Involvement of Tumor Lymphatic System in Translocation of Intratumorally Injected Liposomes

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Received September 7, 2017; accepted January 1, 2018

The tumor microenvironment is one of the key factors contributing to the efficiency of drug delivery to a tumor. It has been reported that lymphangiogenesis is induced in certain tumors. Because the lymphatic system functions as a drainage one, it is possible that tumor lymphatic vessels alter not only the tumor microenvironment, but also the distribution of drug nanocarriers accumulated in the tumor tissue. Herein, we aimed to elucidate the involvement of the tumor lymphatic system in the translocation of intratumoral liposomes to regional lymph nodes by using vascular endothelial growth factor (VEGF)-C-overexpressing B16F10 tumor-bearing mice (B16/VEGF-C). When the amount of polyethylene glycol (PEG)-modified liposomes in lymph nodes (cervical, brachial, axillary, and inguinal lymph nodes) was measured after the radiolabeled liposomes had been intratumorally injected into B16/VEGF-C-bearing mice or wild-type B16-bearing mice, the accumulation of liposomes in the axillary and inguinal lymph nodes was significantly higher on the tumor-implanted side of B16/VEGF-C-bearing mice than on that of the B16-bearing ones. On the other hand, the accumulation of liposomes in these lymph nodes on the control side (no implantation) of either type of tumor-bearing mice was very low; and no difference could be observed between the 2 sides. Furthermore, the intratumoral distribution of liposomes was observed to be located near the lymphatic vessels. These results indicate that the tumor lymphatic system contributed to the extrusion of a portion of PEG-modified liposomes from the tumor tissue, suggesting that tumor lymphangiogenesis would be one of the key factors to determine the intratumoral distribution of liposomes and their subsequent fate.

Key words liposome; lymphangiogenesis; lymph node; tumor; vascular endothelial growth factor-C

MATERIALS AND METHODS

Plasmid Expression Vector cDNA encoding the mouse VEGF-C open reading flame (ORF) was introduced into the multiple cloning sites of the pcDNA3.1/Hygro+ plasmid vector (pcDNA/Mock, Life Technologies, Japan Ltd., Japan) between HindIII and BamHI regions. The VEGF-C plasmid DNA (pcDNA/VEGF-C) was amplified in DH5α Escherichia coli and purified by using a QIAGEN plasmid purification kit. The sequence of the insert region was analyzed by use of Gene-tyx® software (Tokyo, Japan).

Cell Culture and Gene Transfection Murine melanoma B16F10 cells (B16) were purchased from the American Type

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Culture Collection and cultured at 37°C under 5% CO₂ in a humid atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 unit/mL), and streptomycin (100 µg/mL).

Transfection of B16 cells with pcDNA/Mock or pcDNA/VEGF-C was performed according to the manual of Lipo- fectamine® 2000. For selection of the transfected cells (B16/ Mock or B16/VEGF-C), the cells were cultured in 10% FBS-DMEM medium containing hygromycin (500 µg/mL).

**Preparation of Tumor-Bearing Mice** Five-week-old C57BL/6 male mice were purchased from Japan SLC Inc. (Shizuoka, Japan). All animals used in this study were fed and handled subject to the Animal Facility Guidelines of the University of Shizuoka. All animal procedures were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

B16 or B16/VEGF-C cells (1×10⁶ cells/mouse, 50 µL) were subcutaneously implanted into the left leg of the mice. When the larger diameter of the solid tumor that formed reached about 8 mm, the tumor-bearing mice were used in each experiment.

**RNA Extraction and Real-Time PCR** Total RNAs of B16, B16/Mock or B16/VEGF-C cells were extracted by using an RNeasy Plus Mini Kit (QIAGEN) following homogeniza- tion of the cells with a QIAshredder. After the amount of RNAs had been adjusted, the first-strand cDNAs were prepared with a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare UK Ltd., England). Next, the cDNAs were mixed with the primers for mouse VEGF-C or β-actin (TaKaRa Bio Inc., Shiga, Japan); and SYBR Green I PCR assay was then performed with SYBR Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan); and SYBR Green I PCR assay was then performed with SYBR Premix Ex Taq II (TaKaRa Bio Inc.) on a Thermal Cycler Dice Real Time System (TP-800, TaKaRa Bio Inc., U.S.A.). Donkey anti-rabbit immunoglobulin G (IgG) antibodies used were rabbit anti-mouse VEGF-C antibody (PerkinElmer, Inc., U.S.A.) was mixed in the lipid/chloroform solution before the evaporation. For fluorescence labeling of liposomes, 1,1'-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiICh, Life Technolo- logies) was added in the same manner.

**Quantitative Analysis of Liposome Translocation to Lymph Nodes** [3H]-Labeled PEG-modified liposomes (10 µL, 18.5 kBq) were directly injected into the solid tumors of B16 or B16/VEGF-C tumor-bearing mice at the injection speed of 2 µL/min with an infusion pump (Harvard Pump 11 Plus Advanced Dual Syringe with Dual RS-232, Harvard Appara- tus). After 3 h, the mice were sacrificed; and the lymph nodes (cervical, brachial, axillary, and inguinal) were removed and completely lysed with Solvable® (PerkinElmer, Inc.). The result- ing solution was mixed with Hionic-Flour® (PerkinElmer, Inc.), and the radioactivity was measured by using a liquid scintillation counter (LSC-7400 Hitachi-Aloka Medical, Ltd.).

