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Original Articles

Restoration of natural killer cell cytotoxicity by VEGFR-3 inhibition in myelogenous leukemia

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ABSTRACT

Acute myeloid leukemia (AML) cells *in vivo* are constantly exposed to lymphangiogenic cytokines such as VEGF-C. However, it is poorly understood how the VEGF-C signaling modulates the immune functions in the tumor microenvironment. We have previously reported that natural killer (NK) cells in AML patients strongly upregulated VEGFR-3, the major VEGF-C receptor, and that the VEGFR-3 expression level in NK cells inversely correlates with their cytotoxic potential. These findings have led us to hypothesize that VEGFR-3 inhibition may reinstate the cytotoxic capacity of the AML-associated NK cells. To address this hypothesis, we employed a pharmaceutical approach to block the VEGFR-3 function in the murine model of syngeneic myelogenous leukemia. Using various molecular and cellular analyses, we assessed the correlation between VEGFR-3 inhibition and NK cell cytotoxicity. Indeed, we found that leukemic environment is highly enriched with lymphangiogenic stimuli, and that VEGFR-3 inhibition restored NK cell killing function with an increased IFN-γ level, providing a therapeutic implication of VEGFR-3 against AML. Together, we demonstrate the therapeutic value of functional modulation of NK cells by blocking VEGFR-3, and provide a possibility of advanced therapeutic approaches using immune cells against myelogenous leukemia.

Introduction

Acute myeloid leukemia (AML) is an intricate hematologic disorder, which includes exposure to a damaged immune network. Forty-five percent of young adults and 90% of older patients have no improvement in conventional treatment yet. In general, patients in the high-risk groups, such as elderly, specific subtypes, and dysfunctional immune system are suffering from relapse. Although serial chemotherapies and hematopoietic stem cell transplantation are commonly used to treat AML [1], emerging evidence has strongly emphasized the importance of immunotherapy [2]. Many trials have demonstrated the importance of immune reinforcement in cancer diseases; thus, restoration of the immune system to kill the cancer has been a fundamental goal of therapy in AML [3,4]. Vascular endothelial growth factor receptor (VEGFR)-3 is a representative mediator of lymphangiogenesis via its ligand VEGF-C or D [5]. Previous papers have shown that VEGFR-3 is expressed on AML blasts and is also involved in various cancer diseases [6,7]. AML blasts also highly express VEGF-C, and the axis of VEGF-C and VEGFR-3 involves leukemic cell proliferation and resistance to chemotherapy [8,9]. De Jonge et al. reported correlation between high VEGF-C and poor outcome in AML patients [10]. VEGFR-3 has been used as a marker for sinusoidal endothelium, which plays important roles in the bone marrow (BM) [11]. Main factors for lymphangiogenesis, VEGF-C and VEGFR-3 play important roles in AML. Based on this, we previously reported that high VEGFR-3-expressing natural killer (NK) cells in AML patients have a low killing potential with low level of interferon (IFN)-γ [12,13]. It suggested a relevance of VEGFR-3 in NK cell function under AML. Kirkin et al. demonstrated that the VEGFR-3 antagonist, MAZ51, preferentially inhibit VEGFR-3 in low concentration, 5 MM, but not other receptor tyrosine kinases (RTKs) including VEGFR-2 as well as antitumor effects, such as reducing tumor sizes *in vivo* [14,15]. Sorensen et al. also reported that VEGFR-3 is suppressed by IFN-γ, which is a major producer of interleukin (IL)-12 in tumor vessels, implying a central role for IFN-γ in VEGFR-3 inhibition in tumor models [16]. The aforementioned papers led us to question whether cytotoxic NK cells are affected by a lymphangiogenic-promoted condition with VEGFR-3. To gain a better understanding of the role of VEGFR-3 in the leukemic microenvironment, a syngeneic leukemic mouse model using murine leukemic cell line was used for this study. The utilization of animal models for cancer is critical to studying the microenvironmental biology of cancer, including gene aberrations and preclinical therapeutic agents [17]. Moreover, the fields of research that relate to leukemia are highly dependent on *in vivo* experimentation due to the lack of mimic phenomena in *in vitro*

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systems [18]. Syngeneic, immunocompetent mouse models of leukemia allow for the validation of crucial findings from *in vivo* immune cell studies. Because of experiements involving immune cells is not possible using immunodeficient mice such as NOD-Scid IL-2Rg^{Null}, which are depleted of T, B and NK cells. Thus, we developed a syngeneic leukemic mouse model and found that VEGF-C and VEGFR-3 are highly expressed in various organs of the leukemic mouse and VEGFR-3 is enriched in low functional NK cells, suggesting that NK cells in AML are affected by lymphangiogenic promoted conditions. Moreover, this study showed that the restoration of cytotoxic function on NK cells by VEGFR-3 inhibition *in vivo* system was detected with the high expression of IFN-γ and provided possibility as a therapeutic source in the preclinical stage.

