Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts

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Abstract In bone development and regeneration, angiogenesis and bone/cartilage resorption are essential processes and are closely associated with each other, suggesting a common mediator for these two biological events. To address this interrelationship, we examined the effect of vascular endothelial growth factor (VEGF), the most critical growth factor for angiogenesis, on osteoclastic bone-resorbing activity in a culture of highly purified rabbit mature osteoclasts. VEGF caused a dose- and time-dependent increase in the area of bone resorption pits excavated by the isolated osteoclasts, partially by enhancing the survival of the cells. Two distinct VEGF receptors, KDR/Flk-1 and Flt-1, were detectable in osteoclasts at the gene and protein levels, and VEGF induced tyrosine phosphorylation of proteins in osteoclasts. Thus, osteoclastic function and angiogenesis are up-regulated by a common mediator such as VEGF.

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Key words: Vascular endothelial growth factor; Osteoclast; Bone resorption; KDR/Flk-1; Flt-1

1. Introduction

Angiogenesis is required for the development, remodeling and repair of most tissue including bone. In skeleton, the appearance of bone/cartilage-resorbing cells such as osteoclasts and chondroclasts coincides with blood vessel invasion, and the formation of new capillaries and the resorption of mineralized matrices are essential events for bone morphogenesis and growth [1]. This intimate interrelationship between the bone/cartilage resorption and the angiogenesis also occurs in pathological bone disorders including bone metastasis and rheumatoid arthritis [2,3]. Thus, the simultaneous appearance of the bone/cartilage-resorbing and the invasion by blood vessels suggests that there may be a common modulator that regulates both angiogenesis and bone/cartilage resorption.

Vascular endothelial growth factor (VEGF) is the most important essential mediator of angiogenesis [4]. Although VEGF is secreted by many cell types, osteoblasts and chondrocytes have been reported to produce this cytokine in the skeleton [5,6]. Besides the stimulation of angiogenesis, VEGF has also been demonstrated to be involved in early hematopoietic development and chemotaxis of monocytes [7,8]. A recent immunohistological study revealed the expression of VEGF receptors on chondroclasts as well as on endothelial cells [6]. In addition, it has most recently been demonstrated that VEGF induced osteoclastogenesis with a combination of receptor activator of NF-kB ligand (RANKL) [9]. These findings suggest that VEGF may regulate osteoclast differentiation as well as angiogenesis. However, there is no information about the direct action of VEGF on the function of fully differentiated osteoclasts in vitro.

In this study, using a population of functional osteoclasts of extremely high purity (more than 95%) from rabbit bones [10], we examined if VEGF directly acts on osteoclast function and if VEGF receptors are expressed in mature osteoclasts. Here we report our findings indicating that VEGF directly enhances the osteoclastic bone resorption probably through KDR/Flk-1 and/or Flt-1 expressed in mature osteoclasts.

2. Materials and methods

2.1. Isolation of mature osteoclasts and pit assay

Purified mature osteoclasts were prepared using 10-day-old rabbits as described previously [10]. Briefly, long bones from 10-day-old rabbits were minced with scissors and agitated with a vortex mixer. An aliquot of unfractonated bone cells was seeded onto 0.24% collagen gel (Nitta Gelatin, Tokyo) coated on 100-mm tissue culture dishes and incubated. Two hours later, the non-adherent cells were washed off and the remaining osteoclasts were then removed from the gels with 0.1% collagenase solution (Wako Pure Chemicals Co.). By staining with tartrate resistant acid phosphatase (TRAP), we ascertained that more than 95% of isolated cells were multinucleate osteoclasts. Isolated osteoclasts (200 cells/well) were cultured on a dentine slice placed in each well of 96-well plates containing 0.1 ml of α-MEM/5% fetal bovine serum (FBS). After 24-h incubation, the medium was then replaced with the fresh medium with or without recombinant human VEGF (165-amino acid form, Genzyme/Techne, Cambridge, MA, USA). After culture, the cells were scraped off the dentine slices, and the slices were stained with acid hematoxylin [11]. The stained pit areas were determined under a microscope at ×100 magnification by counting the number of mesh squares (100×100 μm) covering the pit to evaluate possible osteoclastic bone resorption. In the case of TRAP staining, after fixation, the osteoclasts were stained for TRAP activity with a leukocyte acid phosphatase kit (Sigma Chemical Co., St. Louis, MO, USA), and the number of adherent TRAP-positive osteoclasts was counted.

For Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR), we purified rabbit osteoclasts on plastic dishes by the method of Tezuka et al. [12]. Briefly, the above unfractonated bone cells were plated into 100-mm tissue culture dishes, and after an overnight culture, the non-adherent cells were removed by several washes with phosphate-buffered saline (PBS). We used the remaining adherent cells for immunocytochemical detection of VEGF receptors (KDR/Flk-1 and Flt-1). The adherent cells were incubated in PBS containing 0.001% pronase E and 0.02% EDTA for
10 min at room temperature. By this incubation, cells other than osteoclasts became detached from dishes and were washed off. The purity of the remaining osteoclastic multinucleate cells on the plastic dishes was also more than 95%.

2.2. Normal human umbilical vein endothelial cell (HUVEC) cultures
HUVECs, purchased from Bio Whittaker (Walkersville, MD, USA), were cultured in the manufacturer’s recommended medium (EBM-2) supplemented with EGM-2 SingleQuots (2% FBS, 0.4 µg/ml hydrocortisone, 4 µg/ml recombinant human bFGF, 1 µg/ml VEGF, 1 µg/ml R-IGF-1, 1 µg/ml ascorbic acid, 1 µg/ml recombinant human EGF, 1 µg/ml GA-1000, 1 µg/ml heparin) at 37°C in a 5% CO2 incubator. We used these cells at early passages (2–3) for RT-PCR, immunocytochemistry and immunoblot analysis as a positive control.

2.3. Assay for tyrosine phosphorylation of cellular proteins
The isolated osteoclasts on plastic dishes were cultured in α-MEM supplemented with 5% FBS for 3 h. Then, the osteoclasts were treated with various concentrations VEGF for various periods. The cells were quickly washed with ice-cold PBS containing 5 mM EDTA and 1 mM Na3VO4, and lysed with a lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 mM Na2HPO4, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM Na3VO4, 1 mM aminoethyl-benzenesulfonyl fluoride (ABSF), 10 µg/ml aprotinin and 10 µg/ml leupeptin. The protein concentration in the cell lysate was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA). Each cell lysate containing equal amounts of protein was electrophoresed through 8% SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blockage with 5% bovine serum albumin, the membrane was incubated with 0.5 µg/ml of anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) and subsequently with 0.2 µg/ml of peroxidase-conjugated anti-mouse IgG antibody. Phosphotyrosine-containing proteins were visualized with the use of Western blot chemiluminescence reagents (Dupont New England Nuclear Products, Boston, MA, USA) following the manufacturer’s instructions.

2.4. RT-PCR
Total RNA was extracted from rabbit osteoclasts prepared as mentioned above, from rabbit aorta freshly obtained from a 7-day-old rabbit, and from HUVECs by acid guanidium thiocyanate–phenol–chloroform extraction with TRIZOL® reagent (Gibco BRL, Gaithersburg, MD, USA). The first-strand cDNA was synthesized from 1 µg of total RNA using a supercript II preamplification system (Gibco BRL). Primers were designed on the basis of the reported human Flt-1 and KDR/Flk-1 sequences (GenBank databases: AF063657 and AF063658, respectively). Sequences of the primers used for PCR amplification were 5′-CAAGTGCTAAGGGCATGGA-3′; KDR/Flk-1 reverse, 5′-CAGCACTCCAGCATGTC-3′; Flk-1 forward, 5′-CAAGTGCTAAGGGCATGGA-3′; KDR/Flk-1 reverse, 5′-CAGCACTCCAGCATGTC-3′. Amplification was carried out for 30 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 1 min for each in a 50-µl reaction solution containing 5 µl of each cDNA, 1 µM of sense and antisense primers, 1.25 U of Taq DNA polymerase (Quanta GmbH, Hilden, Germany) and 0.2 mM of each dNTP. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The amplified cDNAs for KDR/Flk-1 and Flt-1 from rabbit osteoclasts were subcloned into the pPCR 3.1 vector (Invitrogen Corp., San Diego, CA, USA) according to the manufacturer’s instructions. Each cDNA insert was sequenced by the dideoxynucleotide chain termination method, using a BioDye Terminator Cycle Sequencing kit and an ABI PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA).

