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Original article

Suppression of lymphangiogenesis by soluble vascular endothelial growth factor receptor-2 in a mouse lung cancer model

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ABSTRACT

The vascular endothelial growth factor (VEGF) family has a key role in the formation of blood vessels and lymphatics. Among the members of this family, VEGF-C is one of the most important factors involved in lymphangiogenesis *via* binding with two receptors (vascular endothelial growth factor receptor-2 and -3: VEGFR-2 and VEGFR-3). Soluble VEGFR-2 (sVEGFR-2) has a role in maintaining the alymphatic state of the cornea associated with binding to VEGF-C, and selectively inhibits lymphangiogenesis but not angiogenesis. In this study, we introduced sVEGFR-2 into lung cancer cells and evaluated the influence on tumor progression and on genes regulating lymphatic formation and metastasis *in vivo*. A retroviral vector was used to introduce the sVEGFR-2 gene into Lewis lung carcinoma cells (LLC), which were designated as LLC-sVEGFR-2 cells. Proteins secreted into the culture supernatant by these cells were detected by western blotting using specific antibodies. To examine lymphangiogenesis by primary lung cancer *in vivo*, LLC-sVEGFR-2 cells were subcutaneously injected into C57BL/6 mice. At 14 days after injection, immunohistochemistry was performed using an antibody directed against lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), a marker of lymphatics. Expression of mRNA for VEGFR-2, VEGFR-3 and matrix metalloproteinases (MMPs) was also determined by real-time PCR. Furthermore, LLC-sVEGFR-2 cells were directly inoculated into the left lung in C57BL/6 mice and the number of micro-metastases in pulmonary lymph nodes was determined. Introduction of sVEGFR-2 into LLC cells resulted in secretion of sVEGFR-2 protein into the culture supernatant. There were fewer LYVE-1 positive lymphatics after inoculation of LLC-sVEGFR-2 into mice compared with the control group. In addition, VEGFR-2, VEGFR-3, and MMPs gene expression was suppressed in the primary tumors of the LLC-sVEGFR-2 group compared with the control group. Furthermore, there were fewer micro-metastases in the pulmonary lymph nodes of the LLC-sVEGFR-2 group compared with the control group after cells were directly inoculated into the lung. These findings indicate that introduction of sVEGFR-2 suppressed lymphangiogenesis in primary lung cancer and also suppressed lymphogenic metastasis by inhibiting VEGF-C, followed by down-regulation of VEGFR-2, VEGFR-3 and MMPs. Accordingly, sVEGFR-2 might be a promising target for treatment of cancer by regulating lymphangiogenesis and lymphogenic metastasis.

1. Introduction

Vascular endothelial growth factor (VEGF) has an important role in modulating both angiogenesis and lymphangiogenesis, two processes that are essential for tumor progression and metastasis [1]. A correlation has been reported between lymphangiogenesis and a poor prognosis of non-small-cell lung cancer (NSCLC), which accounts for approximately 80% of all lung cancer [2]. While lymph node metastasis and tumor lymphangiogenesis are clinically useful

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prognostic indicators of tumor progression, the origin and role of lymphatic endothelial cells in tumor tissue are still unclear.

VEGF-A and VEGF-C are considered to mainly be involved in angiogenesis and lymphangiogenesis, respectively [3,4]. The VEGF receptors (VEGFRs), including VEGFR-2 and VEGFR-3, are members of the tyrosine kinase receptor family, and VEGFR-mediated signaling induces autophosphorylation of tyrosine residues and activation of the MAP kinase pathway [5]. It has been reported that these signals induce tumor metastasis accompanied by endothelial cell proliferation, angiogenesis and lymphangiogenesis [6]. A positive correlation was also reported between VEGF-C expression and lymphatic metastasis in gastric cancer, intestinal cancer, prostate cancer, thyroid cancer, and melanoma [7–11]. Furthermore, production of VEGF-C by cancer cells was reported to promote lymphangiogenesis, resulting in the progression of metastasis [12].

Soluble VEGFR-2 (sVEGFR-2) is expressed as an alternative splicing variant of VEGFR-2, and is thought to act as an endogenous inhibitor of corneal VEGF-C that maintains the alymphatic state of the cornea. Membrane-bound VEGFR-2 has a secretory signal domain that binds with the membrane after this protein is transferred to the cell membrane. In contrast, sVEGFR-2 lacks membrane and extracellular domains, which means that it does not bind to the cell membrane and is secreted extracellularly. Subsequently, sVEGFR-2 binds with VEGF-C and inhibits its signaling. Because of its high affinity for VEGF-C, but not VEGF-A, sVEGFR-2 has been reported to be a selective inhibitor of lymphangiogenesis without inhibiting angiogenesis [13].

Several factors contribute to tumor progression, including angiogenesis, escape of tumor cells from the primary lesion, and infiltration into the stroma. Matrix metalloproteinases (MMPs) play an important role in the process of cancer invasion. Among the various MMPs, MMP-2 and MMP-9 are involved in degradation of type IV collagen and contribute to tumor invasion/metastasis by breakdown of the surrounding stroma [14].

