VASCULAR ENDOTHELIAL GROWTH FACTOR-D ACTIVATES VEGFR-3 EXPRESSED IN OSTEObLASTS INDUCING THEIR DIFFERENTIATION*

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Running Title: VEGF-D/VEGFR-3 signaling induces osteoblast differentiation

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Vascular endothelial growth factor (VEGF)-D is a member of the VEGF family of angiogenic growth factors that recognizes and activates the vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 on blood and/or lymphatic vessels. Here we show that in the long bones of newborn mice Vegf-d and Vegfr-3 are expressed in the osteoblasts of the growing plate. The treatment of primary human osteoblasts with recombinant VEGF-D induces the expression of osteocalcin and the formation of mineralized nodules in a dose-dependent manner. A monoclonal neutralizing antibody anti-VEGF-D or VEGFR-3 silencing, by lentiviral-mediated expression of VEGFR-3 small hairpin RNA (shRNA), affect VEGF-D-dependent osteocalcin expression and nodule formation. Moreover, in primary human osteoblasts VEGF-D expression is under the control of VEGF and inhibition of VEGF-D/VEGFR-3 signaling, by monoclonal antibodies or VEGFR-3 silencing, affects VEGF-dependent osteoblast differentiation. These experiments establish that VEGF-D/VEGFR-3 signaling plays a critical role in osteoblast maturation and suggest that VEGF-D is a downstream effector of VEGF in osteogenesis.
critical role. VEGF was first identified as an angiogenic factor that is expressed in different splicing forms. It binds and activates VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as KDR or Flk1) on endothelial cells and their expression is required for vascular development and adult angiogenesis (6,7). During skeletal development VEGF has been shown to play a role in blood vessel invasion that is essential for coupling resorption of cartilage with mineralization of the extracellular matrix and bone formation (8-10) and synergizes with BMP2 to promote bone formation and bone healing via modulation of angiogenesis (11). VEGF is essential not only for normal angiogenesis, but also to allow normal differentiation of hypertrophic chondrocytes, osteoblasts, and osteoclasts (5,12-18). VEGF plays a role in bone repair. In mouse femur fracture treatment with exogenous VEGF enhanced not only blood vessel formation, but also ossification and new bone maturation, while VEGF inhibition decreased bone formation and callus mineralization (19). Although experiments in osteoblasts demonstrated an increase of cell migration in response to VEGF (14), no functional data of the activity of VEGFRs in osteoblasts has been reported.

During mouse development, the expression of another member of the VEGF family, VEGF-D, was detected in the periosteum/osteoblast layer of the developing vertebral column, the limb buds, and the dental mesenchyme close to the enamel epithelium (20). As in mouse VEGF-D only recognizes murine VEGFR-3 (21) its pattern of expression suggests that VEGF-D/VEGFR-3 signaling plays a role in bone development. VEGFR-3 has been previously shown to be involved in vascular development, lymphatic maintenance and tumor angiogenesis (22-28).

Here we investigated the involvement of the angiogenic growth factor VEGF-D and its receptor VEGFR-3 in osteoblasts. We show that osteoblasts of the long bones of newborn mice and primary human osteoblasts express VEGFR-3. Osteoblasts treated with recombinant VEGF-D respond with VEGFR-3 auto-phosphorylation, osteocalcin expression and nodule formation. Moreover, VEGF treatment induces VEGF-D expression in these cells. Accordingly, the inactivation of VEGF-D activity by neutralizing antibodies or VEGFR-3 silencing inhibited both VEGF- and VEGF-D-dependent nodule formation in osteoblasts. Our data demonstrate the involvement of VEGF-D in maturation and regulation of osteoblastic activity via VEGFR-3.

**EXPERIMENTAL PROCEDURES**

**Primary Human Osteoblast Cultures** - Bone samples were obtained from 10 women and 4 men (aged 56-78 years, with a mean age of 66 years) who underwent total hip replacement surgery for degenerative joint disease. After extensive washes of trabecular bone explants, small bone chips were placed in flasks with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cultures were incubated at 37° C in a humidified, 5% CO₂ atmosphere. For differentiation assays, osteoblasts were seeded in 6-well plates. Upon confluence, recombinant human VEGF-D (0-50 ng/ml) with or without the mouse monoclonal anti-human VEGF-D neutralizing antibody (MAb 3.11A25) at a concentration of 5-36 µg/ml were added. Media were changed every two days. The mineralized nodule formation was determined after 15 days by using Alizarin Red S staining as previously described (29).