2.5. Immunocytochemistry and immunoblot analysis for KDR/Flk-1 and Flt-1
Unfractionated bone cells were fixed with ice-cold fixation solution consisting of 25 mM sodium phosphate (pH 7.5), 45% acetone and 9% formaldehyde for 3 min at 4°C. After having been blocked with 5% skim milk and 0.1% Triton X-100 in PBS, the fixed cells were immunoreacted with 1 µg/ml of monoclonal mouse anti-KDR/Flk-1 IgG (Santa Cruz Biotechnology, Inc., San Diego, CA, USA) or polyclonal rabbit anti-Flt-1 IgG (Upstate Biotechnology, Lake Placid, NY, USA) or non-immune mouse IgG or rabbit IgG, and subsequently with 7.5 µg/ml of biotin-conjugated anti-mouse and anti-rabbit IgGs, respectively. The immunoreactive cells were visualized by the use of avidin–biotin–peroxidase complex immunostaining reagents (Vector Laboratories, Inc., Burlingame, CA, USA) following the manufacturer’s instructions.

In the case of Western blot analysis, the isolated osteoclasts on plastic dishes and HUVECs were lysed by sonication in a lysis buffer consisting of 10 mM Tris–HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA, 2 mM ABSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin. The cell lysates were then centrifuged at 30000g for 30 min and the obtained pellets were dissolved in Laemmli’s sample buffer. The samples were electrophoresed through 7.5% SDS–PAGE and transferred onto a PVDF membrane. After blockage with 5% skim milk, the membrane was immunoreacted with 1 µg/ml of anti-KDR/Flk-1 and anti-Flt-1 antibodies, subsequently with peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies, respectively. The proteins immunoreactive with anti-KDR/Flk-1 or anti-Flt-1 antibody were visualized with Western blot chemiluminescence reagents as mentioned above.

3. Results
The osteoclastic bone resorption in response to VEGF treatment was first determined in cultures of rabbit isolated osteoclasts with various concentrations of VEGF for 24 h (A), or incubated without (open circle) or with VEGF (100 ng/ml, closed circle) for the indicated times (B). Then, the areas of pits excavated by the isolated osteoclasts were measured. The numbers of TRAP-positive osteoclasts on the dentine slices were counted, and the percentage to the number of initially seeded osteoclasts was indicated as survival rate. The experiments were performed four times, and the values are means ± S.D. for five cultures in a representative experiment. *P < 0.01 vs. untreated cells.

Fig. 1. Dose- and time-dependent effect of VEGF on bone resorption by mature osteoclasts. Isolated mature osteoclasts (200 cells) were incubated with various concentrations of VEGF for 24 h (A), or incubated without (open circle) or with VEGF (100 ng/ml, closed circle) for the indicated times (B). Then, the areas of pits excavated by the isolated osteoclasts were measured. The numbers of TRAP-positive osteoclasts on the dentine slices were counted, and the percentage to the number of initially seeded osteoclasts was indicated as survival rate. The experiments were performed four times, and the values are means ± S.D. for five cultures in a representative experiment. *P < 0.01 vs. untreated cells.
osteoclasts. When the isolated osteoclasts were incubated with VEGF on dentine slices for 18 h, VEGF dose- and time-dependently stimulated the bone resorption by these cells (Fig. 1). The pit area at 100 ng/ml was about 1.7-fold over the control value. In addition, VEGF increased the survival rate of the isolated osteoclasts on the dentine slices in the same concentration range, suggesting that the stimulation of osteoclastic bone resorption is mediated in part by enhancing the osteoclast survival.

Osteoclasts are a member of the hematopoietic cell family [13,14], and various types of hematopoietic cells have been demonstrated to express VEGF receptors, which receptors play roles in regulating the proliferation, development, chemotaxis and survival of hematopoietic cells [7,8,15]. Taken together with the previous findings, the above results suggest the presence of VEGF receptors in the osteoclasts. Therefore, we examined the expression of receptors of VEGF in isolated osteoclasts. As shown in Fig. 2, RT-PCR analysis demonstrated that cDNAs for KDR/Flk-1 and Flt-1 were amplified in the sample obtained from isolated osteoclasts as well as in those samples from HUVECs and rabbit aorta, whereas the amplification was not shown in any samples not subjected to the reverse transcriptase reaction. The nucleotide sequences of the amplified rabbit cDNAs revealed a high homology to those of human and mouse KDR/Flk-1 (75.7% and 77.1% identical, respectively) and Flt-1 (90.6% and 90.7% identical, respectively).

To detect expression of VEGF receptors on osteoclasts at the protein level, using anti-KDR/Flk-1 and anti-Flt-1 antibodies, we performed immunocytochemical staining of unfractionated bone cells. As shown in Fig. 3, multinucleate osteoclasts were stained with the anti-KDR/Flk-1 and anti-Flt-1 antibodies, whereas other bone cells were not. In addition, the protein expression of KDR/Flk-1 and Flt-1 on osteoclasts was also confirmed by Western blotting analysis of a cytoplasmic membrane fraction from isolated osteoclasts (Fig. 3E). We found two immunoreactive protein bands with molecular masses of 200 kDa and 230 kDa corresponding to mature forms of glycosylated KDR/Flk-1 in the osteoclastic membrane fraction. In addition, the immunoreactive Flt-1 with a molecular mass of 180 kDa was also detected.

