Therapy with siRNA for Vegf-c but Not for Vegf-d Suppresses Wide-spectrum Organ Metastasis in an Immunocompetent Xenograft Model of Metastatic Mammary Cancer

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Abstract. Cancer metastasis contributes significantly to cancer mortality and is facilitated by lymphangiogenesis and angiogenesis. Vascular endothelial growth factors-C and D (VEGF-C and VEGF-D) are heavily involved in lymphangiogenesis. Using small interfering RNA (siRNA) against mouse Vegf-c, and Vegf-d, we sought to inhibit metastasis in a model of metastatic murine mammary cancer. BJMC3879Luc2 cell-induced mammary carcinomas received direct intratumoral injections in vivo of either plasmid VEGF-C/D siRNA (psiVEGF-C, psiVEGF-D) or a vector control followed by in vivo gene electrotransfer weekly for seven weeks. Treatment with psiVEGF-C and with psiVEGF-D resulted in lower tumor volumes as compared to the controls. Treatment with psiVEGF-C suppressed wide-spectrum organ metastasis involving lung and lymph nodes. Treatment with psiVEGF-C further reduced the number of dilated lymphatic vessels with invading cancer cells and inhibited tumor blood microvessel density. In contrast, although treatment with psiVEGF-D was not effective and gave equivocal results, it did induce some insignificant reduction in tumor volume increment, average numbers of lymph node metastases and average number of intratumoral dilated lymphatic vessels. In conclusion, specific silencing of the Vegf-c gene suppresses wide-spectrum organ metastasis, including the lung and lymph nodes. However, therapy with siRNA for Vegf-d was not adequately effective in this murine system.

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Materials and Methods

Cell line. The BJMC3879 mammary adenocarcinoma cell line was derived from a metastatic focus within a lymph node of an inoculated BALB/c mouse in an earlier study. When inoculated into BALB/c mice, the line continues to retain a high metastatic propensity, especially to lymph nodes and lungs (21-23). In addition, BJMC3879 cells have been reported to carry the p53 mutation, as inferred by immunohistochemistry (24). The BJMC3879Luc2 mammary carcinoma cell line used in our investigations was generated by stable transfection of the luc2 gene (an improved firefly luciferase gene) into the parent BJMC3879 cell line (25). BJMC3879Luc2 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum with streptomycin/penicillin at 37°C under 5% CO₂.

Animals. Thirty-six-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were housed five per plastic cage on wood chip bedding with free access to water and food under controlled temperature (21±2°C), humidity (50±10%), and lighting (12-12 h light-dark cycle) conditions. All animals were held for a one-week acclimatization period before study commencement. This animal experiment was approved by the Animal Experiment Committee of Osaka Medical College (approved no. 19017). Mice were treated in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals at Osaka Medical College, the Japanese Government Animal Protection and Management Law (No.105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No.6).

siRNA sequences for silencing mouse Vegf-c or Vegf-d. Four different sequences each of siRNA oligonucleotides targeting Vegf-c and Vegf-d, were synthesized using the Qiagen kit and protocol (Qiagen, Hilden, Germany). The four siRNA sequences for mouse Vegf-c were as follows: Vegf-C1, 5’-GAAUGACUAUAUUAAUUTt-3’ and 5’-AUAAUUUAUUAUGUCUAtt-3’; Vegf-C2, 5’-CACAGAAGUGUC UUCUUUAtt-3’ and 5’-UAAGGAAGACACUUCUGUG; Vegf-C3, GACGUUGUUGAUAUUAUAtt-3’ and 5’-UGUAAU UCUC AACAACGCUtt-3’. The scrambled negative siRNA control was purchased from Qiagen.

siRNA expression vectors. To construct short hairpin RNAs (shRNA) targeting mouse Vegf-c or Vegf-d, the following oligonucleotides were designed: Vegf-C1 siRNA, 5’-GATCCGAATGAC TATATAATTTATTTATATATATATATATATTTT TTA-3’; and Vegf-D1 siRNA, 5’-GATCCGGGCAATGTCT ATGAGTATTatgctgtgtgtTAACTCATGACGATTC CCTTCTTTT A-3’. The lower case letters in each sequence above indicate the 15-nucleotide spacer separating the antisense segments. Each complementary oligonucleotide was annealed and ligated into a pBAsi-mU6PurDNAvector (Takara Bio, Inc., Otsu, Japan). This vector contains an RNA polymerase III promoter, the mouse U6 promoter, which can generate high amounts of shRNAs. A control siRNA vector was also purchased from Takara Bio, Inc. This control siRNA vector contains a scrambled sequence with no homology to any human or mouse mRNA. The control siRNA sequence was as follows: 5’-GATCCGCTTTATTCGCGTATAAACGCUACGUCUGAC GCTTCTATACCGATATAGCTTTTTTTTTTT A-3’. For simplicity, in this article, the therapeutic vectors are referred to as psiVEGF-C, psiVEGF-D, and psiSCR (scrambled control).

