VEGF induces osteoclast differentiation via FLT-1 and FLK-1.

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Abstract

It has been reported that vascular endothelial growth factor (VEGF) 165 and placenta growth factor (PlGF) can induce differentiation of osteoclasts via Flt-1, because Flt-1 is the common receptor of these two factors. However, little is known about osteoclast differentiation via Flk-1, which is one of the main receptors for VEGF165. VEGF-E showed potent stimulatory activity to endothelial cell growth vascular permeability similar to those of VEGF165 via Flk-1 but not Flt-1. The purpose of this study was to clarify whether VEGF-E can induce osteoclast and the receptors of VEGF families for osteoclast differentiation. Ten μg VEGF-E was injected into 11-day-old op/op mice and the number of osteoclasts was observed 3 days after the injection. The effects of both Flt-1- and Flk-1- neutralizing antibodies against the injection of VEGF165 were also observed. Finally, the expression of these receptors in osteoclast precursors (OCP) was examined. After VEGF-E injection into op/op mice, many osteoclasts were induced. However, the number was less than that induced by VEGF165. The number of osteoclasts induced by VEGF165 was significantly reduced by the injection of either Flt-1- or Flk-1- neutralizing antibodies. Flt-1 and Flk-1 were clearly detected in OCP at both the mRNA and protein levels. We concluded that VEGF-E can also induce many osteoclasts in op/op mice. Furthermore, it is suggested that the VEGF165 signal for osteoclast differentiation is mediated via both Flt-1 and Flk-1.

Introduction

Osteopetrotic (op/op) mice are characterized by a deficiency in osteoclasts, monocytes, and macrophages caused by the absence of functional macrophage-colony stimulating factor (M-CSF) [1]. It has been reported that osteopetrosis in mutant mice can be cured by continuous injections of recombinant human (rh) M-CSF [2,3]. Thus, it was suggested that M-CSF is an important factor for osteoclast differentiation.

Receptor activator of NF-kappaB ligands (RANKL) is expressed on the plasma membrane of osteoblasts/bone marrow stromal cells. A soluble form of opacity distribution function (ODF) has induced osteoclast-like multinucleated cells in the presence of M-CSF in mouse spleen cell cultures [4]. A TNF family molecule, osteoprotegerin ligand (osteoprotegerin ligand (OPGL); also known as TNF-related activation-induced cytokine (TRANCE), RANKL and ODF) was identified as a potential osteoclast differentiation factor and mice with a disrupted OPGL gene showed severe osteopetrosis as a result of an inability of osteoblasts to support osteoclastogenesis [5]. These results suggest that both M-CSF and OPGL are essential for osteoclast differentiation. However, it is also well known that osteoclasts in op/op mice increase progressively with age [6]. Niida et al., demonstrated that vascular endothelial growth factor 165 (VEGF165) and placenta growth factor (PlGF) can substitute for M-CSF in osteoclast induction in the mutant mice [7]. VEGF165 is highly specific mitogens for vascular endothelial cells [3,8]. The 165-amino acid (VEGF165), having 165 amino acids, is the major gene product found in human tissue and is the most effective angiogenic factor in the VEGF family [9,10,11]. Because osteoclasts predominantly express Flt-1 which is one of the major receptors for VEGF165 and also the only receptor for PI GF [12,13], it has been suggested that VEGF165 and PI GF may induce osteoclasts through Flt-1. On the other hand, it has been
shown that osteoclasts expresses another VEGF receptor, Flk-1 as well as Flt-1, and VEGF165 can support osteoclastic bone resorption and survival of mature osteoclasts [14]. From these findings, we hypothesized that both Flt-1 and Flk-1 may act as receptors of VEGF165 for osteoclast differentiation. Recently, Ogawa et al. identified a gene encoding a polypeptide with an approximately 25% amino acid similarity to mammalian VEGF165 in the genome of Orf virus and proposed the name VEGF-E for this protein [15]. The purpose of this study was to clarify the receptors of VEGF families for osteoclast differentiation. Therefore, we investigated the effect of VEGF-E on osteoclast differentiation in op/op mice and examined the influence of Flt-1- and Flk-1-neutralizing antibodies on osteoclast induction by VEGF165 in op/op mice. Furthermore, we examined Flt-1 and Flk-1 expression at both the mRNA and protein levels in osteoclast precursors (OCP).

