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Paracrine signaling by VEGF-C promotes non-small cell lung cancer cell metastasis via recruitment of tumor-associated macrophages

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ABSTRACT

High expression of tumoral vascular endothelial growth factor C (VEGF-C) is correlated with clinical non-small cell lung cancer (NSCLC) metastasis and patient survival. Nevertheless, the comprehensive mechanisms accounting for VEGF-C-mediated cancer progression remain largely unclear. The present study found that VEGF-C expression was upregulated in various NSCLC cell lines. By utilizing transwell migration assay, we found that both recombinant VEGF-C protein and overexpression of VEGF-C in NSCLC cells (A549 and H441 cell lines) could efficiently enhance RAW264.7 cell (murine macrophages) migration. However, recombinant VEGF-C treatment had no effects on both CD206 (an M2 macrophage marker) expression and M1/M2 cytokine profiles of macrophages. Furthermore, additional treatment of recombinant Flt-4/Fc, the specific VEGFR-3 inhibitor or the specific VEGFR-2 inhibitor significantly suppressed macrophage migration compared with A549-CM (conditioned medium) or H441-CM alone group, confirming that NSCLC cells-derived VEGF-C is sufficient to promote macrophage migration. Interestingly, VEGF-C could stimulate the Src/p38 signaling via VEGFR-2/3 axis in macrophages, and inhibition of Src/p38 signaling obviously reversed the enhancement effect of VEGF-C on macrophage migration. Finally, the functional importance of macrophage infiltration induced by tumoral VEGF-C in promoting metastasis was established in a mouse model. In conclusion, our results highlight a novel function of tumoral VEGF-C that paracrinely induces macrophage recruitment, and resultantly promotes NSCLC cell metastasis. Therefore, VEGF-C/VEGFR-2/3 axis may be a promising microenvironmental target against progression of NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer–related deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for up to 80% of all lung cancer cases [1]. Tumor-associated macrophages (TAMs) are a major component of the leukocyte infiltration of many solid tumors, and the infiltration of TAMs correlates with cancer metastasis process and poor prognosis in most types of human cancer including NSCLC [2,3]. However, the mechanism of cancer-mediated macrophage infiltration remains unclear.

Vascular endothelial growth factors (VEGFs) are a family of secreted polypeptides with a highly conserved receptor-binding cystine-knot structure, comprising several different proteins, including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor [4]. Expression of VEGFs is commonly observed in most aggressive tumors, and profoundly associates with the poor prognosis of cancer patients [5]. VEGF-C is the relatively newly defined VEGFs family member, and compared with the well-characterised VEGF-A, many functions and molecular mechanisms involved in the cancer progression mediated by the VEGF-C remain undefined.

VEGF-C functions through interacting with its receptors, VEGFR-3 (also called Flt-4) and VEGFR-2 [6]. In NSCLC, a previous report indicated that high expression levels of both VEGF-C and its receptor Flt-4 show the poor prognosis of cancer patients [7]. Besides, another work indicated that VEGF-C is frequently upregulated in cancer tissues, and interestingly, high tumoral but not stromal VEGF-C status shows poor prognosis in NSCLC patients [8]. Specifically, recent studies showed that the specific VEGFR-3-TK inhibitor (SAR131675) could significantly reduce TAMs infiltration and aggregation in 4T1 (a murine breast cancer cell line) tumor-beard mice [9,10]. As VEGFR-3 was confirmed

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to be abundantly expressed in monocytes and TAMs [11,12], these findings strongly suggest that VEGF-C/VEGFR-3 axis mediates TAMs infiltration of cancer tissues. Nevertheless, the definite role of tumoral VEGF-C in NSCLC-induced TAMs infiltration and the resultant impact on cancer metastasis remain still unexplored.

In the present study, we demonstrate that paracrine VEGF-C from NSCLC cells can increase macrophage migration in vitro and promote macrophages infiltration in vivo, mainly via VEGFR2/3/Src/p38 pathway in macrophages, thus facilitating cancer cell metastasis. Therefore, the VEGF-C/VEGFR-2/3 axis may be a promising micro-environmental intervention target in NSCLC therapy.

2. Materials and methods

2.1. Reagents

Recombinant VEGF-C and VEGFR-3 Fc chimera recombinant protein (Flt-4/Fc) were purchased from R&D. SAR131675 (VEGFR-3 inhibitor), SB203580 (p38 inhibitor), saracatinib (Src inhibitor) and SKLB1002 (VEGFR-2 inhibitor) were from Selleck Chemicals. Cell culture reagents were obtained from Invitrogen. All other reagents were from Sigma unless stated otherwise.