Transfection of siRNAs into mammary cancer cells. BJMC3879Luc2 cells were plated in 24-well plates at 8×10⁴ cells/well and transfected with 25 nM siRNA-Vegf-C1 to -C4, siRNA-Vegf-D1 to -D4 or 25 nM negative siRNA control using the Hyperfect transfection reagent (Qiagen). At 24 h post-transfection, we measured the size of each treated mammary cancer cell line using the Hyperfect transfection reagent (HyperFect; Qiagen). The four siRNA sequences for mouse Vegf-D were as follows: Vegf-D1, 5’-GGGCAAAUGUCAUGAGUUt-3’ and 5’-UAACUAUGAGC AUUGCCCat-3’; Vegf-D2, 5’-CGGCAAAUGUCAUGAGUUt-3’ and 5’-UAACUAUGAGC AUUGCCCat-3’; Vegf-D3, 5’-CGAGGUAGU GGCGUGUAAGAUGAGUUt-3’ and 5’-UAACUAUGAGC AUUGCCCat-3’; and Vegf-D4, 5’-GGGAUAAACCAAUGAUGAAUtt-3’ and 5’-UAACUAUGAGC AUUGCCCat-3’. The scrambled negative siRNA vector was also provided as psiSCR (scrambled control).

Vector injection and gene electrotransfer were performed once a week for seven weeks. As the tumors grew, a volume of ≤150 μl of each, as the psiVEGF-C, psiVEGF-D or psiSCR (control) groups. Two weeks post-inoculation, when the resulting tumors had reached 0.3-0.5 cm in diameter, we injected psiVEGF-C, psiVEGF-D or psiSCR (0.5 μg/μl saline) directly into the tumors using a 27-gauge needle while the animals were under isoflurane anesthesia. In vivo gene electrotransfer was performed immediately after by applying a conductive gel (Echo Jelly; Aloka Co., Ltd., Tokyo, Japan) to the unshaved skin over the tumor and to the surface of small conductive gel (Echo Jelly; Aloka Co., Ltd., Tokyo, Japan) topically with a pulse length of 20 milliseconds at 100 volts (22, 27-29).

Vector injection and gene electrotransfer were performed once a week for seven weeks. As the tumors grew, a volume of ≤150 μl of each vector solution was introduced into larger masses, while smaller tumors of 0.6-0.7 cm in diameter were infused until we detected leakage of the vector solution. We hoped to reliably administer of 50-75 μg plasmid/tumor, dependent on tumor size.

Using calipers, we measured the size of each treated mammary tumor weekly and calculated tumor volumes using the formula: maximum diameter × (minimum diameter)² / 2. As the tumors grew, a volume of ≤150 μl of each vector solution was introduced into larger masses, while smaller tumors of 0.6-0.7 cm in diameter were infused until we detected leakage of the vector solution. We hoped to reliably administer of 50-75 μg plasmid/tumor, dependent on tumor size.
immediately frozen in liquid nitrogen for molecular analysis. Lungs were routinely inflated with the fixative, excised, and immersed in the fixative. We subsequently trimmed and examined all lobes for metastatic foci before processing for histology, where they were cut into 4-μm slices and stained with hematoxylin and eosin (H&E) for histopathological examination.

**In vivo bioluminescent imaging.** At experimental week 7, we anesthetized four or five mice from each group using isoflurane inhalation administered via the SBH Scientific anesthesia system (SBH Designs, Inc., Windsor, Ontario, Canada). Each anesthetized mouse received an intraperitoneal injection of 3 mg of D-luciferin potassium salts (Wako Pure Chemical Industries, Osaka, Japan) for bioluminescent screening with a Photon Imager (Biospace Lab, Paris, France). We quantified the bioluminescent signals received during the 6-min acquisition time with the Photovision software (Biospace Lab).