**Immunofluorescent Microscopy** - For histological analysis, newborn SWISS mice were sacrificed and limbs were dissected and embedded in OCT (Tissue-Tek®). 10 µm cryostat sections were cut and fixed in 3%
paraformaldehyde for 20 min at room temperature. Human osteoblasts were grown on glass coverslips and, after treatment, fixed in 3% paraformaldehyde for 15 min at room temperature. For permeabilization, cryostat sections were incubated with 0.5% Triton X-100 in PBS for 5 min at 4°C. For the staining of cultured human osteoblast cells were not permeabilized. Specimens were washed twice in PBS, blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and incubated for 1 h at 37°C with the following primary antibodies: rabbit polyclonal anti-VEGF-D (30), goat polyclonal anti-PECAM-1 (Santa Cruz Biotechnology), rat monoclonal anti-VEGFR-3 (eBioscience), goat polyclonal anti-osteocalcin (Santa Cruz Biotechnology), and goat polyclonal anti-VEGFR-3 (R&D Systems), diluted in 1% BSA/PBS. After washing, specimens were incubated for 1 h at 37°C with Alexa Fluor-568 or Alexa Fluor-488 secondary antibodies (Molecular Probes), and mounted in Mowiol 4-88 (Calbiochem). Fluorescent images were captured using a Leica TCS SP2 laser scanning confocal microscope.

**Cloning, Purification, and Folding of Recombinant Human VEGF-D** - To generate the 6xHis epitope-tagged human VEGF-D (amino acids 90 to 203, GenBank™/EBI Data Bank accession number NM_004469), a cDNA clone containing the complete sequence of the VEGF-D gene (31) was PCR-amplified with a forward primer containing a NdeI restriction site and a reverse primer containing a SalI site (Table I). The PCR fragment was then cloned into the NdeI SalI sites of the bacterial expression vector pET-22b (Novagen). The construct was checked by automated sequencing. VEGF-D transformed BL21-DE3 E. coli cells were grown for 3 h at 37°C after IPTG induction, pelleted, and solubilized in 6 M guanidium chloride. VEGF-D was purified by Immobilized Metal Affinity Chromatography (IMAC) under denaturing conditions (8 M Urea) in the presence of 1 mM Tris-(2-carboxyethyl)phosphine-HCl using an AKTA purifier (Amersham Biosciences). To obtain a VEGF-D dimer, the monomer (0.25 mg/ml) was dialyzed against: a) 6 M Urea, 0.1 M NaH₂PO₄, 10 mM Tris-HCl, 5 mM GSH, pH 8.5; b) 50 mM Tris-HCl, 5 mM NaCl, 0.5 M L-Arginine, 5 mM GSH, 1 mM GSSG, 2 M Urea, pH 8.5; c) in the same buffer as b without 2 M Urea; d) 50 mM Tris-HCl, pH8. Each dialysis was performed for 16 h. To eliminate aggregate forms, VEGF-D was loaded onto a His-TRAP affinity column in non-denaturing conditions and eluted with 250 mM imidazole. Imidazole was removed on a HiTrap desalting column and the dimer formation was checked by gel filtration using a Superdex 75 HR Column. All the columns were from Amersham Biosciences.

**Monoclonal Antibody Production and Characterization** - Monoclonal antibodies (MAbs) against human recombinant VEGF-D were generated using a standard fusion protocol (32). Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA). Antibody-secreting hybridomas were cloned and inoculated into pristane-primed BALB/c mice for production of ascitic fluid. The isotypes were determined using the Mouse monoclonal antibody isotyping kit (Amersham Biosciences). Antibodies were purified by affinity chromatography and characterized by immunoprecipitation and cell proliferation assays.

**Cell proliferation and viability assays** – Human umbilical vein endothelial cells (HUVEC) were grown in M199 culture medium containing 20% fetal bovine serum and growth supplements. 5 x 10³ cells were plated in a 96-well plate and starved for 24 h in DMEM containing 2% fetal bovine serum and 1% BSA. Cells were treated with 25 ng/ml VEGF-D in the presence of different concentrations of an anti-VEGF-D monoclonal antibody, and 0.5 µCi/well [methyl-³²H]thymidine (Amersham Biosciences). After 20 h cell proliferation was measured as
thymidine uptake by a β-counter. The assays for cell viability were performed by using thiazolyl blue tetrazolium bromide (MTT, Sigma Chemical) according to manufacturer’s instructions.

