity against DOX was approximately 100-fold lower than that of normal 4T1 cells (Figure S5A). This resistance was canceled by Verapamil, a known P-glycoprotein (Figure S5B).8 Although we previously reported that siVEGFR2 encapsulated in RGD-LNP could inhibit tumor growth in primary renal cell tumors,6 the survival for the lung metastasis model was not prolonged (Figure S6). This result might indicate that the supply of oxygen, nutrients, and growth factors needed by the metastatic tumor depends not on angiogenesis but vascular co-option, which means cancer cells invaded the pre-existing vessels of the metastasized host organ.12

We then used siRNA against the delta-like ligand (DLL) 4, which is a well-known endothelial gene that ultimately exerts an inhibitory effect on tumor growth since the inhibition of DLL4 resulted in non-productive angiogenesis.29,30 This inhibitory effect was reported to be caused by a chaotic vascular network.31,32 When RGD-LNP encapsulating anti-DLL4 siRNA (siDll4) was injected 8 times at a dose of 2.0 mg/kg, the overall survival of the lung metastasis mouse model was moderately prolonged (Figure 5), but, unfortunately, the results were not statistically significant (p = 0.0508, non-treatment [NT] versus RGD-LNP) because of the small sample number. On the other hand, the PEG-LNP (not RGD-modified LNP) and DOX-loaded liposomes exhibited no therapeutic effect. Additionally, whenDll4 was suppressed in an in vitro study with 4T1 cells, no difference on the viability between siDll4 and siRNA against human polo-like kinase 1 (siControl) (Figure S7) was found.

Although DLL4 expression might be also decreased in cancer cells (Figures 4A and 4B), these results suggest that this therapeutic effect can be attributed to a decrease ofDll4 expression in TECs caused by the RGD-LNP. In addition, in the case of DOX-sensitive 4T1 cells, not only RGD-LNP but also DOX-loaded liposomes showed a therapeutic effect (Figure S8), indicating that the therapeutic effect in the experimental lung metastasis model did not reflect the amount of nanoparticles loaded with the anti-cancer drug that had accumulated. Concerning the primary tumor site, it was reported that DLL4 inhibition caused by the antibody induced a significant delay in primary metastasis.
tumor growth. Accordingly, a therapeutic effect against a primary tumor with RGD-LNP would be expected because of our previous report on a gene-silencing effect in primary tumors with RGD-LNP. Further study will be required before anti-cancer drug-loaded nanoparticles can be used with an experimental lung metastasis model.

MATERIALS AND METHODS

General
Distearyl-sn-glycerophosphocholine (DSPC), PEG-distearyl-sn-glycerophosphoethanolamine (DSPE), PEG-dimyristoyl-glycerol (DMG), and PEG-distearyl-glycerol (DSG) were purchased from NOF (Tokyo, Japan). YSK05 was synthesized as previously reported. Cyclic RGD (cRGD) was synthesized by Peptides International (Louisville, KY, USA). RPMI-1640, cholesterol (chol), and Hoescht33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). siRNAs were synthesized by Hokkaido (siVEGFR2: sense, cAA ccA GAG Acc cuG uuTdTsTdT; anti-sense, AAA CGA GGG UGU CUG GGU GdTsTdT; siDLL4: sense, uGc uAuG GGA cuG uuTdTsTdT; anti-sense, AAA GAU GUC CcA uAc AGG AdTsTdT; lower-case denotes 2’-OMe-modified RNA, “s” denotes phosphorothioate, and “d” denotes DNA). These sequences were previously reported. PBS without Ca²⁺ and Mg²⁺ (PBS [−]) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animal Model
BALB/c (4-week-old, female) mice were purchased from SLC Japan (Shizuoka, Japan). The murine breast cancer 4T1 cells expressing fluorescent protein TdTomato and luciferase (4T1-TdTomato/luc2) were systemically administered via the tail vein at a density of 1.0 × 10⁶ cells. To establish DOX-resistant 4T1 cells, 4T1 cells were incubated in RPMI containing 800 nM DOX for over 4 weeks, and the cells were then subjected to single-cell cloning. For maintenance, the obtained DOX-resistant cells were cultured in RPMI containing 800 nM DOX. All animal experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as stated by the NIH, and they were approved by the Hokkaido University Animal Committee.

Preparation of LNPs
First, 2,100 nmol YSK05, 900 nmol chol, and 90 nmol PEG-DMG were dissolved in 400 μL t-BuOH. PEG-DMG was altered with PEG-DSG to form PEG-LNP, which was delivered to the tumor site via the EPR effect. In the next step, 200 μL siRNA solution (40–160 μg siRNA in 2 mM citrate buffer [pH 4.0]) was added to the lipid mixture under vigorous mixing. The siRNA and lipid mixture was next diluted with 2.0 mL citrate buffer, and the solution was then rapidly diluted with 4.0 mL PBS (−). The resulting preparation was subjected to twice ultrafiltration with an Amicon Ultra-15 against PBS (−) to remove un-encapsulated siRNA and to reduce the concentration of t-BuOH. The solution was mixed with 18 nmol RGD-PEG-DSPE (3 mol% of total lipids) in 7.5% EtOH (v/v, 20 mM citrate buffer [pH 5.5]) for 30 min at 60°C. RGD modification was not carried out in the case of the PEG-LNP. The siRNA recovery ratio and encapsulation efficiency of each of the LNPs were determined by means of a RiboGreen assay, as previously reported. LNPs were characterized using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, Worcestershire, UK). To labeled LNPs with fluorescence, 0.5 mol% DiD was added to the first lipid mixture.