**Histopathological analysis.** All mice were euthanized and necropsied at week 7. Mammary tumors, lungs, lymph nodes, and abnormal organs/tissues were removed from each mouse, fixed in 10% phosphate buffered formaldehyde solution, paraffin-embedded and cut at 4 μm. Mounted tissue sections were stained with either H&E for histopathological examination or left unstained for immunohistochemical analysis. We routinely removed lymph nodes from the axillary and femoral regions, as well as any lymph nodes that appeared abnormal. Lungs were inflated with formaldehyde solution prior to excision and fixation and each lung lobe examined individually for metastatic foci prior to paraffin embedding.

**Microvascular density in mammary tumors.** We quantitatively assessed lymphatic and blood microvessel density in primary mammary carcinomas by immunohistochemistry (IHC) using the avidin-biotin complex (ABC) method (LSAB kit; Dako, Glostrup, Denmark) with hamster anti-podoplanin monoclonal antibody (AngioBio Co., Del Mar, CA, USA), a lymphatic endothelium marker, and rabbit anti-CD31 polyclonal antibody (Lab Vision Co., CA, USA), a specific marker for blood vessel endothelium. Since histological confirmation of lymphatic vessel invasion by tumor cells has prognostic value in various malignancies, we counted the number of podoplanin-positive lymphatic vessels containing intraluminal tumor cells and expressed them as the mean±SD. We additionally counted the number of CD31-positive blood microvessels by first scanning the slides at low magnification (×100) to locate areas of highest vessel density and then selecting five sites within those areas to count at higher magnification (×200-400). Values obtained were expressed as the mean±SD (31).

**Statistical analyses.** Significant quantitative differences in intergroup data were analyzed using Welch’s Student’s t-test, which provides for insufficient homogeneity of variance. The variations in metastatic incidence were examined by Fisher’s exact probability test, with p<0.05 or p<0.01 considered to represent a statistically significant difference.

**Results**

Selection of functional siRNA sequence for mouse Vegf-c or Vegf-d. Figures 1A and B illustrate the knockdown ratios of each Vegf-c and Vegf-d siRNA oligonucleotides tested in BJMC3879Luc2 cells. Out of the siRNAs targeted to these growth factors, the strongest silencing was achieved with oligonucleotides Vegf-C1 and Vegf-D1 (66% and 67% respectively).
Body weights and tumor growth of animals under Vegf-c or Vegf-d siRNA treatment. At seven weeks, one animal from the psiVEGF-C group, and three animals from the psiVEGF-D group died due to metastasis vs. five animals in the control psiSCR group. As shown in Figure 2A, body weights did not differ statistically between all mice throughout the experiment until week 7, when there was a decrease in the weights of control and psiVEGF-D-treated animals (psiVEGF-C: 20.9±2.0 g; psiVEGF-D: 19.8±2.4 g; psiSCR: 19.4±1.9 g).

Tumor volumes are presented in Figure 2B. As compared to the psiSCR-treated mice, tumor volume increase in the psiVEGF-C-treated group significantly slowed from weeks 2 through 6. Increases in volumes of psiVEGF-D-treated tumors also tended to be lower in relation to controls, but the differences were not significant.

Bioluminescent imaging of transfected mice. Bioluminescent imaging revealed signals indicative of metastatic growth in mandibular, axillary, and inguinal lymphatic regions in all groups; however, less metastatic expansion occurred in mice from both the psiVEGF-C- and -D-treated groups as compared to control animals (Figure 3), with a more pronounced reduction in VEGF-C-treated mice.

