Inhibition of Prostate Tumor Growth and Bone Remodeling by the Vascular Targeting Agent VEGF_{121}/rGel

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Abstract

The pathophysiology of tumor growth following skeletal metastases and the poor response of this type of lesion to therapeutic intervention remains incompletely understood. Vascular endothelial growth factor (VEGF)-A and its receptors play a role in both osteoclastogenesis and tumor growth. Systemic (i.v.) treatment of nude mice bearing intramoferal prostate (PC-3) tumors with the vascular ablative agent VEGF_{121}/recombinant gelonin (rGel) strongly inhibited tumor growth. Fifty percent of treated animals had complete regression of bone tumors with no development of lytic bone lesions. Immunohistochemical analysis showed that VEGF_{121}/rGel treatment suppressed tumor-mediated osteoclastogenesis in vivo. In vitro treatment of murine osteoclast precursors, both cell line (RAW264.7) and bone marrow-derived monocytes (BMM), revealed that VEGF_{121}/rGel was selectively cytotoxic to osteoclast precursor cells rather than mature osteoclasts. VEGF_{121}/rGel cytotoxicity was mediated by Flt-1, which was down-regulated during osteoclast differentiation. Analysis by flow cytometry and reverse transcription-PCR showed that both BMM and RAW264.7 cells display high levels of Flt-1 but low levels of Flk-1. Internalization of VEGF_{121}/rGel into osteoclast precursor cells was suppressed by pretreatment with an Flt-1 neutralizing antibody or by placenta growth factor but not with an Flk-1 neutralizing antibody. Thus, VEGF_{121}/rGel inhibits osteoclast maturation in vivo and it seems that this process is important in the resulting suppression of skeletal osteolytic lesions. This is a novel and unique mechanism of action for this class of agents and suggests a potentially new approach for treatment or prevention of tumor growth in bone. (Cancer Res 2006; 66(22): 10919-28)

Introduction

Prostate cancer is highly metastatic to bone. The survival rate of patients with prostate cancer metastases is about 31%, compared with a ~100% 5-year survival rate if the cancer is locally confined. Skeletal metastases of prostate tumors are, therefore, a major source of mortality and morbidity. One of the properties characteristic of prostate cancer is that metastases to bone produce pure osteoblastic lesions (1, 2). Osteoblastic/osteolytic lesions late in the disease process may also form. However, despite the osteoblastic nature of most prostate cancer skeletal metastasis, several studies suggest that simultaneous up-regulation of osteolytic bone resorption also occurs (3). Osteoclast precursor cells may be stimulated to differentiate or may be activated directly by tumor-secreted soluble factors, such as macrophage colony-stimulating factor (M-CSF), interleukins (IL), transforming growth factor-β, and vascular endothelial growth factor (VEGF) among others (4–6). The secretion of some of these factors by cancer cells regulates expression on the surface of stromal osteoblasts of receptor activator of nuclear factor-κB ligand (RANKL), a maturation factor and dominant mediator of osteoclastogenesis, thereby increasing osteoclast-mediated bone resorption (7).

Molecular engineering has enabled the synthesis of novel chimeric molecules having therapeutic potential. Chimeric fusion constructs targeting the receptors for IL-2, EGF, VEGF, and other growth factor have been described (8–11). Receptor kinase inhibitors (12), antibodies targeting the receptors for VEGF (13), and other approaches to reduce VEGF expression or its interaction to its receptors (14–17) are under development to disrupt tumor angiogenesis.

We previously developed and characterized VEGF_{121}/recombinant gelonin (rGel), a novel growth factor fusion construct composed of VEGF_{121} and the highly cytotoxic plant toxin gelonin (rGel; ref. 18). VEGF_{121} binds only to Flt-1/FLT-1 [VEGF receptor (VEGFR)-1] and Flk-1/KDR (VEGFR-2). Both receptors are overexpressed on the endothelium of tumor vasculature (19–21), including lung, brain, breast, colon, prostate, skin, and ovarian cancers. In contrast, these receptors are almost undetectable by immunohistochemistry in normal tissues other than pancreas and kidney (22). These receptors have also been identified on osteoclasts (23–25), and Flt-1/FLT-1 may play a role in recruitment of osteoclast precursors to the site of bone resorption (24). These receptors, therefore, seem to be excellent targets for the development of therapeutic agents that inhibit tumor growth and metastatic spread through inhibition of tumor neovascularization or through inhibition of osteoclastogenesis.