Materials and methods

Mice and cell lines

C57Bl/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were maintained under pathogen-free conditions in accordance with ethical guidelines for the care of these mice at the Department of Research, Catholic University of Korea. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of the Catholic University of Korea. For the syngeneic model, C1498 (a murine myelogenous leukemia cell line, 3×10^6 cells in 200 ml PBS) were intravenously (i.v.) injected via the tail veins of C57Bl/6J. Beginning at 1 week post-injection, tissues from sacrificed tumor-bearing mice were collected and subjected to immunohistochemistry and RT-PCR after confirmation of engraftment or infiltration of leukemic cells via detection of a lymphatic marker. For MAZ51 treatment (EMD-Calbiochem, San Diego, CA, USA), C1498 cells were first injected into C57Bl/6J mice (five mice per group) via the tail vein. The MAZ51-treated group was injected (intraperitoneal injection, 200 μl in dPBS; stock in DMSO) daily with 8 mg/kg for 3 days. A control group was injected with the same amount of DMSO after injection with C1498 cells. The murine C1498 and YAC-1 cell lines were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were grown in DMEM (Dulbecco's modified Eagle's medium) (GIBCO, Grand Island, NY, USA) and were supplemented with 10% fetal bovine serum (FBS; GIBCO) and antibiotics (Invitrogen, Carlsbad, CA, USA). For cytotoxicity assays, YAC-1 cells were cultured in 10% FBS RPMI medium in a humidified atmosphere containing 5% CO_2 at 37 °C. All cell lines were purchased from ATCC.

Silencing of VEGFR-3 gene by siRNA transfection

A total of 1×10^6 splenocytes were cultured in complete RPMI 1640 medium supplemented with IL-2 (1000 U/ml, Prospec, cyt-209), non-essential amino acids and sodium pyruvate for 4 days. Then, these cells were transfected with murine VEGFR-3 siRNA or scrambled control siRNA (Invitrogen). siRNA and LipofectAMINE RANiMAX (Invitrogen) was diluted into RPMI media with reduced serum concentration according to the instruction of the supplier. The diluents were mixed and incubated for 20 min at room temperature for formation of transfection complex. Cells were incubated with the transfection complex to a final RNA concentration of 35 nmol/l for 6 h and then replaced by fresh medium. After incubation of 72 h, low concentration of MAZ51 (5 MM) was treated in siRNA transfected cells for 4 h at 7 days, then these cells were subjected to FACS analysis and cytotoxicity assay.

Cytotoxicity assay

To generate lymphokine-activated killer (LAK) cells, splenocytes (3×10^6/ml) from mice were cultured in complete RPMI 1640 medium supplemented with IL-2 (1000 U/ml, Prospec, cyt-209), non-essential amino acids and sodium pyruvate for 7 days. YAC-1 cells were used as target cells. Immune cells from leukemic mice were used as control. The target cells were labeled with a BATDA solution (DELFIA® cytotoxicity assay; PerkinElmer, Waltham, MA, USA) for 10 min, washed twice and placed into 96-well V-bottom plates at approximately 5×10^3 target cells per well. Effector cells were added to the wells and incubated for at least 6 h. After the reaction, the supernatants were collected and reacted with a europium solution for 5 min. Fluorescence intensity was measured using an EnVision system. Cytotoxicity was calculated as follows: percentage of target cell lysis = [(cpm of experimental release – cpm of spontaneous release)/(cpm of maximum release – cpm of spontaneous release)] × 100. At least four independent experiments were performed in duplicate.