**Intratumoral Distribution of PEG-Modified Liposomes** DiIC₁₅-labeled liposomes were intratumorally injected into B16 or B16/VEGF-C tumor-bearing mice at the injection speed of 2 µL/min with an infusion pump. After 3 h, the solid tumors were excised, and frozen sections of them were prepared. Finally, immunofluorescence staining of LYVE-1 was performed to visualize lymphatic vessels; and the distribution of fluorescence was confirmed by using a confocal laser-scanning microscope.

**RESULTS AND DISCUSSION**

To prepare VEGF-C-overexpressing tumor cells and we selected the B16F10 melanoma cells. Isaka et al. has previ- ously produced VEGF-C-expressing tumors by introducing VEGF-C gene into B16F10 cells and used the transfectant as a lymphatic metastasis tumor model in order to clearly visualize the lymphatic vessels induced by B16F10 cells and analyze its function. In the present study, we also prepared VEGF-C-expressing B16F10 cells. The results of quantitative analysis by real-time RT-PCR indicated that the expression level of VEGF-C mRNA in B16/VEGF-C cells was much higher than...
that in either B16 or B16/Mock cells (Fig. 1A). Furthermore, the protein expression of VEGF-C was obviously increased in B16/VEGF-C cells; and the secreted form of VEGF-C was also detected (Fig. 1B). To visualize the lymphatic vessels in B16/VEGF-C solid tumors, we immunostained them with anti-LYVE-1 antibodies. As the result, a larger number of lymphatic vessels could be observed in the B16/VEGF-C tumor sections than in the B16 tumor ones (Fig. S1). The real-time RT-PCR analysis also demonstrated that the expression levels of not only VEGF-C but also LYVE-1 in the B16/VEGF-C solid tumor were obviously higher than those in the B16 solid tumor (Fig. 1C). These observations indicate that the preparation of this solid-tumor model having lymphatic vessels was successful. However, the expression level of VEGF-C was not completely corresponded with that of LYVE-1 in the B16/VEGF-C tumor. We speculate this relatively lower expression of LYVE-1 than VEGF-C in the tumor caused from the lymphatic vessel formation in the limited region: The immunostaining images in Fig. S1 showed the lymphatic vessels were mainly observed in the edge of B16/VEGF-C tumor section, and rarely observed in the central region (data not shown). It is well known that formation of vascular systems including blood vessels in most of solid tumors was heterogeneous and limited in the edge of tumor tissue, because the tissue fluid pressure in the central region of tumor tissue tends to be quite high. These evidences support our speculation that lymphatic
vessel formation is also affected by high tissue fluid pressure in the tumor.

Then, \(^{3}\text{H}\)-labeled PEG-modified liposomes were intratumorally injected into B16 or B16/VEGF-C tumors, and their translocation to major lymph nodes was quantitatively evaluated. Since the site of tumor implantation was the left leg of the C57BL/6 mouse, the sentinel lymph nodes of the tumor would be expected to be the left inguinal and axillary ones. As the result, the accumulation of liposomes in the left inguinal and axillary lymph nodes in the B16/VEGF-C-bearing mice was significantly higher than that in the B16-bearing ones (Fig. 2). Additionally, on the non-implanted right side, the liposome accumulation was scarcely observed in all lymphatic nodes; and there were no differences between the right side of either type of tumor-bearing mice (Fig. 2). Furthermore, microscopic observation of the liposome distribution revealed that the fluorescently labeled liposomes were located near the lymphatic vessels in the B16/VEGF-C tumor (Fig. 3). These results suggest that a portion of intratumoral liposomes was extruded from the tumor tissue via the lymphatic vessels newly formed by stimulation from tumor-secreted VEGF-C and translocated to regional lymph nodes such as the inguinal ones. In clinical surgery to remove metastatic breast cancer, a near-infrared fluorescence dye, indocyaningreen (ICG), is often injected into the primary lesion of the tumor tissue in order to fluorometrically identify its sentinel lymph nodes; because ICG is extruded from the tumor tissue via the tumor lymphatic vessels and accumulates in the sentinel lymph nodes.\(^9\) Furthermore, Proulx et al.\(^{10}\) earlier demonstrated that liposomal ICG accumulates much more in the sentinel lymph nodes of a metastatic tumor after the intratumoral injection, because liposomes have the potential to be retained in these lymph nodes for a long time.\(^{10}\) These lines of evidence support our results showing that the location of intratumoral liposomes was altered by the tumor lymphatic system and that a portion of them was easily translocated to the regional lymph nodes. On the other hand, in the VEGF-C-overexpressed solid tumor, the blood vessel formation in the tumor tissue was enhanced, which was similar to lymphatic vessel one, because the expression of PECAM in B16/VEGF-C tumor was increased about 10-fold compared with that in B16 tumor (Fig. 1). In fact, proangiogenic effect of VEGF-C was previously reported by Cao et al.\(^{11}\) The distribution of blood vessels in the tumor tissue might affect the behavior of liposomes after tumor accumulation, because the existence of tumor blood vessels changes the microenvironment of tumor tissue.

We suggest that liposomal drugs could apply for not only the detection of sentinel lymph nodes during the surgical operation for cancer diagnosis, but also the treatment of lymph node metastasis, because intratumorally injected liposomes translocated to the sentinel lymph nodes via the lymphatic vessels newly constructed by VEGF-C stimulation.

In conclusion, we have demonstrated that the tumor lymphatic system altered the intratumoral distribution of PEG-modified liposomes and facilitated the translocation of them to the regional lymph nodes via the tumor lymphatic vessels. The next step will be to clarify the effect of the lymphatic system on the therapeutic efficacy of liposomal anti-tumor drugs.

**Acknowledgment** This study was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (No. 20790041).

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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