Several reports (26–28) have implicated VEGF in prostate carcinogenesis and in metastatic spread in addition to its well-established role in angiogenesis. VEGF is also produced by tumor cells to facilitate nesting of metastatic cells in bone and to promote neovascularization (23), which are two critical events necessary for the successful formation of skeletal metastases. Previous studies (29, 30) have suggested a link between VEGF expression and metastatic spread (including skeletal metastases) in prostate
tumors. However, the relative contribution of neovascularature versus osteoclast activity in the development and maturation of skeletal metastasis is not well understood. Kitagawa et al. (31) showed recently the activity of PTK787, a tyrosine kinase inhibitor designed to bind to the ATP-binding sites of VEGFRs, against prostate cancer–induced osteolytic lesions in bone. In the current study, we used the vascular ablative fusion toxin VEGF121/rGel to inhibit prostate cancer tumor growth in an osteolytic model and show that targeting VEGFR-1 on osteoclast precursor cells is important in the resulting suppression of skeletal osteolytic lesions.

Materials and Methods

Bacterial strains, pET bacterial expression plasmids, and recombinant enterokinase were obtained from Novagen (Madison, WI). Rabbit anti-gelonin antisera were obtained from the Veterinary Medicine Core Facility at M.D. Anderson Cancer Center (Houston, TX). Anti-Flt-1 and anti-Flk-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). R-phycoerythrin (R-PE)–conjugated Flk-1 monoclonal antibody and Alexa Fluor 488–conjugated CD11b (Mac-1) monoclonal antibody were purchased from BD Pharmingen (San Diego, CA). R-PE–conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Neutralizing antibodies to Flt-1 (AF471), Flk-1 (AF644), recombinant M-CSF, and recombinant placenta growth factor (PFGF) were purchased from R&D Systems (Minneapolis, MN).

Cell lines. Porcine aortic endothelial cells transfected with the human KDR (PAE/KDR) or FLT-1 (PAE/FLT-1) receptors were a generous gift from Dr. J. Waltenberger (University Hospital, Maastricht, Netherlands). Mouse brain endothelial (bEnd3) cells, mouse osteoclast precursor cells (RAW264.7), and the prostate cancer cell line PC-3 were obtained from American Type Culture Collection (Manassas, VA). Bone marrow–derived monocytes (BMM) were harvested as described below.

Primary bone marrow cell culture. BMM cells were harvested from the tibia and femora of 8- to 12-week-old mice. Bone marrow cells were washed and plated in complete αMEM overnight. Nonadherent cells were then washed, resuspended in α-MEM, and plated in 96-well or 24-well plates in the presence of M-CSF (10 ng/mL) as described below. After 3 days, cells were washed and treated as described below.

Animals. Male athymic BALB/c nude mice (National Cancer Institute, Frederick, MD) and black 6 mice (C57BL/6J, Charles River, Wilmington, MA) were maintained in a laminar airflow cabinet under specific pathogen-free conditions according to American Association for Accreditation of Laboratory Animal Care (AAALAC) standards.

Expression and purification of VEGF121/rGel. The construction, expression, and purification of VEGF121/rGel has been described previously (18). The fusion toxin was stored in sterile PBS at −20°C.

Cytotoxicity of VEGF121/rGel and rGel. Cytotoxicity of VEGF121/rGel and rGel against log-phase PC-3, RAW264.7, and BMM cells was done as described (18). Log-phase cells (3 × 10^4 PC-3, 5 × 10^4 RAW264.7, or 2.5 × 10^4 BMM) were plated in 96-well flat-bottomed tissue culture plates and allowed to attach overnight. Purified VEGF121/rGel and rGel were diluted in culture medium and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson’s buffer [0.1 mol/L sodium citrate (pH 4.2) in 50% ethanol]. Absorbance was measured at 630 nm. Competition assays were done by plating RAW264.7 (5 × 10^5) and BMM (2.5 × 10^5) cells in 96-well plates and by preincubating the cells for 1 hour with 25 nmol/L PFGF before addition of 40 nmol/L RAW264.7 or 8 nmol/L (BMM) VEGF121/rGel. Results were analyzed by staining the remaining adherent cells with crystal violet as described above.

In vitro osteoclast differentiation. BMM and RAW264.7 cells were cultured in 96-well plates at a density of 5 × 10^3 per well and 3 × 10^3 per well, respectively. Cell cultures were initially treated with 100 ng/mL RANKL and 10 ng/mL M-CSF (for BMM) and also subject to a medium change on day 3. Osteoclast differentiation was assessed by counting the total number of multinucleated (more than three nuclei), tartrate-resistant acid phosphatase (TRAP)–positive cells per well 96 hours after treatment using the Leukocyte Acid Phosphatase kit (Sigma-Aldrich, St. Louis, MO).