Immunoprecipitation and Immunoblotting Assays - For osteoblast immunoprecipitation analysis, cultures were starved in serum-free DMEM containing 1% BSA for 24 h. Before stimulation, cells were incubated with 0.1 mM Na$_3$VO$_4$ for 10 min to inhibit phosphatase activity. Cells were stimulated for 30 min with 25 ng/ml VEGF-D at 37°C, washed with ice-cold PBS containing 0.1 mM Na$_3$VO$_4$, and lysed in 1 ml of lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton-X 100, 1.5 mM MgCl$_2$, 1mM EGTA, 10mM Na$_2$PO$_4$, 100 mM NaF, 10 mM DTT, 1 mM Na$_3$VO$_4$, protease inhibitors, Sigma Chemical). Cells lysates were incubated on ice for 10 min, centrifuged at 10,000 x g for 15 min, and the supernatants were incubated with anti-VEGFR-3 antibodies. For immunoprecipitation analysis of VEGF family growth factors, HEK293 stable clones expressing human VEGF 164, VEGF-C, and VEGF-D were generated (M. B. unpublished data). 400 µl of supernatant from serum starved cells were immunoprecipitated with 2 µg of MAb 3.11A25 in 50 mM Tris-Hcl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40. Immunoprecipitates were analysed by 6-15% SDS-PAGE. Immunoblot analysis were performed as previously described (33). The following primary antibodies were used: mouse monoclonal anti-VEGFR-3, anti-P-Y, anti-VEGF, and anti-β-tubulin (Santa Cruz Biotechnology); anti-P-p44 MAPK and anti-p44/42 MAPK (Cell Signaling); anti-VEGF-C (R&D Systems); anti-VEGF-D (MAb 197). The blots were washed, incubated with HRP-labeled secondary antibodies and developed by using the enhanced chemiluminescence substrate (Amersham Bioscience).

VEGFR-3 RNA Interference – Small hairpin (shRNA) cassette was cloned and the recombinant lentiviruses were produced as previously described (34). Briefly, oligonucleotides coding for human VEGFR-3 and unrelated shRNA were designed to contain a sense strand 5’-GAGACAAGGACAGCGAGGACA-3’ (VEGFR-3 D clone), 5’-GTACATCAAGGCACGCATCGA-3’ (VEGFR-3 F clone), and 5’-GCCACAAGTTCAGCGTGTC-3’ (unrelated) followed by a spacer (5’-TTCAAGAGA-3’) and their reverse complementary strand followed by 5 thymidines as an RNA polymerase III transcriptional stop signal. The complementary oligonucleotides were phosphorylated, annealed, and cloned into lentiviral vector. HEK293 cells were transiently transfected using PolyFect transfection reagent (Qiagen) according to the manufacturer instructions. The lentiviruses were harvested 24 and 48 h later and filtered through 0.22-µm pore cellulose acetate filters. Recombinant lentiviruses were concentrated by ultracentrifugation for 2 hours at 50,000 x g. Vector infectivity was evaluated by infecting cells with a GFP vector and titrating shRNA-expressing virus by real-time quantitative RT-PCR of a common lentiviral genome region compared with the GFP vector.

Real-time quantitative RT-PCR analysis - Total RNA was isolated from cells by the guanidinium thiocyanate method, quantified, and integrity was tested by gel electrophoresis. The gene expression analysis was performed using a LightCycler apparatus, and data were analyzed with the LightCycler software version 3.5 (Roche Applied Science). The RT-PCR reactions were set up in microcapillary tubes using the LightCycler RNA Amplification Kit SYBR Green I (Roche Applied Science) following the manufacturer's instructions. For each sample, triplicate determinations were made, and the gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the same sample. The primer pairs used are reported in Table I.
RESULTS