Materials and Methods

VEGF-E Injection

B6C3Fe-a/a-op/+ male and female mice, as a breeding pair, were obtained from Jackson Laboratory (Bar Harbor, USA), and normal and op/op genotypes were raised in our laboratory. The op/op mice were identified by failure of incisor eruption at about 10 days after birth. Ten μg recombinant human (rh) VEGF-E (Relia Tech, Braun- schweig, Germany) was injected into the peritoneal cavities of 11-day-old op/op mice. Non-injected op/op mice were used as a control group (n=5 each). Three days after the injection, the mice were killed under general anesthesia.

Injection of Flt-1 and Flk-1 neutralizing antibodies

To investigate the effect of Flt-1 and Flk-1 neutralizing antibodies on osteoclast induction, fifteen 11-day-old op/op mice were used and divided into three groups. Group 1: 0.5 μg rhVEGF165 only (Pepro Tech, London, UK) was injected. Group 2: 50 μg Flt-1 neutralizing antibodies (R & D Systems, Minneapolis, USA) were injected at 6 hours before 0.5 μg rhVEGF165 injection and 6 hours before sacrifice (total 100 μg). Group 3: 50 μg Flk-1 neutralizing antibodies (R & D Systems, Minneapolis, USA) were injected at 6 hours before 0.5 μg rhVEGF165 injection and 6 hours before sacrifice (total 100 μg). Five animals were used for each group respectively (total 15 animals). Three days after the VEGF165 injection, the mice were killed under general anesthesia. The animals were treated under ethical regulations as defined by the Ethics Committee, Hiroshima University Faculty of Dentistry.

Histological Examination

Three days after the injection, the mice were killed under general anesthesia with sodium pentobarbital, fixed in 4% paraformaldehyde. The femora were removed from op/op and the specimens were decalcified in 14% EDTA (pH 7.4) for 14 days and embedded in paraffin and cut into longitudinal sections of 7 μm thickness. The five sections from each mouse were stained with tartrate-resistant acid phosphatase (TRAP), which is a generally acknowledged cytochemical characteristic of osteoclasts [8]. Osteoclasts were identified by staining with TRAP, as multi-nucleated cells on the bone surface.

Cell Culture

Murine osteoclast precursor cells (OCP) were obtained from Cell Garage (Tokyo, Japan). The cells were cultured in α-modified MEM (Sigma, Saint Louis, USA) containing 10% FCS (Biological Industries, Kibbutz Beit-Haemek, Israel), 32 U/mL penicillin G (Meiji Seika, Tokyo, Japan), 250 μg/ml amphotericin B (Nacalai Tesque, Kyoto, Japan) and 60 μg/ml kanamycin (Meiji Seika, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO₂.

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from the cell cultures with a Quickprep Total RNA extraction kit (Amersham Biosciences, Tokyo, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA by use of Oligo (dT) 20 primer (Toyobo, Osaka, Japan) and a ReverTra Ace-a first strand cDNA synthesis kit (Toyobo).

Primers and RT-PCR

We used the primers for mouse Flt-1 and Flk-1 obtained from R & D Systems (Minneapolis Inc., Minneapolis, USA). A reverse transcriptase-polymerase chain reaction (RT-PCR) was performed from 1 μl of sample cDNA and the amplification was carried out for 33 cycles (45 s at 94°C, 45 s at 54°C, 45 s at 72°C). The PCR products were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Non-RT samples were used as negative controls.