Time-dependent cytotoxicity and effect on osteoclastogenesis. Nonadherent, primary monocytes (30 × 10^5 per well) were cultured overnight in 24-well plates. Cells were then treated with RANKL (100 ng/mL) in the absence or presence of various concentrations of VEGF121/rGel or rGel added either simultaneously with RANKL or 60 hours after RANKL stimulation. TRAP staining was done at 60 or 96 hours after stimulation with RANKL. Cells were fixed and stained for TRAP and TRAP-positive multinucleated cells were counted in each condition.

RNA extraction. BMM and RAW264.7 cells were treated with their respective IC_{50} VEGF121/rGel doses in the absence and presence of RANKL (and presence of M-CSF for BMM cells) for 24 to 96 hours. Control cells, including bEnd3, PAE/KDR, and PAE/FLT-1 cells, were treated with PBS. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) and its integrity was verified by electrophoresis on a denaturing formaldehyde agarose gel and on a 2100 Bioanalyzer (Agilent, Foster City, CA).

Reverse transcription-PCR analysis. Levels of Flt-1/FLT-1, Flk-1/KDR, and VEGF-A were assessed by reverse transcription-PCR (RT-PCR) analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as controls. In addition to primers for Flt-1/FLT-1, Flk-1/KDR, and GAPDH described previously (22), the following primers were also used: Flk-1, 5′-CATGCAACAGTCTAGCCAACC (forward) and 5′-GCGAACATGTCTTAC-ACCTCGGT (reverse); Flt-1, 5′-ACTGAAATAGGCACATCGCTTGG (forward) and 5′-GGGATCTGTACAGTGCTGCTG (reverse); and VEGF-A, 5′-TGAAGT-GATCAAGTCTTGAGCACT (forward) and 5′-TACCGCGCTTGGTGTTCG (reverse). Amplified RT-PCR products were subjected to densitometric analysis using Alpha Innotech FluorChem 8900 (San Leandro, CA).

Western blot analysis. Total cell extracts of PAE/KDR and PAE/FLT-1 cells were obtained as described previously (22). Blots were incubated overnight with appropriate antibodies (1:200 in 5% milk) followed by incubation with goat anti-rabbit IgG horseradish peroxidase, developed using the Amersham (Piscataway, NJ) enhanced chemiluminescence detection system and exposed to X-ray film.

Fluorescence-activated cell sorting analysis. RAW264.7 and BMM cells were harvested and washed with PBS and resuspended in staining buffer (PBS + 2% fetal bovine serum) at a concentration of 10 × 10^6 cells/mL. One hundred microliter aliquots of RAW264.7 and BMM cells were incubated with 2 and 0.5 μg anti-Flt-1 or R-PE-conjugated anti-Flk-1 antibody, respectively. Cells were incubated with primary antibodies for 1 hour at 4°C. In addition, BMM cells were incubated for 1 hour (4°C) with 0.2 μg Alexa Fluor 488–conjugated CD11b antibody for detection of the monocye/macrophage population. R-PE-conjugated secondary antibody (0.5 μg) was used to detect anti Flt-1. Samples were analyzed on a BD FACS calibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro acquisition software (BD Biosciences). Instrumentation setup and electronic compensation for spectral overlap was done using cell samples single-stained with FITC or R-PE. At least 10,000 events were collected for each sample.

Internalization of VEGF121/rGel into RAW264.7 and BMM cells. Cells were incubated with various concentrations of VEGF121/rGel or rGel at the time points indicated. To show receptor specificity, Cells were pretreated with Flt-1 or Flk-1 neutralizing antibodies for 1 hour before treatment with VEGF121/rGel or rGel. Glycine buffer [500 mmol/L NaCl, 0.1 mol/L glycine (pH 2.5)] was used to strip the cell surface of noninternalized VEGF121/rGel. Cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Nonspecific binding sites were blocked with 5% bovine serum albumin in PBS. Cells were then incubated with a rabbit anti-gelonin polyclonal antibody (1:200) followed by a FITC-conjugated anti-rabbit secondary antibody (1:80). Nuclei were stained with propidium iodide (1 μg/mL) in PBS. The slides were fixed with DABCO medium, mounted, and visualized under fluorescence (Nikon Eclipse TS1000, Melville, NY) and confocal (Zeiss LSM 510, Thornwood, NY) microscopes.
**Intrabone injections.** The PC-3 cells were harvested by a 1-minute treatment with trypsin/EDTA. The culture flask was tapped to detach the cells. The cells were washed in PBS and resuspended in PBS in preparation for implantation into the mice. Animals (10 mice per group) were anesthetized with i.m. injections of ketamine (100 mg/kg) plus acepromazine (2.5 mg/kg). Aliquots of $5 \times 10^4$ of PC-3 cells were diluted in 5 μL growth medium and then injected into the distal epiphysis of the right femur of each mouse using a 28-gauge Hamilton needle. The contralateral femur was used as an internal control. Twenty mice were randomized into two treatment groups. Treatment began 1 week after tumor placement. A maximum tolerated dose of 45 mg/kg for VEGF121/rGel given i.v. under the conditions described below has been established previously (22). Accordingly, the animals were treated (i.v.) with the following protocol: group 1, 200 μL saline every other day for nine days (5 treatments) and group 2, 180 μg VEGF121/rGel in 200 μL saline every other day for nine days (5 treatments). Mice were monitored weekly for tumor bulk and bone loss. Mice were killed in case of excessive bone loss as per AAALAC guidelines and pathologic examination of the subject bones was done.