Analysis of Tumor Vasculature Structure by Immunofluorescence Microscopy
Lung tissues were collected from mice that had been sacrificed under a CO₂ atmosphere. They were filled with PBS (−) at optimal cutting temperature (OCT) compound (1:1), and the resulting lung tissues were embedded in OCT compound. The resulting tissues were sliced at a thickness of 5 μm. After placing the slices on M5S-coated glass slides (Matsunami, Tokyo, Japan), they were then incubated with the first antibody solution using a Cy3-labeled anti-αSMA antibody (Sigma-Aldrich, C6198), anti-mouse CD31 Armenian Hamster immunoglobulin G (IgG) (MA31505, Thermo Fisher Scientific), anti-mouse VEcad rat IgG (138001, BioLegend), and anti-bromodeoxyuridine (BrdU) mouse IgG (364101, BioLegend). The slices were then washed twice with PBS (−) and immersed in a second antibody solution containing DyLight488-labeled anti-Armenian hamster IgG goat IgG (405503, BioLegend) for CD31 and AlexaFluor647-labeled anti-Rat IgG goat IgG (A21247, Thermo Fisher Scientific) for 30 min. The slices were covered with a coverslip (Matsunami, 1s) after washing with...
PBS (–). The tumor slices were observed with a Nikon A1R system (Nikon, Tokyo, Japan) using CFI Plan Apo Lambda 20× or CFI Plan Apo VC 60× water immersion objective lens.

**Detection of Proliferating Endothelial Cells in Metastasized Lungs**
Mice were intraperitoneally treated with 150 μL 10 mg/mL BrdU solution 2 hr before the metastasized lung was resected. The resected lung was embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and 5-μm-thick slices were produced with a CM3050S (Leica Microsystems, Nussloch, Germany). For denaturing genomic DNA, the lung sections were treated with 1N HCl for 10 min and then with 2N HCl for 10 min at room temperature. After removing the 2N HCl, the sections were immersed in 0.1 M borate buffer for 12 min. The slice was then washed 3 times with 1% Triton X-100 in 0.1 M PBS (pH 7.4) for 5 min each. After allowing the slice to stand in 1.5% Triton X-100 in 0.1 M PBS (pH 7.4) for 1 hr, it was incubated in anti-BrdU rat IgG (BioLegend) overnight at 4°C, and then a Goat anti-Rat IgG (H+L) Secondary Antibody (Thermo Fisher Scientific) for 30 min.

**Observation of the Accumulation of Evans Blue Dye**
To observe Evans Blue dye in the metastasis lung, 100 μL 5% solution of Evans Blue was systemically administered to normal or metastasized mice. The lungs were then excised and observed or minced with scissors in 200 μL PBS (–) 30 min after the injection. The extracted Evans Blue was then measured.

**Quantification of mRNA Expression**
Using scissors, the cancerous and non-cancerous regions of the lungs were visually isolated and collected 24 hr after the injection of the LNPs. The tissues were then homogenized in TRI Reagent with a PreCelly24 (Bertin Technologies, Montigny-le-Bretonneux, France). The total extracted RNA was then subjected to reverse transcription with a High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. The obtained cDNA was diluted to an appropriate concentration (typically 50-fold), and qPCR was carried out using the diluted cDNA. To clarify whether cancerous regions could be distinguished from non-cancerous regions of lungs, nested PCR was performed.

**Quantification of the Accumulation of LNPs by Radioisotope**
To label LNPs with a radioisotope, cholesteryl hexadecyl ether (CHE) was added to the lipid composition at the preparation step. Lungs were collected 24 hr after the LNPs containing approximately 1,000,000 dpm had been intravenously administered via the tail vein. The excised lungs were minced with scissors and then dissolved in Soluene-350 (PerkinElmer, Boston, MA) at 50°C overnight. A 20 mL portion of HionicFluor (PerkinElmer) was added to the dissolved solution, and the radioactivity was then measured using an LSC-6100 counter (ALOKA, Tokyo, Japan).

**Statistical Analysis**
The Student’s t test was carried out for pairwise comparison. For comparison among three or more groups, the non-repeated ANOVA (nrANOVA), followed by Bonferroni test or Student-Newman-Keuls (SNK) test, was used. p value < 0.05 was regarded as being a statistically significant difference.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes eight figures and can be found with this article online at https://doi.org/10.1016/j.omto.2018.10.004.

**AUTHOR CONTRIBUTIONS**
Y.S. and H.H. designed the whole study. T.H. was involved in almost all of the experiments. A.K. and W.M. performed in vitro experiments. Y.H. carried out the in vivo competitive inhibition assay.

**CONFLICTS OF INTEREST**
The authors have no conflicts of interest directly relevant to the content of this article.

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