Comparison of VEGF-D and VEGFR-3 expression in newborn mice – Immunohistochemical staining for murine VEGF-D performed on neonatal radius showed the expression of VEGF-D in correspondence of osteoblasts adjacent to hypertrophic chondrocytes (Fig. 1 C). To verify whether VEGF-D expression was compatible with a role in bone formation, we analyzed the expression of its receptor VEGFR-3, as in mouse VEGF-D only recognizes VEGFR-3 (21). VEGFR-3 showed expression in the growth plate at the interface between the forming bone and the terminal hypertrophic chondrocytes (Fig. 1). This is a bone developing region characterized by new blood vessel formation and differentiating osteoblasts. VEGFR-3 is transiently expressed in endothelial cells during active angiogenesis (22,27,35). Consistent with this, we observed low levels of VEGFR-3 expression in endothelial cells in the radius of new born mice, reflecting the fact that a peak of angiogenesis in the growing bone takes place before birth. Double staining between VEGFR-3 and PECAM1 (CD31), a marker of endothelial cells, revealed a partial overlapping of these signals demonstrating that VEGFR-3 is still expressed in endothelial cells at this stage (Fig. 1 D-F). A more consistent VEGFR-3 signal was observed in osteocalcin positive cells (Fig. 1, G-L). These data demonstrate that VEGFR-3 is expressed in mouse osteoblasts.

VEGF-D activates VEGFR-3 on primary human osteoblasts - To address whether VEGF-D/VEGFR-3 signaling plays a functional role in osteoblasts, we obtained from trabecular bone explants primary human osteoblasts and analyzed VEGF-D response on these cells. We detected VEGFR-3 expression in primary human osteoblasts by immunofluorescence analysis and by Western blot (Fig. 2 A-D), while we could not detect VEGFR-2 in these cells or VEGFR-3 in fibroblasts (data not shown). To analyze VEGFR-3 auto-phosphorylation, human osteoblasts were treated with recombinant VEGF-D and whole cell lysates were immunoprecipitated with an antibody recognizing VEGFR-3 C-terminus and immunoblotted with anti-phophotyrosine antibodies. This analysis revealed that VEGF-D treatment induced VEGFR-3 auto-phosphorylation in osteoblasts (Fig. 2 E). VEGFR-3 activation was leading to an intracellular signaling cascade as we observed that VEGF-D treatment was followed by an increase of the phosphorylation of the intracellular protein ERK1/2 (Fig. 2 E). These experiments demonstrate that VEGF-D recognizes and activates VEGFR-3 expressed in primary human osteoblasts.

VEGF-D induces nodule formation and osteocalcin expression - To investigate whether VEGF-D affects osteoblast differentiation, primary human osteoblasts were grown in complete medium for 14 days in the presence of different concentrations of VEGF-D. As shown in Figure 3, VEGF-D significantly increased the number of mineralized nodules in a dose-dependent manner (Fig. 3 A). In addition, the nodules grew bigger and better mineralized than those of the control cultures that showed less and poorly mineralized nodules (Fig. 3 B). To verify whether the neutralization of VEGF-D activity affects osteoblast differentiation, we used the MAb 3.11A25 (isotype IgG2a) able to selectively immunoprecipitate VEGF-D (Fig. 3 C), and to inhibit VEGF-D-dependent proliferation of endothelial cells (Fig. 3 D). The treatment of osteoblast cultures with MAb 3.11.A25 decreased nodule formation to values of untreated cells while a non correlated antibody did not influence nodule formation even at high concentrations (Fig. 3 E). Importantly, the treatment of osteoblasts for 14 days with high concentrations of MAb 3.11A25 did not alter osteoblasts viability (Fig.
Taken together these data demonstrated that VEGF-D induces osteoblasts to form mineralization nodules. Quantitative real-time RT-PCR analysis of the expression of RUNX2, a marker of early differentiation of osteoblast, and osteocalcin, a marker of late osteoblast differentiation, showed that VEGF-D treatment did not influence RUNX2 expression, while osteocalcin mRNA was significantly increased in a dose-dependent manner (Fig. 4 A). Moreover, osteocalcin induction was inhibited by the anti-VEGF-D neutralizing antibody (Fig. 4 B). Immunofluorescence analysis also showed that VEGF-D treatment increased osteocalcin secretion with respect to untreated cells (Fig. 4 C-F) demonstrating that VEGF-D plays a role in osteoblast differentiation. Inhibition of VEGFR-3 signaling reduces nodule formation - To investigate whether VEGF-D-dependent osteoblast differentiation acts via VEGFR-3 signaling, we generated two lentiviral vectors (clones D and F) expressing small hairpin RNA (shRNA) designed to inhibit VEGFR-3. Primary human osteoblasts infected with either lentivirus expressing VEGFR-3 shRNA, but not an unrelated shRNA, showed a reduced VEGFR-3 protein expression and affected VEGF-D-dependent ERK1/2 activation (Fig. 5 A). Osteoblasts knockdown for VEGFR-3, showed significant impairment of nodule formation and osteocalcin production following VEGF-D treatment (Fig. 5 B, C).