Histopathological analysis of mammary tumors. Mammary tumors induced by BJMC3879Luc2 cell inoculations uniformly proved to be moderately-differentiated adenocarcinomas; we found no histopathological differences among groups. However, we did note treatment-induced variations in metastatic incidence and spread. Figure 4 summarizes the incidence and average number of metastatic lesions counted across multiple organs, and Figure 5 illustrates the histopathological presentation. Lymph node metastasis occurred in 100% of mice in the control psiSCR- and
Figure 4. Quantitative analysis of metastasis in lymph nodes, lung and other organs. A: Lymph node metastasis occurred in 100% of psiSCR- and psiVEGF-D-treated mice, while metastasis was reduced to 67% that of the control with psiVEGF-C treatment. B: The average number of metastatic lymph nodes per mouse was significantly lower in mice receiving psiVEGF-C, as compared to those receiving psiSCR. A tendency for decreased nodal involvement was seen in psiVEGF-D-treated mice, but this was not statistically significant. C: psiVEGF-C-treated mice also exhibited decreased incidence of lung metastasis, while treatment with psiVEGF-D had no effect. B: Development of larger metastatic lung nodules >250 µm was significantly reduced only in the psiVEGF-C-treated group. E: psiVEGF-C treatment appeared to reduce the total overall metastatic burden; Silencing Vegf-d had minimal effect. F: The average number of organs with metastases per mouse was significantly reduced only in the psiVEGF-C-treated group. Data represent the mean±SD. Number of animals examined was nine in the psiSCR-treated group, nine in the psiVEGF-C-treated group and ten in the psiVEGF-D-treated group. *p<0.05; **p<0.01 compared with the psiSCR-treated control.
Figure 5. Continued
psiVEGF-D-treated groups, but in only 67% of mice treated with psiVEGF-C (Figure 4A; Figure 5A-C); furthermore, treatment with psiVEGF-C caused a significant reduction in the average number of cancerous nodes per mouse (Figure 4B). Some insignificant reduction also occurred in the psiVEGF-D-treated group as compared to the psiSCR-treated group.

Metastasis to the lungs followed a similar pattern, as illustrated in Figures 4C-D and 5D-F. Lung metastasis developed in ~89% of mice in both the psiSCR- and psiVEGF-D-treated groups, while treatment with psiVEGF-C reduced the incidence to 44% (Figure 4C). This degree of reduction was not statistically significant. However, down-regulation of Vegf-c did significantly reduce the number of large (>250 μm) metastatic nodules (Figure 5E) as compared to the control (Figure 4D; Figure 5D and F).

Metastatic foci were also observed in kidneys, adrenals, ovaries and uterus (Figure 5G-J). With respect to bilateral organs, metastasis to the only unilateral organ was counted as one, and metastases to the bilateral organs were counted as two. The multiplicity of overall metastasis is presented in Figure 4E and F. The total number of organs affected per group tended to be much lower in mice treated with psiVEGF-C as compared to mice in both the control and the psiVEGF-D treatment groups (Figure 4E); similarly, the average number of all organs with metastasis per mouse was significantly lower in the psiVEGF-C-treated group as compared to the psiSCR-treated group (Figure 4F).

Lymphatic and blood microvessel density in treated mammary tumors. Intratumoral podoplanin-positive lymphatic microvessels are demonstrated in Figure 6A-C. Lymphatic vessels were well-developed in the outer, superficial layers of the mammary tumors in all groups, and tumor cells were frequently observed within the lumina of dilated vessels in both control (Figure 6A) and treated animals (Figure 6B and C). As shown in Figure 7A, significantly fewer vessels containing cancer cells were detected in psiVEGF-C-treated vs. psiSCR-treated tumors, suggesting suppression of lymphatic migration. Fewer vessels harboring migrating cells were also found in psiVEGF-D-treated vs. control tumors, but the difference was significant.

Blood microvessel density, as determined by immunohistochemical analysis for the blood vessel endothelial cell marker CD31 (Figure 6D-F), was significantly lower in the group given siRNA therapy targeting Vegf-c compared to the psiSCR-treated group (Figure 7B).