KDR/Flk-1 and Flt-1 belong to the family of receptor tyrosine kinases [16–18], and the action of VEGF is mediated by the activation of intracellular tyrosine kinase through the autophosphorylation of its receptors. Thus, we finally examined the relationship between tyrosine phosphorylation and VEGF-induced osteoclastic bone-resorbing activity. The addition of VEGF to the isolated osteoclasts induced the stimula-

![Fig. 2. RT-PCR analysis for the expression of VEGF receptors in mature osteoclasts.](image-url)

![Fig. 3. Expression of VEGF receptor proteins in mature osteoclasts.](image-url)

![Fig. 4. Involvement of protein tyrosine phosphorylation in the stimulatory effect of VEGF on osteoclastic bone resorption.](image-url)
tion of tyrosine phosphorylation of numerous cellular proteins in a short-term exposure (Fig. 4).

4. Discussion

Our findings presented here indicate that a potent angiogenic factor, VEGF, stimulated the bone-resorbing activity of isolated rabbit osteoclasts, at least in part by enhancing the survival. This is the first report indicating the direct action of VEGF on the function of mature osteoclasts. Because the isolated osteoclasts expressed two isoforms of VEGF receptors, the stimulatory effect could have been mediated through either VEGF receptor, or through both of them. So, it remains to be elucidated which receptor, KDR/Flik-1 or Flt-1, is important to mediate the stimulatory effect of VEGF in osteoclasts. The method employed for isolation of rabbit mature osteoclasts in this study is based on the much stronger adherence of osteoclasts than that of non-osteoclastic cells to a collagen gel, such that osteoclast cultures can be deleted of stromal cells [10]. The isolated rabbit mature osteoclasts are capable of resorbing dentine even in the absence of bone-derived osteoblastic/stromal cells. Thereby, direct actions of osteotropic hormones and local factors on mature osteoclasts in vitro can be precisely estimated without the influence of non-osteoclastic cells [19–21], and the culture system of rabbit isolated osteoclasts on dentine slices provides us more information in regulating osteoclastic function.

Recently, Niida et al. [9] demonstrated by several histochemical analyses in vivo that VEGF can support the osteoclast recruitment in osteopetrotic op/op mice lacking production of functional M-CSF. In addition, the action may be mediated by Flt-1, suggesting that VEGF can substitute for M-CSF in osteoclastogenesis under some circumstances. Using the culture system eliminating any cells other than osteoclasts, we obviously demonstrated the direct action of VEGF on mature osteoclasts to enhance their function and survival in vivo. The action of VEGF is close to that of M-CSF that enhances osteoclast survival [22]. Taken together, the data indicate that VEGF is an important regulator of osteoclastic bone resorption.

A recent study demonstrated that at the bone/cartilage junction site in the growth plate, administration of a soluble VEGF receptor chimeric protein (mFlt-1-Fc) into mice caused a complete suppression of blood vessel invasion, concomitant with impaired trabecular bone formation, expansion of the hypertrophic chondrocyte zone, and a decrease in cartilage resorption followed by a reduction of recruitment and/or function of chondroclasts [6]. It is well established that hypoxia is one of the principal stimuli for the expression of VEGF [23]. Because hypertrophic chondrocytes are in an avascular environment, these cells are a potential source of this cytokine within the growth plate [6]. In that study, it was also reported that chondroclasts and osteoclasts as well as epithelial cells expressed KDR/Flik-1 and Flt-1 [10]. These findings suggest that VEGF-mediated capillary invasion plays a central role in growth plate morphogenesis by acting in a paracrine manner. In addition, VEGF is also produced by osteoblasts in the skeleton [5], and the expression of VEGF was up-regulated by certain osteotropic factors such as 1,25-dihydroxyvitamin D3 and prostaglandin E2, both of which are potent stimulators of bone resorption [24,25]. Thus, VEGF produced by osteoblasts in response to these osteotropic fac-

tors may be involved in the stimulation of osteoclastic bone resorption. Finally, these results presented here provide a new insight into the multifunctional roles played by VEGF in the relationship between bone/cartilage resorption and angiogenesis.

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