Regulation of expression level of fms-like tyrosine kinase-4 is related to osteoclast differentiation

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Abstract

Introduction: The aim of this study is to determine whether regulation of the expression level of fms-like tyrosine kinase-4 (Flt-4) is related to osteoclast differentiation.
Material and methods: Osteoclast formation and differentiation of mouse bone marrow cells and RAW264.7 cells were performed. To induce osteoclast differentiation, RANKL (50 ng/ml) with or without vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D) was added to mouse bone marrow cells and RAW264.7 cells. Then cells were examined under a microscope. TRAP-positive cells with 3 nuclei or more were considered as osteoclasts and counted. The Flt-4 gene was knocked down by transfection of siRNAs against Flt-4. Immunoblot analyses were performed.
Results: The osteoclast formation assay indicated that VEGF-C resulted in 500 or 450 vs. 100 ($p < 0.05$) of osteoclasts in mouse bone marrow cells and RAW264.7 cells, respectively. Vascular endothelial growth factor-D resulted in about 600 or 630 vs. 100 ($p < 0.05$) of osteoclasts for both mouse bone marrow cells and RAW264.7 cells. The knock-down of Flt-4 expression abolished the induction by VEGF-C or VEGF-D, resulting in induction similar to that of the negative control PBS.
Conclusions: Both VEGF-C and VEGF-D can induce osteoclast differentiation in the presence of the receptor activator of nuclear factor κB ligand. Down-regulation of expression level of Flt-4 protein abolishes osteoclast differentiation induced by VEGF-C or VEGF-D.

Key words: fms-like tyrosine kinase-4, osteoclast differentiation, vascular endothelial growth factor-C, vascular endothelial growth factor-D.

Introduction

Strength of the human skeleton is dependent on the balance between bone resorption by osteoclasts and bone formation by osteoblasts. In bone metabolisms, bone is resorbed by osteoclasts and replaced by new bone generated by osteoblasts [1]. Therefore, the balance between functions of osteoclasts and osteoblasts contributes to the maintenance of correct bone structure, bone mass and bone qualities. However, this important balance can be affected by pathological increases in osteoclast

numbers, since it will lead to a net loss of bone. Osteoclasts are derived from pluripotent hematopoietic stem cells, which generate myeloid stem cells that then further differentiate into osteoclasts, megakaryocytes, granulocytes, and so on. Numerous hormones and cytokines generated in the bone microenvironment can regulate osteoclast differentiation and their functions [2–5]. The detailed mechanisms underlying regulation of osteoclast differentiation are not well understood but are of great clinical importance.

Vascular endothelial growth factors (VEGFs) are proteins produced by cells that stimulate vasculogenesis and angiogenesis. Vascular endothelial growth factors contributes to formation of new blood vessels during embryonic development or after injury. Vascular endothelial growth factors, including VEGF-A, -B, -C, -D, were found to be involved in many cellular processes [6–9]. Vascular endothelial growth factor-C and VEGF-D are ligands for the vascular endothelial growth factor receptor-3, which mediates lymphangiogenesis [10–14].

VEGF receptor-3 is an fms-like tyrosine kinase [15]; it is also termed fms-like tyrosine kinase-4 (Flt-4). Flt-4 is important for many signaling pathways. Interaction of Flt-4 and the focal adhesion kinase is related to survival of human neuroblastoma cell lines [16]. Mutations in Flt-4 result in Milroy disease, which is an autosomal dominant condition associated with congenital lymphedema [17]. In addition, it is reported that mutation in VEGF-C is associated with the autosomal dominant Milroy-like primary lymphedema [18].

In this study, we used VEGF-C and VEGF-D to induce osteoclast differentiation in cultured human peripheral blood mononuclear cells. We also used transfection of siRNA against Flt-4 to knock-down expression of Flt-4. It was found that decreases in expression of Flt-4 reduce osteoclast differentiation.

Material and methods

Cell lines and reagents

The mouse bone marrow cells and the macrophage/pre-osteoclast cell line RAW264.7 (purchased from ATCC, Manassas, VA, USA) were maintained in α-minimal essential medium (MEM; Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; CSL Biosciences, VIC, Australia), 0.005% penicillin (10,000 U/ml)/streptomycin (10,000 U/ml) (Life Technologies, USA), L-glutamine (Life Technologies) and HEPES (Sigma-Aldrich, USA). Cells were cultured at 37°C with 5% CO_2 and 100% humidity. Recombinant soluble murine receptor activator of nuclear factor κB ligand (RANKL) was purchased from the Oriental Yeast Co. (Tokyo, Japan). For tartrate resistant acid phosphatase (TRAP) staining, naphthol AMX phosphate, fast red violet LB Salt F-1625, and dimethylformamide were purchased from Sigma-Aldrich (St Louis, MO, USA). The human macrophage-colony stimulating factor (M-CSF) was purchased from R&D Systems (Minneapolis, MN, USA). The rhVEGF-C and rhVEGF-D were purchased from R&D Systems.