Recent studies have suggested that lymphangiogenesis precedes lymphatic metastasis of cancer. Thus, members of the VEGF family are thought to be key molecules controlling pathological angiogenesis and lymphangiogenesis, and investigation of mechanisms inhibiting these molecules is expected to lead to new treatments for cancer.

In the present study, we demonstrated that the introduction of sVEGFR-2 suppresses *in vivo* tumor lymphangiogenesis and lymphatic metastasis in a mouse lung cancer model. Accordingly, sVEGFR-2 might be a promising therapeutic target for prevention of tumor progression and metastasis.

2. Materials and methods

2.1. Cells

Lewis lung carcinoma (LLC) cells and NIH/3T3 fibroblasts were kindly provided by Dr. Majima. PT67 cells were purchased from Clontech (*via* Takara Bio Inc., Shiga, Japan). All cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco by Thermo FisherScientific, Waltham, NY, USA) and 100 units/ml penicillin plus 10 μg/ml streptomycin (Gibco) under a humidified atmosphere of 95% air and 5% CO_2.

2.2. Cloning of mouse soluble VEGFR-2 and introduction into LLC via a retroviral vector

The extracellular domain of mouse sVEGFR-2 cDNA was amplified from a mouse lung cDNA library (Takara) by using specific primers targeting the *Xho* I and *Bam* HI restriction enzyme sites [cloning primers F and B, respectively (pos. 199-2084, Table 1)] and a Hot Star High Fidelity Polymerase Kit (Qiagen, Venlo, The Netherlands). Then the amplified cDNA was inserted into the pBlueScript SK II vector (Agilent Technologies, Santa, CA, USA).

The unique C-terminal region of sVEGFR-2 cDNA (pos. 1981-2312) was synthesized by Medical & Biological Laboratories (BML) (Aichi, Japan), followed by attachment of the *Xho* I and *Bam* HI restriction enzyme sites to the 5′ and 3′ ends, respectively. The *Hind* III site (pos. 2037) was used to combine the extracellular domain (pos. 199-2084) and the synthesized C-terminal region (pos. 1981-2312). The C-terminal region digested by *Hind* III and *Bam* HI was cloned into the extracellular domain region containing pBlueScript digested by the same restriction enzymes (Fig. 1A).

Finally, the complete sVEGFR-2 cDNA (199-2312) was digested by *Apa* I and *Bam* HI, and was inserted into the *Apa* I and *Bam* HI sites of a deficient retrovirus vector (pDON-5 Neo, Takara), which was designated as pDON-sVEGFR-2 (Fig. 1B). The complete sequence of sVEGFR-2 cDNA was verified by comparison with a database (accession no. EU884114) using an ABI 310 genetic analyzer (Applied Biosystems by ThermoFisher Scientific, Waltham, NY, USA).

pDON-sVEGFR-2 or the pDON-5 Neo empty vector was transfected into PT67 cells, followed by selection with G418 (Roche, Mannheim, Germany), and the retroviral infectious titer was estimated by infection of NIH/3T3 cells, followed by selection with G418 (approximate 1×10^3 cfu/ml). Then the retroviral vector was used to infect LLC cells, followed by selection with G418, and the resulting cell lines were designated as LLC-sVEGFR-2 and LLC-EV, respectively.

2.3. Gene expression analysis

Total RNA was extracted and purified from cells or tumor tissues by using an RNeasy Plus Mini Kit (Qiagen) and TURBO DNA-free Kit (Ambion, ThermoFisher Scientific). Then 2 μg of total RNA was employed for synthesis of cDNA using an Omniscript RT Kit (Qiagen).

An aliquot of the cDNA (1 μl of 20 μl) was then subjected to RT-PCR in total volume of 50 μl using a thermal cycler (GeneAmpPCR System 9700: Applied Biosystems). Initialization at 94 °C for 15 min was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C

Table 1
Primer sequences.

Target gene and product length
mouse sVEGFR-2(199-2084) 1886 bp
F: TTCTCGAGCGCATACCGCCTCTGTGACTTC
B: TTGGATCCTGTGAGTGATTCGCCCATGTGGAC
GAPDH 162 bp
F: CTGACGTGCCGCCTGGAGAAAC
B: CCCGGCATCGAAGGTGGAAGAGT
sVEGFR-2 extracellular domain 150 bp
F: GGGGCTTGATTTCACCTGGCACTC
B: TCCCCTTGGTCACTCTTGGTCACC
VEGFR-2 Intracellular domain 129 bp
F: CTCTCCCTGCCTACCTCACCTGTT
B: CACTGGCCGGCTCTTTCGCTTACT
VEGFR-3 199 bp
F: GCGCCAAAGCTGATAGGAGGAGAC
B: TTGCGGGAAGCCAGGAACT
MMP-2 145 bp
F: TTCCCCGCGCCCAGTG
B: GAGAAAAGCGCAGCGGAGTGACG
MMP-9 170 bp
F: TGCCCCATGTCACTTTCCCTTCA
B: CTCCGTTGCCGTGCTCCGTGTAGA