Immunoblot Analysis

For immunoblot analysis, OCP were collected and washed with cold PBS. The cell pellet was lysed in a cell extraction buffer consisting of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₃P₂O₇, 2 mM Na₂VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate. The extract was centrifuged at 13000 rpm for 10 min at 4°C. The
samples were electrophoresed through 7.5% acrylamide gel and the protein was transferred onto a nitrocellulose blotting membrane. The membrane was treated with 10 μg/ml of rabbit anti-Flt-1 and Flk-1 polyclonal antibodies (LAB VISION, Fremont, CA, USA). As a peroxidase-labeled secondary antibody, a goat anti-rabbit IgG (1 μg/ml) was used. Then, the membrane was stained with TMB Membrane Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MA, USA).

**Statistical Analysis**

Statistical significance was evaluated by analysis of variance (ANOVA) and multiple-comparison test (Scheffé). A confidence level of $p < 0.05$ was defined as statistically significant. We performed each experiment three times for statistical analysis.

**Results**

**Changes in the numbers of osteoclasts resulting from rhVEGF-E injection into op/op mice**

First, we examined whether VEGF-E can induce osteoclasts in op/op mice, because Flk-1 is the only specific receptor for VEGF-E. Many osteoclasts appeared in rhVEGF-E injected op/op mice, and there were lacunae of resorption by osteoclasts (Fig. 1A), while only a few osteoclasts were detected in non-injected op/op mice and the bone marrow cavity was filled with excessive bone trabeculae (Fig. 1B). The number of osteoclasts in the rhVEGF-E injected op/op mice was significantly larger than in non-injected mice ($p < 0.01$, Fig. 2).

**Figure 1: Histological examination of the femora of the rhVEGF-E injection op/op mice.**

**The effect of Flt-1 and Flk-1 neutralizing antibodies on osteoclast induction after rhVEGF165 injection into normal mice**

After injection of 0.5 μg rhVEGF165, many osteoclasts appeared in the femora of op/op mice. In contrast, the injection of Flt-1 or Flk-1 neutralizing antibodies significantly suppressed the increase of osteoclast number induced by rhVEGF165 (Fig. 3, $p < 0.01$). No significant difference was found between the injection of Flt-1 and Flk-1 antibodies.

**Figure 2: Changes in the number of osteoclasts in the femora after injection of rhVEGF-E into op/op mice.**

**Figure 3: Changes in the number of osteoclasts in the femora after injection of rhVEGF-E into op/op mice.**

**Expression of Flt-1 and Flk-1 in OCP**

We hypothesized that OCP possess both Flt-1 and Flk-1, and that osteoclast differentiation is mediated via these receptors. Therefore, to determine the expression of VEGF165 receptors at the mRNA level, RT-PCRs for Flt-1 and Flk-1 were performed. The cDNAs for Flt-1 and
Flk-1 were amplified at a position corresponding to 569 bp for Flt-1 and 302 bp for Flk-1 products (Fig. 4A). No PCR product was detected for negative controls (Fig. 4A). We also confirmed the expression of the two receptors at the protein level by Western blot analysis. With the anti-Flt-1 antibody, we found an immunoreactive protein band of 180 kDa corresponding to Flt-1. A band of 180 kDa corresponding to Flk-1 protein was also detected with anti-Flk-1 antibody (Fig. 4B).

**Figure 4:** RT-PCR (A) and Western blotting analysis (B) for the expression of VEGF receptors in osteoclast precursor (OCP)