Flow cytometry

The FACS staining and analysis of MNCs were performed as previously described [19]. Briefly, cells were resuspended in 100 Ml of rinsing buffer and incubated with antibodies. After washing, the cells were analyzed using a FACSCalibur flow cytometer equipped with Cell Quest software (BD Biosciences). For primary antibodies, we used phycoerythrin (PE)-conjugated mouse anti-mouse NK1.1 (553165, BD Biosciences), allophycocyanin (APC)-conjugated anti mouse CD3e (553066, BD Pharminogen), rabbit anti-mouse VEGF-C (NBP2-20853, Norvus Biologicals), FITC-conjugated anti mouse CD4 (553729, BD Pharminogen), PerCP-Cy™ 5.5-conjugated anti-mouse B220 (552771), PerCP-conjugated rat anti-mouse CD8a (553034, BD Biosciences), FITC-conjugated anti-mouse podoplanin (53–5381-82, eBioscience), biotinylated anti-mouse VEGF-R3 Ab (NBP1-43502, Norvus Biologicals) and anti-mouse IFN-r (NB100-78214, Norvus Biologicals), APC-conjugated anti-mouse CD11b (553312, BD Pharminogen). For secondary Abs, FITC-conjugated anti-mouse IgG (sc-2010, Santa Cruz Biotech), PE-conjugated anti rabbit IgG (Cy3-conjugated anti-rabbit IgG, Jackson Immunoresearch), and APC-conjugated streptavidin (17–4317-82, eBioscience) were used to detect biotinylated signals. Flow cytometric data were analyzed using appropriate controls with proper isotype-matched IgG and unstained controls.

Statistical analysis

All results are presented as the means ± SE. Data for compared groups was compared by Mann–Whitney U test. The GraphPad Prism ver. 4 software (GraphPad Software, La Jolla, CA, USA) was used for the analyses. Values of $P < 0.05$ were considered to denote statistical significance.

Results

Leukemic mouse model revealed high expression of VEGF-C and VEGFR-3, with a loss of IFN-γ in tissues

To confirm induction of leukemic mouse, gross examination and histological changes of major organs such as liver, ovary and BM tissues from the mice were investigated. Gross appearance unequivocally revealed that a significant phenotype of tumor-bearing mice was stably established when 3×10^6 C1498 cells were injected into C57Bl/6J mice. Aberrant-pigmented livers and enlarged ovaries were observed from myelogenous leukemia C1498-injected mice, demonstrating the infiltration of leukemic cells (Fig. 1A). Tissues were first observed for cancer cell engraftment or infiltration via H&E staining. Tumor clusters in liver and spindle-shaped cells, unlike the typical morphology of hematopoietic cells, were clearly observed in the vascular regions of BM from myelogenous

Fig. 1. Established leukemic mouse models displayed expression of VEGF-C and VEGFR-3. (A) Gross appearance of leukemic mice with leukemic properties. An ovary with a large tumor cluster from a C1498 cell-transplanted mouse (yellow boxes), and metastasis in leukemic mice were detected in liver. (B) H&E staining. Livers and BM from C1498 cell-injected mice also exhibited successful engraftment of leukemic cells in BM and infiltration in the liver (yellow lines). (C) Double staining from paraffin blocks also showed that VEGF-C positive cells (pinkish color) are closely located in VEGFR-3 positive cells (brown color, red arrow) magnification, × 20. (D) BM flushed cells from femur were subjected to immunocytochemistry staining for VEGFR-3 (magenta, white arrow), VEGF-C (green), and DiI (red) stain in C1498 cells (yellow arrow). Confocal imaging demonstrated DiI stained VEGF-C$^+$ cells, VEGF-C$^+$ cells without DiI and VEGFR-3$^+$ cells were detected in BM. DAPI (blue) was used for nuclear staining. Scale bar, 20 μm. (E) Expression of VEGFR-3 was highly increased in various organs from leukemic mice. Panels illustrate BM, livers, and spleens from iso control, wild-type, and C1498 cell-injected mice, respectively. Mouse models exhibited increased VEGFR-3 expression levels in peritumoral regions in various types of tissues. Immunostaining clearly displayed VEGFR-3 positive staining in cells as well as in sinusoidal vessels, compared to iso type control. Yellow arrows indicate VEGFR-3 single cells. Magnification, × 40. (F) BM-MNCs were isolated and subjected to qRT-PCR. The expression levels of lymphatic marker genes, especially *Vegfr-3*, were significantly increased in leukemic mouse tissues, including liver and BM. Genes were normalized to GAPDH. Data shown represent the means from three independent experiments (n = 4). Bars in F and G represent SEM, and asterisks depict statistically significant differences compared to normal donor groups ($^*P < 0.05$, $^{**}P < 0.01$). (G) Prepared PB-MNCs were subjected to FACS analysis. Data shown represent the means of independent experiments (n = 6). Podoplanin and VEGFR-3 in C1498 cell-injected mice gradually increased their expression depending on day. (H) VEGF-C$^+$ cells were counted from BM. Data from at least three independent experiments for each animal model are shown $^{**}P < 0.01$ (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)