**Processing of bone tissue samples.** Formalin-fixed, paraffin-embedded tissue samples from the tumors were prepared as described (32) and stained with H&E. TRAP staining was done as described by the manufacturer of the kit (Sigma-Aldrich) and the total number of osteoclasts was determined by counting the number of multinucleated (more than three nuclei), TRAP-positive cells.

**Results**

VEGF121/rGel inhibits growth of intrafemoral PC-3 tumors and reduces the number of tumor-induced osteoclasts. The antitumoreffect of the fusion protein VEGF121/rGel was evaluated in a prostate cancer bone model by injecting PC-3 tumor cells into the distal epiphysis of the right femur of athymic nude mice. Tumor growth was monitored by X-ray analysis and animals with large osteolytic lesions or bone lysis were sacrificed. One hundred percent of tumor-inoculated mice treated with saline developed osteolytic lesions (Fig. 1A, left) and 50% survival occurred 40 days after tumor placement ($P < 0.05$; Fig. 1B). In contrast, treatment with VEGF121/rGel resulted in suppression of intrafemoral growth of tumor osteolytic lesions as assessed radiologically (Fig. 1A, right); 50% of the VEGF121/rGel-treated mice survived past 140 days without sign of osteolysis (Fig. 1B). H&E staining showed PC-3 cells throughout the bone marrow of mice treated with saline (Fig. 1C, left) but not with VEGF121/rGel (Fig. 1C, middle, arrows). Right, representative bone section, in which PC-3 tumor cells had been placed from a mouse treated with VEGF121/rGel, showing absence of tumor. T, tumor cells; M, normal marrow cells. D, effect of VEGF121/rGel on the number of osteoclasts in bone sections of nude mice with PC-3 tumor cells. VEGF121/rGel reduces the number of osteoclasts ($P < 0.05$) and inhibits tumor growth in vivo.

**Figure 1.** VEGF121/rGel inhibits tumor growth and osteolysis in the PC-3 xenograft model and increases the survival of mice. A, effect of VEGF121/rGel in nude mice with PC-3 tumors in bone. Mice were injected with $5 \times 10^4$ PC-3 cells into the distal epiphysis of the right femur, using the contralateral femur as an internal control. Mice received five treatments with either saline or 180 μg VEGF121/rGel 2 days apart, starting 2 days after PC-3 tumor placement. Animals were analyzed by X-ray, and radiograms are representative of 20 mice. Arrows, location of osteolytic lesion, which were only found in the saline-treated animals (left) but not in the majority of VEGF121/rGel-treated animals (right). B, survival curve of the mice in this study ($P < 0.05$). All control mice were sacrificed by day 67. Asterisk, one mouse (without tumor) that did not recover from anesthesia. C, sections of femurs 2 weeks after injection of PC-3 tumor cells were stained for H&E to analyze the presence of PC-3 cell burden. Mice treated with saline show proliferation of PC-3 tumor cells (left). In contrast, mice treated with VEGF121/rGel show isolated pockets of PC-3 tumor cells (middle, arrows). Right, representative bone section, in which PC-3 tumor cells had been placed from a mouse treated with VEGF121/rGel, showing absence of tumor. T, tumor cells; M, normal marrow cells. D, effect of VEGF121/rGel on the number of osteoclasts in bone sections of nude mice with PC-3 tumor cells. VEGF121/rGel reduces the number of osteoclasts ($P < 0.05$) and inhibits tumor growth in vivo.
with saline (Fig. 1C, left). In contrast, isolated pockets of PC-3 cells were visible in some bone marrow sections from VEGF121/rGel-treated mice (Fig. 1C, middle, arrows). Tumor cells were not visible in some bone sections from mice treated with VEGF121/rGel (Fig. 1C, right). Thus, VEGF121/rGel significantly prevented osteolysis and tumor growth in bone compared with saline-treated animals.