Discussion

As the presence and degree of metastasis is a significant prognostic indicator for survival in most types of cancer, anti-metastatic therapies are of paramount importance. Dissemination of cancer cells can occur in different ways e.g. local tissue invasion and/or migration via blood and lymphatic vessels. Malignant cells also move into the bloodstream to reach distant sites, and lymph/blood crossover occurs; lung metastasis may initially occur through the lymphatics, but cancer cells can influx into the thoracic duct, the left subclavian vein, through the right ventricle and settle into lung tissue. The most common pathway of initial dissemination of many solid malignancies is via the lymphatics, with varying
Figure 6. Immunohistochemical (IHC) analysis of lymphangiogenesis and angiogenesis in mammary tumors transfected with psiSCR, psiVEGF-C, or psiVEGF-D. Lymphatic vessels were often dilated and frequently contained migrating tumor cells (arrows) within the lumina. A: Podoplanin-positive lymphatic endothelium in a control mouse with multiple intraluminal cells. B: Notice fewer dilated lymphatic vessels with migrating cancer cells in a mouse treated with psiVEGF-C. C: Intratumoral dilated lymphatic vessels of a mouse treated with psiVEGF-D. D: Representative section of a tumor transfected with psiSCR, showing a high density of well-developed and CD31-positive blood microvessels. E: CD31-positive blood microvessels tended to be fewer in tumors transfected with psiVEGF-C than with control or psiVEGF-D. F: Tumors treated with psiVEGF-D appeared similar to those in the psiSCR group. A-C: Anti-podoplanin IHC, original magnification x200. D-F: anti-CD31 IHC, original magnification x100.
patterns of spread via afferent ducts (32). Lymphatic and blood capillaries present in tumors and surrounding tissues provide migrating cells entrance to the system. Involvement of the nearby sentinel nodes precedes involvement of more distal nodes and subsequent seeding of distant organs.

Expression of VEGF-C and VEGF-D correlates with lymph node metastasis in a variety of human cancer types, including breast neoplasms (19, 33, 34), although, in contrast to VEGF-C, the role of VEGF-D is more equivocal. In at least one study, no association between VEGF-D and lymph node metastasis was found in human breast cancer (35), while another group of investigators linked high expression of VEGF-A and VEGF-C, but not VEGF-D, with poor prognosis (36). More recently, a clinical study demonstrated that only tumor-derived VEGF-C induced pre-metastatic sentinel node lymphangionogenesis in primary breast cancer (37).

In many animal models of cancer, overexpression of both VEGF-C and VEGF-D evidently enhanced tumor lymphangiogenesis and nodal/distant organ metastasis (10-16), and siRNA down-regulation of VEGF-C reduced lymph node and lung metastases in murine mammary models (21, 38). An endogenous soluble isoform of the VEGF-C receptor, sVEGFR-2 was identified and shown to be a specific inhibitor of lymphatic vessel growth (39); a subsequent study showed sVEGFR-2 suppressed tumor growth and lymph node metastasis in a mouse mammary model specifically through inhibition of lymphangiogenesis (23). VEGF-R-3, the co-receptor for both VEGF-C and VEGF-D, is predominantly expressed on lymphatic endothelial cells (40), and VEGF-C/D-dependent receptor activation stimulates both lymph endothelial cells and lymphatic vessel development (8, 9). Using soluble VEGFR-3 to sequester VEGF-C and VEGF-D effectively blocked VEGFR-3 signaling and inhibited lymphangiogenesis and lymph node metastasis in animal models (41-43), while an antibody to VEGF-D suppressed VEGF-D-induced lymphatic spread (14).

We attempted to refine Vegf-c/d down-regulation by first isolating the most effective silencing siRNA sequences and then directly treating highly metastatic mammary tumors in vivo with those sequences in a transfectable plasmid vector. Although intratumoral transfection with psiVEGF-C or psiVEGF-D reduced the average number of metastatic lymph nodes, statistically significant reduction was obtained only with psiVEGF-C treatment. Silencing Vegf-c also resulted in significant reductions in treated tumor volumes, in the average number of metastatic nodules of the lungs, and in the average number of other organs affected by metastasis. In contrast, siRNA therapy targeting Vegf-d gave equivocal results; it was ineffective in modulating overall organ metastasis, but did show some reduction of tumor volume increment, average number of lymph node metastases and average number of dilated lymphatic vessels with invading cancer cells. Carter et al. reported that once human breast carcinomas reach 4 cm or larger, the chance of tumor recurrence and metastasis increases dramatically (44). The reduction in tumor volume and size with psiVEGF-C could thus have clinical significance. A similar pattern emerged when analyzing blood microvessel density and lymphatic vessel invasion; transfection with psiVEGF-D tended to reduce both parameters, but psiVEGF-C treatment was significantly more effective. This was a much stronger anti-metastatic effect than we had previously obtained (21), due, we believe, to the selection and use of a more effective Vegf-c siRNA sequence.
In conclusion, treatment with psiVEGF-C, but not psiVEGF-D, significantly suppressed wide-spectrum organ metastasis and several parameters of tumor metastasis in a mouse model with prognostic significance in human cancer, suggesting a potential clinical therapeutic option in the treatment of human metastatic disease.

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