2.2. Cell lines, conditioned medium preparation and transfection

Murine macrophage cell line RAW264.7, normal lung epithelial cell line BEAS-2B cells and NSCLC cell lines (A549, H460, H358, H441 and HCC827) were originally from ATCC. NSCLC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA). BEAS-2B cells were cultured in epithelia cell medium (Gibco-BRL, Gaithersburg, MD, USA). All of them were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were cultured in a standard humidified incubator at 37 °C in a 5% CO2 atmosphere. To obtain the conditioned medium (CM) , NSCLC cell lines (A549 and H441) were seeded at 2×10^6 cells/75 cm^2. Then, the supernatants were harvested at 24 h of further incubation.

VEGF-C in pcDNA3.1 (+) vector (Invitrogen, The Netherlands) or empty vector were transfected into A549 and H441 cells using liposomes according to a previous study [13]. The VEGF-C-overexpressing cells were selected for stable transfection over 14 days using normal culture medium with 600 μg/mL G418 sulphate (Calbiochem, Germany).

2.3. Western blotting and ELISA

Western blotting protocol was according to our previous report [14]. Primary antibodies were anti-VEGF-C (1: 1000; CST #2445), Src (1: 1000; CST #2109), p-Src (1:1000; CST #12432), p38 (1: 1000; CST #8690), p-p38 (1:1000; CST #4511), and β-tubulin (1:5000; ab6046). The secondary antibody was Goat anti-rabbit IgG (1:7500; Proteintech, USA, SA00001-2). The bands were detected by ECL detection reagent. The supernatants of macrophage culture were centrifuged before ELISA. IL-10, IL-12 and IL-23 were measured by commercial ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Real-time RT-PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen), and then complementary DNA (cDNA) was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Japan, FSQ-301) according to the manufacturer's instructions. Real-time RT-PCR was performed with the SYBR Green Realtime PCR Master Mix (TOYOBO, Japan, QPK-201) on an iCycler (Bio-Rad) following the manufacturer's instructions. The primer sequences were as follows: VEGF-C forward primer: GCAGTTACGGTCTGTGTCCA; VEGF-C reverse primer: TTGAG GTTGGCCTGTTCTCT; GAPDH forward primer: GGAGTCAACGGATTT GGT; GAPDH reverse primer: GTGATGGGATTTCCATTGAT. The gene expression levels for each amplicon were calculated by the ΔΔCT method [15] and normalized against GAPDH mRNA.

2.5. In vitro migration assay

The RAW264.7 cell migration was assayed using the Falcon TM Cell Culture Inserts containing polycarbonate membranes with pore sizes of 8 μm. Briefly, Macrophages were seeded (1×10^5 cells/well) in the upper chamber of a transwell and placed it on the 24-well plate. The conditioned medium of A549 or H441 cells were added into the lower chamber. In the coculture experiments, A549 or H441 cells (5×10^4 cells/ insert) were seeded into the lower chamber of 24-well plates. After 10 h, the cell suspension in the upper chamber was aspirated, and the upper surface of the filter was carefully cleaned with cotton plugs. After migration through the polycarbonate membrane, macrophages were stained with crystal violet and images from five representative fields of each membrane were taken. The migratory cells within the lower chamber were counted.

2.6. In vivo metastasis model

BALB/C nude mice (6–8 weeks old) were housed under standard conditions according to the institutional guidelines for animal care. For experimental metastasis , A549 or VEGF-C-overexpressing A549 cells (5×10^6 per mouse) were injected into the tail vein of nude mice. For therapeutic experiments, mice were oral administration of SAR131675 (100 mg/Kg/day) after 5 days post injection of A549 cells. In another therapeutic experiment group, mice were received clodronate encapsulated in liposome nanoparticles (5 mg/mL) to deplete macrophages in mice according to another report [16] before tail-injection of VEGF-C-overexpressing A549 cells. Mice were euthanized after 4 weeks and the livers were harvested, and the number - of visible surface metastases was counted. The liver tissues were either processed for immunohistochemistry.

2.7. Immunohistochemistry

The liver tissue sections were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling the sections in low-pH citrate buffer for 15 min. The sections were stained and visualized by BBI Science Life ABC kit. Primary antibody was rat anti-mouse F4/80 at a 1:200 dilution (Novus). Images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). The F4/80 expression levels in the IHC slices were determined by measuring the cumulated integrated optical density (IOD) using IPP software (Media Cybernetics, Inc., USA).

2.8. Statistical analysis

Statistical analysis was evaluated by Student's test for simple comparisons between two groups and one-way ANOVA for comparisons among multiple groups using GraphPad Prism 5. Data are expressed as mean ± S.D. P value of < 0.05 was considered statistically significant.

3. Results

3.1. VEGF-C induces migration of macrophages but not macrophage polarization

To determine the role of tumoral VEGF-C in macrophage recruitment, we first detected the mRNA expression levels of VEGF-C in both NSCLC cell lines and normal lung epithelium cells by qRT-PCR. The VEGF-C mRNA levels in NSCLC cell lines (A549, H460, H358, H441 and HCC827) were significantly higher than those in BEAS-2B cells (Fig. 1A).