We next examined PC-3 tumor cells to identify whether the observed VEGF121/rGel-mediated inhibition of PC-3-induced osteolysis was due to a direct effect on the tumor cells. We have shown previously that the VEGF121/rGel fusion construct is not specifically cytotoxic to PC-3 cells in vitro compared with the rGel toxin alone, suggesting that these cells express an insufficient number of VEGFRs to mediate specific VEGF121/rGel cytotoxicity (18). RT-PCR analysis indicated that PC-3 cells express relatively low levels of FLT-1 and KDR transcript (data not shown).

Because PC-3 tumors result in osteolytic lesions, we analyzed the number of osteoclasts in tissue sections. As expected, TRAP staining revealed a dramatic increase in the number of osteoclasts in the tumor-bearing leg of mice treated with saline (P < 0.05; Fig. 1D). However, bone sections of VEGF121/rGel-treated mice surprisingly showed the same number of osteoclasts as those present in the contralateral (control) leg, suggesting that VEGF121/rGel inhibits tumor-mediated osteoclast proliferation and/or differentiation.

**VEGF121/rGel kills osteoclast precursor cells but not terminally differentiated osteoclasts.** To understand the effect of VEGF121/rGel in the bone microenvironment and test if VEGF121/rGel may be directly targeting osteoclast precursor cells in vivo, we next evaluated the effect of VEGF121/rGel on RANKL-induced osteoclast differentiation of RAW264.7 and BMM cells in vitro. Treatment with increasing concentrations of VEGF121/rGel, but not rGel, showed a dramatic decrease of TRAP-positive multinucleated osteoclasts in both RAW264.7 (Fig. 2A and B) and BMM (Fig. 2C and D) cells. The observed effect was not mediated...
by either VEGF121 or gelonin alone but is a characteristic unique to the combined fusion protein (data not shown). The IC_{50} of VEGF121/rGel on undifferentiated RAW264.7 cells was 40 nmol/L compared with 900 nmol/L for rGel, indicating that the cytotoxicity of VEGF121/rGel was mediated through VEGF121 and suggested the presence of a receptor recognizing VEGF121 (Supplementary Fig. S1A). Similar to the RAW264.7 cells, the IC_{50} of VEGF121/rGel (8 nmol/L) on undifferentiated BMM cells was substantially lower than that of rGel (Supplementary Fig. S1B). VEGF121/rGel showed a greater cytotoxic effect on BMM compared with undifferentiated RAW264.7 cells. In addition, we observed that VEGF121/rGel not only inhibited RANKL-mediated differentiation of osteoclast precursors but also exhibited cytotoxicity toward undifferentiated cells in a targeted manner.

The observed inhibitory effect of VEGF121/rGel on osteoclastogenesis could be due to a cytotoxic effect of VEGF121/rGel on osteoclast progenitor cells. Insufficient number of osteoclast progenitor cells may lead to impaired contact between committed progenitors, leading to inability to form multinucleated osteoclasts. Therefore, we next investigated the susceptibility of primary mouse monocytes and their terminally differentiated counterparts to VEGF121/rGel cytotoxicity. BMM cells were treated with VEGF121/rGel or rGel at various times after RANKL and M-CSF stimulation. Addition of VEGF121/rGel with RANKL and M-CSF resulted in inhibition of osteoclastogenesis. The degree of inhibition was dependent on the concentration of VEGF121/rGel (Fig. 3A and B). However, osteoclastogenesis proceeded normally if BMM cells were first allowed to differentiate for 60 hours before the addition of VEGF121/rGel (Fig. 3C). The apparent cytotoxicity of VEGF121/rGel to osteoclast precursor cells, but not to mature osteoclasts, was further investigated by adding VEGF121/rGel at different time points after RANKL stimulation of both RAW264.7 and BMM cells (Table 1). For both cells, the increase in the IC_{50} of VEGF121/rGel corresponded to the length of time the cells were allowed to