Osteoclast differentiation

Osteoclast formation and differentiation experiments were performed as previously described [19, 20]. Briefly, mouse bone marrow cells (1×10^5 cells/well) and RAW264.7 cells (1×10^4 cells/well) were cultured in tissue culture wells (6 mm diameter) containing 0.2 ml of medium (MEM/FBS). To induce osteoclast differentiation, 50 ng/ml RANKL were added to the cells and the medium was replaced with fresh medium every 3 days. On day 8 of incubation, cells were fixed in 4% buffered formalin for 12 min and then washed with a mixture of methanol and acetone with a ratio of 1 : 1. The samples were air dried and histochemically stained for TRAP detection [21]. The TRAP-positive cells with 3 nuclei or more as examined by microscopy were considered as osteoclasts and counted.

For osteoclast survival assays, osteoclasts were dispersed using non-enzymic cell dispersion buffer (Sigma, cat. C5914, USA), rinsed with PBS, resuspended in MEM/10% FBS and plated in 6 mm culture wells (10^5 cells/well). Cells were cultured for 48 h with RANKL in combination with other indicated treatments (PBS, VEGF-C, VEGF-D, or M-CSF). Cells were then fixed and histochemically stained for TRAP examination and the osteoclast numbers were counted. Data were expressed as relative to positive control cultures stimulated by RANKL alone.

siRNA experiments

RAW264.7 cells were transfected with 80 pmol of siRNA against the mouse Flt-4 gene message (cat no. sc-35398; Santa Cruz Biotechnology) or a negative control siRNA (cat no. sc-37007, Santa Cruz Biotechnology) using X-tremeGENE (Roche, USA). After 4 h, cells were then treated with Hsp90 inhibitors or vehicle control. Two days later, total protein was harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses.

Immunoblot assays

Total proteins were harvested from cells, separated on 10% SDS/PAGE gels, and then subjected to immunoblot analyses. The primary antibodies against Flt-4 and β-actin were purchased from Santa Cruz, USA (anti-Flt-4, cat # sc- sc-365748, 1 : 200; anti-β-actin, cat# sc-130301, 1 : 10,000). Secondary antibodies were horseradish-peroxi-

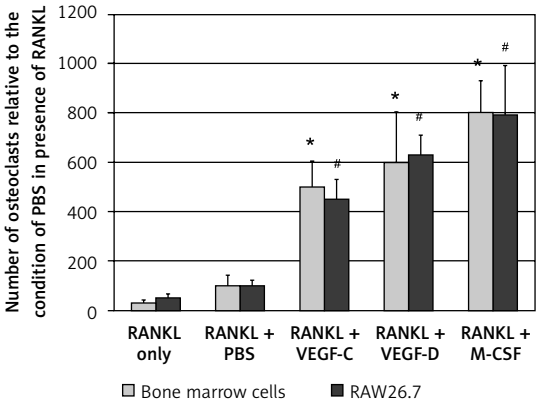

Figure 1. Effects of cytokines on osteoclast production. Osteoclasts of mouse bone marrow cells and RAW264.7 cells were cultured in the absence or presence of RANKL (50 ng/ml), together with PBS, VEGF-C (50 ng/ml), VEGF-D (50 ng/ml), or M-CSF (30 ng/ml). The number of osteoclasts in each condition was counted. All experiments were repeated at least 3 times

*Data are expressed as means ± SD. *Values of p < 0.05, comparisons with RANKL only for osteoclasts of mouse bone marrow cells. # Values of p < 0.05, comparisons with RANKL only for osteoclasts of RAW264.7 cells.*

dase-conjugated secondary anti-mouse IgG (cat # 31430, 1 : 10,000; Pierce Biotechnology). Bound antibodies were detected using the ECL system (Pierce Biotechnology, USA). The experiments were repeated at least 3 times. The mean normalized optical density (OD) of detected protein bands relative to the OD of the β-actin band was calculated.

Statistical analysis

The experimental data are expressed as mean + SD. Statistical software (SPSS 10.0, Chicago, USA) was used for independent sample *t* tests. In all analyses, $p < 0.05$ was considered statistically significant.