As VEGF plays a direct role in osteoblast migration, probably via the activation of VEGFR-1 (14), to provide a functional link between VEGF and VEGF-D-dependent osteoblast differentiation, we analyzed whether in osteoblasts VEGF-D expression depends on VEGF signaling. Quantitative real-time RT-PCR analysis revealed that, following VEGF treatment, osteoblasts responded with an increased expression of VEGF-D, demonstrating that in osteoblasts VEGF-D expression is under the control of VEGF (Fig. 6 A). To analyze whether VEGF-D plays a role in VEGF-dependent osteoblast differentiation, we treated osteoblasts with VEGF in the presence of the monoclonal antibody inhibiting VEGF-D activity and measured the nodule formation induced by VEGF. VEGF treatment of primary human osteoblasts induced a significant number of mineralization nodules. The pretreatment of these cultures with VEGF-D neutralizing antibodies efficiently inhibited VEGF-dependent nodule formation (Fig. 6 B).

This inhibition affected specifically the VEGFR-3 signaling, as we also observed inhibition of VEGF-dependent nodules formation in cells silenced for VEGFR-3 (Fig. 6 C, D). Together these results demonstrate that VEGF induces nodule formation in osteoblasts via the activation of the VEGF-D/VEGFR-3 signaling.

DISCUSSION

This study reveals that VEGF-D/VEGFR-3 signaling induces primary human osteoblast differentiation. Examination of long bones of new-born mice, showed that VEGF-D is expressed together with VEGFR-3 in the osteoblasts of the growth plate, suggesting that VEGF-D/VEGFR-3 signaling might contribute to the bone formation in vivo by an autocrine activation of osteoblasts during their maturation. Accordingly, we found that VEGF-3 is expressed in primary human osteoblasts and VEGF-D stimulates their differentiation measured as osteocalcin induction and mineralized nodule formation. Furthermore, we demonstrated, by VEGFR-3 silencing, that VEGF-3 signaling plays a functional role in VEGF-D-dependent osteoblast maturation. As in mouse VEGF-3 is expressed in osteoblasts and VEGF-D binds only this receptor (21), these results suggest that VEGF-D/VEGFR-3 signaling in bone formation is a common function between mouse and human.
VEGF-D induces angiogenesis, lymphangiogenesis as well as metastatic spread of tumors via lymphatic vessels activating VEGFR-3 on vascular and lymphatic endothelial cells (30,36,37). Besides playing a role in lymphatic vessels homeostasis, VEGFR-3 is implicated in the remodeling of the primary vascular network, and in reorganizing the integrity of endothelial vessels during angiogenesis (22,25-27). On endothelial cells, VEGFR-3 signaling activates proliferation, migration, and survival (38). The data presented in this report demonstrate that, in addition to play a biological function in endothelial cells, VEGF-D/VEGFR-3 signaling is also implicated in osteoblast differentiation. Expression analysis on primary human osteoblasts demonstrated that VEGF-D induces the expression of osteocalcin, a late marker of differentiation, while it has no effect on the early marker RUNX2. RT-PCR analysis in these cells revealed that the inhibitors of RUNX2, Twist 1 and 2 expressed early during osteoblasts differentiation (3), are already down-regulated in osteoblasts before VEGF-D treatment (MO unpublished observation). These data therefore suggest that VEGF-D/VEGFR-3 signaling is not involved in the activation of RUNX2 function, but must be involved in the activation of other yet-unknown regulator(s) of osteoblast differentiation which act at a later stage of osteoblast maturation. This is also confirmed by the fact that VEGF-D induced mineralization in these cells. Hypertrophic chondrocytes secrete a number of growth factors including VEGF, which orchestrate blood vessel formation, chondrocyte maturation, the differentiation of osteoblasts forming the mineralized bone collar as well as the recruitment of osteoclasts into hypertrophic cartilage (16) (and reference therein). In line with these experiments, our results are consistent with a model in which VEGF acts early on bone differentiation by inducing vessel formation, osteoblasts recruitment to the growth plate, and also stimulating VEGF-D production in osteoblasts. VEGF-D in turn acts as a downstream effector of VEGF with autocrine activity on osteoblasts. Therefore these two factors contribute to the process of timely coordinated osteoblast differentiation. These results also imply that VEGF, probably acting on VEGFR-1, stimulates a different cellular response than VEGF-D acting on VEGFR-3. A similar conclusion was previously reached by the analysis of sinusoidal endothelial cells were VEGFR-1 induced these cells to produce the hepatocyte growth factor, while VEGFR-2 induced their proliferation (39). Further analysis of the downstream signaling from these two receptors might enlighten the physiological differences between these two receptors in osteoblasts.