![Figure 3. VEGF121/rGel is cytotoxic to mouse primary monocytes but not mature osteoclasts. The effect of VEGF121/rGel and rGel on primary monocytes was investigated at various time points after RANKL stimulation. Nonadherent, primary monocytes (50 × 10^3 per well) were cultured overnight in 24-well plates in the presence of M-CSF. In the presence of M-CSF, cells were then stimulated to differentiate with RANKL (100 ng/mL). VEGF121/rGel or rGel was added to the cells at the indicated time point. TRAP staining was done 60 or 96 hours after treatment. Cells treated with VEGF121/rGel (*) at 0 hour and TRAP stained (▼) at 96 hours (A), treated with VEGF121/rGel at 0 hour and TRAP stained at 60 hours (B), and treated with VEGF121/rGel at 60 hours and TRAP stained at 96 hours (C). D, number of TRAP-positive multinucleated osteoclasts as a function of VEGF121/rGel treatment time and dose. Cells were fixed and stained for TRAP and multinucleated (more than three nuclei). TRAP-positive cells were counted for each experimental condition. Data are representative of three separate experiments. Columns, mean of each sample done in triplicate; bars, SE. rGel did not have any effect on differentiation at the concentrations used (data not shown).]
differentiate before the addition of VEGF121/rGel. For RAW264.7 cells, the IC$_{50}$ of VEGF121/rGel increased from 30 nmol/L when added simultaneously with RANKL to 300 nmol/L when added 96 hours after RANKL stimulation, whereas the IC$_{50}$ of rGel did not change significantly. The change in IC$_{50}$ of VEGF121/rGel on BMM cells was more dramatic, increasing from 8 to 100 nmol/L if VEGF121/rGel was added 24 hours after RANKL stimulation. Addition of VEGF121/rGel 48 hours after RANKL stimulation increased the IC$_{50}$ to >400 nmol/L.

VEGF$_{121}$/rGel, but not rGel, is internalized into RAW264.7 and BMM cells through a specific mechanism. We next examined by immunostaining whether VEGF$_{121}$/rGel was delivered into the cytoplasm of the osteoclast precursor cells. VEGF$_{121}$/rGel, but not rGel, localized in the cytoplasm of RAW264.7 cells, suggesting that internalization is mediated by VEGF$_{121}$ (Fig. 4). We examined further whether internalization of VEGF$_{121}$/rGel was mediated by VEGF$_{121}$ by examining if VEGF$_{121}$/rGel activated pp44/42, a known downstream target of VEGFR activation (33). Western blot analysis revealed that treatment of RAW264.7 cells with VEGF$_{121}$/rGel resulted in activation of pp44/42 at similar levels as treatment with an equimolar amount of VEGF$_{121}$ (Supplementary Fig. S2). rGel did not induce stimulation of pp44/42, indicating that the effect of VEGF$_{121}$/rGel is mediated by VEGF$_{121}$ rather than a nonspecific mechanism. Treatment of RAW264.7 cells with PlGF, an Flt-1-specific ligand, also resulted in pp44/42 activation, underscoring the presence of this receptor on the surface of osteoclast precursor cells and a possible role in mediating VEGF$_{121}$/rGel internalization.

Localization of VEGF$_{121}$/rGel into RAW264.7 and BMM cells is mediated by Flt-1. To determine the role of VEGF$_{121}$ receptors in VEGF$_{121}$/rGel-mediated cytotoxicity of osteoclast precursor cells, we preincubated RAW264.7 and BMM cells with neutralizing antibodies to Flt-1 and Flk-1 for 1 hour before the addition of VEGF$_{121}$/rGel and monitored internalization of VEGF$_{121}$/rGel. Pretreatment of RAW264.7 and BMM cells with neutralizing antibodies to Flt-1, but not Flk-1, inhibited the localization of VEGF$_{121}$/rGel into these cells (Fig. 5A). We assessed the role of each receptor in VEGF$_{121}$/rGel-mediated cytotoxicity by preincubating RAW264.7 and BMM cells with Flt-1 or Flk-1 neutralizing antibodies or with PlGF for 1 hour before the addition of VEGF$_{121}$/rGel. PlGF was able to inhibit the VEGF$_{121}$/rGel-mediated cytotoxicity in both RAW264.7 (Fig. 5B) and BMM cells (Fig. 5C). Taken together, this indicates that the Flt-1 receptor, but not the Flk-1 receptor, is responsible for mediating VEGF$_{121}$/rGel-induced cytotoxicity in osteoclast progenitor cells.

RAW264.7 and BMM cells express Flt-1. We determined the levels of Flk-1 and Flt-1 in RAW264.7 and BMM cells. RT-PCR analysis indicated low levels of Flt-1, but no Flk-1 transcript, in RAW264.7 cells. Western blot analysis of RAW264.7 cells confirmed this observation (Supplementary Fig. S3A). Fluorescence-activated cell sorting (FACS) analysis indicated that 99% of the RAW264.7 cells expressed Flt-1 (Fig. 6A) and 8% expressed Flk-1 (Fig. 6B). RT-PCR analysis of BMM cells showed an amplification of both Flt-1 and Flk-1 (data not shown) and FACS analysis showed that 41.9% of the CD11b-positive BMM cells expressed Flt-1 and 5.4% expressed Flk-1 (Fig. 6C–E). RT-PCR analysis of BMM cells following stimulation of RANKL-mediated osteoclastogenesis showed no change in the levels of Flk-1, but treatment seemed to down-regulate the Flt-1 transcript (Supplementary Fig. S3B). RT-PCR analysis of mVEGF

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**Table 1. Cytotoxicity of VEGF$_{121}$/rGel and rGel over 72 hours on differentiating RAW264.7 and BMM cells**

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*RAW264.7 cells stimulated with RANKL; BMM cells stimulated with RANKL + M-CSF.