**DISCUSSION**

Osteopetrosis in op/op mice is caused by a severe deficiency of osteoclasts as a result of the absence of functional M-CSF. The mutant mice have a 1-bp insertion within the coding region of the M-CSF gene on chromosome 3 [1]. Previous studies demonstrated that injection of rhM-CSF can induce osteoclast differentiation and cure the disease [2,9]. Kodama et al. established a clonal stromal cell line OP6L7 capable of supporting hemopoiesis from newborn op/op mouse calvaria, and showed that TRAP-positive cells appeared only when bone marrow cells were cultured in contact with OP6L7 cells and rhM-CSF [16], a TNF family molecule, osteoprotegerin ligand (OPGL; also known as TRANCE, RANKL and ODF) was identified as a potential osteoclast differentiation factor and mice with a disrupted OPGL gene showed severe osteopetrosis as a result of an inability of osteoblasts to support osteoclastogenesis [5]. However, it is well accepted that op/op mice show an increase in osteoclast activity with aging, indicating that op/op mice demonstrate an age-related resolution of osteopetrosis [6]. This finding suggests that some alternative factor may substitute for M-CSF in osteoclast differentiation. Niida et al. [7] showed that a single injection of rhVEGF165 and rhPIGF can induce osteoclast recruitment in op/op mice. Most of the osteoclasts induced gradually with age in op/op mice disappeared after injections of anti-VEGF165 antibody, suggesting that endogenous VEGF165 may cause osteoclast differentiation in aged op/op mice [7]. Since Flt-1 is a common receptor for VEGF165 and PIGF, and both factors showed monocyte activation and chemotaxis mediated via Flt-1 [17,18], it is reasonable to suspect that VEGF165 induces osteoclasts via Flt-1. On the other hand, Nakagawa et al. found that VEGF165 stimulated the bone-resorbing activity of isolated mature osteoclasts by enhancing their survival [14]. Mature osteoclasts expressed Flt-1 and Flk-1 at the mRNA and protein levels, indicating that the stimulatory effect of VEGF165 might be mediated through either receptor, or through both of them [12]. However, there was no information available as to which receptor could act individually, especially during osteoclast differentiation.

A previous study showed that VEGF-E demonstrated mitotic activity on primary endothelial cells and vascular permeability activity at the same levels to those of VEGF165 [15]. Since VEGF-E bound Flt-1 but not Flt-1, it was thought that these activities were mediated only through Flk-1 [15,19]. Our present study clearly showed that a single injection of VEGF-E can induce osteoclast recruitment in op/op mice, suggesting that the VEGF-E signal is mediated through Flk-1. Taken together with the previous report that PIGF can also induce many osteoclasts in op/op mice [7], it is suggested that VEGF family may induce osteoclasts via both Flt-1 and Flk-1. Our result showed that around 20 osteoclasts appeared on op/op mice by VEGF-E injection. Niida et al. reported that around 40 osteoclasts were observed in op/op mice by VEGF165 and PIGF injection. From these findings, it is suggested that VEGF-E has almost same effect for osteoclast induction compared with VEGF165 and PIGF.

Next, we examined the effect of neutralizing antibodies for Flt-1 and Flk-1 on osteoclast differentiation in op/op mice. Flt-1 or Flk-1 neutralizing antibodies significantly reduced the number of osteoclasts induced by rhVEGF165. Furthermore, we found that osteoclast precursor (OCP) expressed Flt-1 and Flk-1 at the mRNA and protein levels. These findings confirmed our suggestion that Flt-1 and Flk-1 act as receptors for VEGF family during osteoclast differentiation.

VEGF165, known as a vascular permeability factor [20], is a potent and specific mitogen for vascular endothelial cells and promotes neovascularization [21,22,23]. It is also well known that angiogenesis is necessary for bone remodeling. Recently, it was reported that cyclic tensile forces enhance the expression of VEGF165 in osteoblastic MC3T3-E1 cells, suggesting that osteogenic cells (such as osteoblasts) and angiogenesis are closely related to each other [24]. Moreover, Kodama et al. showed that estrogen deficiency after ovariectomy could induce osteoclast formation in op/op mice. The upregulation of the number of osteoclasts was inhibited by VEGF165-neutralizing antibodies [25]. From these findings, it is thought that VEGF165 may act as both an angiogenic and a bone remodeling factor in several biological conditions mediated through Flt-1 and Flk-1.
Osteoclast induction via VEGF receptors

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