Results

VEGF-C or VEGF-D induces osteoclast differentiation in the presence of RANKL

The VEGF-C and VEGF-D are two growth factors that bind to Flt-4. To determine if VEGF-C or VEGF-D can induce osteoclast differentiation, the mouse bone marrow cells and RAW264.7 cells were cultured in the presence of RANKL, in the presence of VEGF-C, VEGF-D, or M-CSF. The M-CSF served as a positive control and PBS served as a negative control. On day 8 of incubation, cells were fixed in 4% buffered formalin and then washed with a mixture of methanol and acetone. TRAP-positive cells with 3 nuclei or more were considered as osteoclasts and counted as ratios of the negative control (PBS).

As shown in Figure 1, in the absence of RANKL, very few, if any, osteoclasts were produced. When compared with the condition in the presence of RANKL only (RANKL + PBS), VEGF-C resulted in 500 or 450 vs. 100 ($p < 0.05$) of osteoclasts of mouse bone marrow cells and RAW264.7 cells, respectively. VEGF-D resulted in about 600 or 630 vs. 100 ($p < 0.05$) of osteoclasts for both mouse bone marrow cells and RAW264.7 cells. Although lower than the effect of M-CSF on osteoclast induction, the effects of VEGF-C and VEGF-D were obvious (Figure 1). These results suggest that both VEGF-C and VEGF-D can induce osteoclast differentiation in the presence of RANKL.

Knock-down of Flt-4 gene in pre-osteoclast RAW264.7 cells

Since VEGF-C and VEGF-D bind to Flt-4 protein on the surface of cells, we further knocked down the Flt-4 gene in RAW264.7 cells. The cells were transfected with PBS only, the control siRNAs, or siRNA against Flt-4 gene using X-tremeGENE (Roche, USA). Two days later, total proteins were harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses. As shown in Figure 2, the levels of Flt-4 were significantly down-regulated in the RAW264.7 cells. These results suggest that the Flt-4 protein expression levels can be specifically knocked down in the RAW264.7 cells.

Knock-down of Flt-4 protein abolishes the induction of osteoclast production by VEGF-C or VEGF-D

Since we found that both VEGF-C and VEGF-D can induce osteoclast differentiation in the presence of RANKL, the related molecular mechanism was further investigated. The VEGF-C and VEGF-D both bind to Flt-4 protein on the surface of cells. We therefore determined the effects of knock-down of Flt-4 protein on the induction of osteoclast production by VEGF-C or VEGF-D. The Flt-4 gene siRNA-transfected RAW264.7 cells were cultured in the absence or presence of RANKL (50 ng/ml), together with PBS, VEGF-C (50 ng/ml), VEGF-D (50 ng/ml), or M-CSF (30 ng/ml). Eight days later, the number of osteoclasts in each condition was counted. All experiments were repeated at least 3 times. Data are expressed as means ± SD.

As shown in Figure 3, when compared with the condition in the presence of RANKL only (RANKL + PBS), VEGF-C resulted in osteoclast production with a similar level to that of PBS only in RAW264.7 cells. The VEGF-D caused osteoclast production with a similar level to that of PBS only in RAW264.7 cells. However, the effect of M-CSF was still obvious, resulting in a 770 vs. 100 ($p < 0.05$) when compared with the condition of PBS only.