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**Figure 4.** VEGF$_{121}$/rGel is internalized into RAW264.7 cells. RAW264.7 cells were treated with either 10 nmol/L VEGF$_{121}$/rGel or rGel for 24 hours. The cells were fixed, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of rGel (green). The cells were counterstained with propidium iodide (red) to identify nuclei and visualized using a confocal (Zeiss LSM 510) microscope.
isoforms detected low levels of VEGF164 and VEGF120 transcript but no VEGF188 (data not shown). The down-regulation of Flt-1 mRNA in BMM cells following stimulation of osteoclastogenesis by RANKL was confirmed by testing samples at different cycles of the RT-PCR analysis (Supplementary Fig. S3C) and further validated by densitometric analysis as described in Materials and Methods. All bands were individually compared with its internal GAPDH standard loaded in the same fashion to normalize the data. Flk-1 did not show a significant change in expression 96 hours after RANKL stimulation, whereas Flt-1 exhibited a down-regulation of 3.4-fold 96 hours after RANKL stimulation compared with untreated BMM (Supplementary Fig. S3D).

Discussion

Osteoclastogenesis plays a central role in bone development and maintenance. Normal bone requires a close coordination of osteoblastic matrix deposition and osteoclastic resorption (for review, see ref. 34). Interference with the process of osteoclastogenesis alters the kinetics of bone remodeling resulting in abnormal bone development (35, 36). There is general consensus that the hematopoietically derived osteclast is the pivotal cell in the degradation of the bone matrix (37) and stimulation of osteoclastic bone resorption is the primary mechanism responsible for bone destruction in metastatic cancer (38). We tested the efficacy of VEGF121/rGel, a known vascular ablative agent, in a model that mimics tumor growth in bone following skeletal metastases. Our results show that (a) VEGF121/rGel dramatically inhibits prostate tumor growth in bone, (b) VEGF121/rGel treatment suppressed tumor-mediated osteoclastogenesis in vivo, (c) VEGF121/rGel is selectively cytotoxic to osteoclast precursor cells rather than to mature osteoclasts, and (d) VEGF121/rGel cytotoxicity is mediated by the Flt-1 receptor, which is down-regulated as osteoclast precursor cells differentiate to mature osteoclasts.

Although the VEGF-A cytokine family plays an essential role in the regulation of embryonic and postnatal physiologic angiogenic processes, its role in skeletal growth, endochondral bone formation, and differentiation of osteoclast precursor cells is substantially less well understood. Osteoclast precursor cells are recruited to the future site of resorption by VEGF-A and RANKL, two cytokines that are expressed in the immediate vicinity of the bone surface (39, 40). VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate, suggesting that a VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels (23). Treatment with a soluble FLT-1 antibody to block the VEGFR results in almost complete suppression of blood vessel invasion and impaired trabecular bone formation (23), a development that is reversed by cessation of the anti-VEGF treatment.

Figure 5. Flt-1, but not Flk-1, mediates the endocytosis and cytotoxic effect of VEGF121/rGel. 

A, cells were pretreated for 1 hour with 10 μg/mL neutralizing antibodies to either Flt-1 or Flk-1 before the addition of 10 nmol/L VEGF121/rGel. The cells were fixed, acid washed to remove surface-bound material, permeabilized, and immunostained for the presence of rGel (green). The cells were counterstained with propidium iodide (red) to identify nuclei. B, 5 × 10^5 RAW264.7 cells were plated in 96-well plates and incubated overnight. Cells were then incubated with 25 nmol/L recombinant mouse PlGF for 1 hour before the addition of 40 nmol/L VEGF121/rGel for 72 hours. C, nonadherent mouse bone marrow–derived monocytes were isolated from the tibia and femur of mice and plated in 96-well plates (25 × 10^4 per well) and incubated with M-CSF (10 ng/mL). After 3 days, the cells were washed and treated with 25 nmol/L recombinant mouse PlGF for 1 hour before the addition of 8 nmol/L VEGF121/rGel and 10 nmol/L M-CSF for 72 hours. After 3 days, the remaining RAW264.7 and BMM cells were stained with crystal violet to evaluate cytotoxicity of VEGF121/rGel and rGel. Data are representative of two separate experiments. Columns, mean of each concentration done in quadruplicate; bars, SE.
A similar phenotype is observed when Vegf is deleted in the cartilage of developing mice by means of Cre-lox-mediated, tissue-specific gene ablation (41). In addition, examination of VEGF120/120 mice showed delayed recruitment of blood vessels into the perichondrium as well as delayed invasion of vessels into the primary ossification center, indicating a significant role of VEGF at both early and late stages of cartilage vascularization (42). VEGF plays an important role in the vascularization of bone tissues (43); increases osteoclast-mediated bone resorption (25); induces osteoclast chemotaxis and recruits osteoclasts to the site of bone remodeling (40); and can partially rescue M-CSF deficiency in op/op mice (25).

Both of the major receptors of VEGF-A have been observed in osteoclasts (23–25), although some reports cite only the presence of Flt-1 (24, 44). The VEGF-Flt interaction has been implicated in the recruitment process of osteoclast precursor cells from hematopoietic tissue to the site of bone resorption (24, 45). Conflicting data at present suggest that the function and signaling of Flt-1 is complex but dependent on developmental stage and on cell type (46, 47). Flt-1/FLT-1 receptor, not Flk-1/KDR, is associated with inhibition of hematopoietic stem cell cycling, differentiation, and hematopoietic recovery in adults and hematopoietic cell motility (45, 48). Recently, Niida et al. (49) introduced a VEGFR-1 tyrosine kinase domain-deficient mutation into op/op mice and showed that signaling between Flt-1 and the CSF-1 receptor plays a key role in osteoclastogenesis and maintenance of bone marrow functions, including vascularization.

Previous studies in our laboratory have shown that the VEGF121/rGel fusion construct can bind to both Flt-1/FLT-1 and Flk-1/KDR but cytotoxicity and cellular internalization of VEGF121/rGel on vascular endothelial cells is mediated through internalization of Flk-1/KDR and not Flt-1/FLT-1. In addition, endothelial cells that express the KDR receptor below a threshold number (\(~ 2 \times 10^3\) receptors per cell) are several hundred-fold more resistant to VEGF121/rGel than are cells that overexpress KDR (\(~ 1 \times 10^5\) to \(~ 3 \times 10^5\) receptors per cell; ref. 18). Therefore, our finding that VEGF121/rGel can disrupt osteoclast activity was unanticipated. Our observation that the antiangiogenic VEGF121/rGel construct can significantly affect prostate tumor growth in a skeletal setting is a novel finding for agents in this class. The current study shows that Flt-1 and not Flk-1 on osteoclast precursor cells seems to be primarily responsible for mediating the cytotoxic effects of VEGF121/rGel and that the biological role and character of the two receptors for VEGF is different on osteoclasts compared with vascular endothelial cells (46, 47).

VEGFRs have been the focus of attention as therapeutic agents, both by us (18, 22) and others (8, 11), and the context has usually been in terms of vascular targeting and antiangiogenic therapy. The ability of VEGF121/rGel to target osteoclast precursor cells represents a novel application of this fusion protein with significant clinical potential. Because VEGF is involved in both angiogenesis and osteolysis, VEGF121/rGel may disrupt tumor growth both by preventing angiogenesis and by inhibiting the
process of bone remodeling. This in turn prevents further tumor invasion and osteolytic penetration of tumor into bone. We plan to examine the effects of VEGF121/rGel on cancer cell-mediated neoangiogenesis in the bone marrow and delineate the relative contribution of both processes to the development of osteolytic lesions. Because osteolyis is an integral component of osteoblastic lesions as well, it remains to be seen if VEGF121/rGel is as efficacious in these models and xenograft model studies are currently under way to address this issue.

Kaplan et al. (50) showed recently that VEGFR1-positive hematopoietic cells play a key role in the development of metastasis by forming clusters in tumor-specific sites in advance of metastatic lesions. Apparently, VEGF121/rGel may be used to target hematopoietic cells that play a role in preparation of this “premetastatic niche,” thereby impeding, or preventing, metastases from occurring.

Based on our data, the “Trojan horse” approach used by VEGF121/rGel might be useful for the treatment of bone-related malignancies, such as bone metastases and the osteolastic component of Paget’s disease as well as hematopoietic diseases, such as multiple myeloma. Our study also shows that Flt-1/FLT-1 may play an important role in bone biology and warrants further study about its importance in the normal bone remodeling process and in various pathologic states.

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