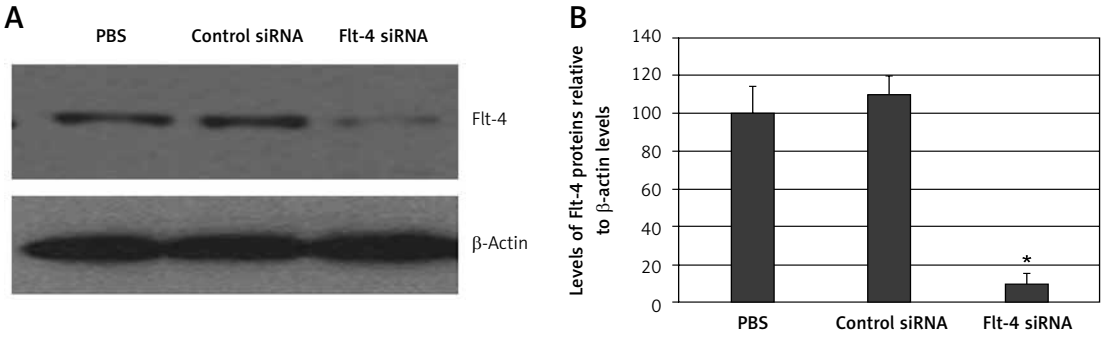

Figure 2. Knock-down of Flt-4 gene in RAW264.7 cells. **A** – RAW264.7 cells were transfected with PBS only, the control siRNAs, or siRNA against Flt-4 gene. RAW264.7 cells were transfected with 80 pmol of siRNA against the mouse Flt-4 gene message or a negative control siRNA using X-tremeGENE (Roche, USA). Two days later, total proteins were harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses. **B** – The bands of Flt-4 proteins were scanned and the ratios (mean ± SD) of the OD values relative to the OD value of β-actin bands were calculated

**Values of p < 0.05, comparisons with cells transfected with the control siRNAs.*

These results suggest that down-regulation of expression level of Flt-4 protein abolishes osteoclast differentiation induced by VEGF-C or VEGF-D.

Discussion

Many cellular factors were involved in the regulation of osteoclast differentiation and their functions [3–5, 22–24]. It have been reported that mutations of Flt-4 lead to Milroy disease, which is an autosomal dominant condition associated with congenital lymphedema [16, 17]. In this study, we found that VEGF-C increased production of osteoclasts of mouse bone marrow cells and RAW264.7 cells. VEGF-D also resulted in about 600 or 630 vs. 100 ($p < 0.05$) of osteoclasts for both mouse bone marrow cells and RAW264.7 cells. Furthermore, the knock-down of Flt-4 abolished the induction by VEGF-C or VEGF-D, resulting in induction similar to that of the negative control PBS. Out findings suggest that down-regulation of the expression level of Flt-4 protein abolishes osteoclast differentiation induced by VEGF-C or VEGF-D.

Since VEGF-C and VEGF-D are important cellular factors involved in many cellular processes, our findings suggest that down-regulation of Flt-4 expression may affect other cellular processes induced by VEGF-C or VEGF-D. Recently, it was reported that VEGF-C and VEGF-D are involved in the inflammatory tumor microenvironment and inhibition of VEGF-C and VEGF-D delays the progression of skin tumors [25]. The serum VEGF-D level may act as a marker facilitating diagnosis of some disease such as lymphangioleiomyomatosis [26]. Therefore, our results suggest that Flt-4, VEGF-C, and VEGF-D might be used in prediction of development of some diseases.

In addition to Flt-4, many cellular proteins were found to be related to osteoclast differentiation. For example, the nuclear factor E2-related factor 2 affects RANKL-dependent osteoclastogenesis via

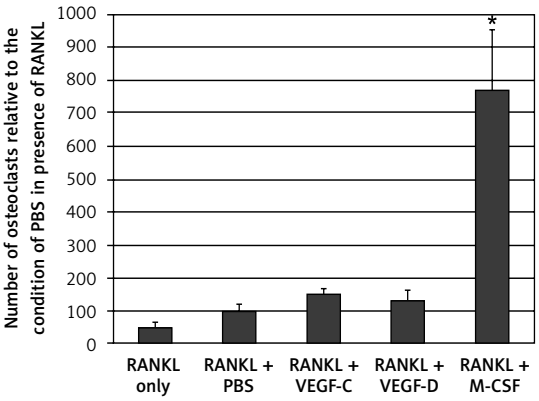

Figure 3. Effects of knock-down of Flt-4 gene on osteoclast production induced by VEGF-C and VEGF-D. The pre-osteoclast RAW264.7 cells were transfected with the control siRNAs or siRNA against the Flt-4 gene by the methods used in Figure 2. The Flt-4 gene siRNA-transfected RAW264.7 cells were cultured in the absence or presence of RANKL (50 ng/ml), together with PBS, VEGF-C (50 ng/ml), VEGF-D (50 ng/ml), or M-CSF (30 ng/ml). The number of osteoclasts in each condition was counted. All experiments were repeated at least 3 times

*Data are expressed as means ± SD. *Values of p < 0.05, comparisons with RANKL only for osteoclasts of RAW264.7 cells.*

alteration of intracellular ROS signaling through expression of cytoprotective enzymes [27]. Enhancement of the mitogen-activated protein kinase pathway and cyclooxygenase-2 expression in RAW264.7 cells increases osteoclast differentiation and activation [28]. Therefore, the findings in this study provide new evidence for the cellular factor-mediated processes induced by VEGF-C [29, 30] and VEGF-D [31].

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Conflict of interest

The authors declare no conflict of interest.

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