Our data suggest that VEGF-D plays a role in the development of long bones. During mouse development VEGF-D expression was also detected in the periosteum layer of the developing vertebral column and in the dental mesenchyme close to the enamel epithelium where dentin and enamel matrices are deposited (20), suggesting that VEGF-D might also play a role in intramembranous bone formation.

REFERENCES


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1 The abbreviation used are: VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; shRNA, small hairpin RNA; MAb, Monoclonal antibody; IMAC, Immobilized Metal Affinity Chromatography; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate buffered saline; BSA, bovine serum albumin; ELISA, Enzyme-Linked Immunosorbtent Assay.

FIGURE LEGENDS

**Fig. 1.** VEGF-D is expressed in the growth plate of developing long bones of newborn mice, and VEGFR-3 is localized in osteocalcin-expressing tissues. A, section through ulna/radius stained with Alizarin Red at P0, showing the developing growth plate (arrowhead). B, transmitted light (TL) interference contrast of a growth plate section, showing osteoblasts stained with Alizarin Red (black). Scale bar, 150 µm. C, immunofluorescence analysis of a distal radius growth plate by using polyclonal anti-VEGF-D antibodies. Scale bar, 75 µm. D and E, the growth plate of a distal radius was analyzed by immunofluorescence using respectively anti-PECAM1 and anti-VEGFR-3 antibodies. F, merge of D and E. Scale bar, 40 µm. G and H, the growth plate of a distal radius analyzed by immunofluorescence, using respectively anti-osteocalcin and anti-VEGFR-3 antibodies. L, merge of G and H. Scale bar, 30 µm.

**Fig. 2.** VEGFR-3 is expressed in primary human osteoblasts and is activated in response to stimulation with recombinant VEGF-D. A and B, human osteoblasts were grown in complete medium and analyzed by immunofluorescence using anti-VEGFR-3 and anti-osteocalcin antibodies. C, TL interference contrast of the images shown in A and B. Scale bar, 25 µm. D, cell extracts from serum-starved osteoblasts treated with VEGF-D and immunoprecipitated with anti-VEGFR-3 antibodies. The immunoprecipitation was analyzed by Western blotting with anti-phospho-Tyr antibodies, and with anti-VEGFR-3 antibodies to confirm equal loading. E, cell extracts from serum-starved osteoblasts treated with VEGF-D were analyzed by Western blotting using anti-P-ERK1/2 and anti-ERK1/2 antibodies to confirm equal loading.

**Fig. 3.** VEGF-D induces nodule formation in primary human osteoblasts. A, nodule formation in osteoblast cultures treated for 14 days with increasing amounts of recombinant VEGF-D. B, mineralized nodule formation in osteoblast cultures in the absence (NT) and presence of 25 ng/ml VEGF-D. The mineralized nodules were stained with Alizarin Red S after 14 days of treatment. Scale bar, 100 µm. C, cell culture supernatants from cells expressing human VEGF-A, VEGF-C, and VEGF-D were analyzed by Western blotting (input) and immunoprecipitated with the MAb 3.11A25. The immunoprecipitates, as the inputs, were analyzed by Western blotting with anti-VEGF, anti-VEGF-C, and anti-VEGF-D antibodies. D, cell proliferation assay on human endothelial cells (HUVEC). Cells were grown in 96-well plates, starved, and treated with 25 ng/ml VEGF-D and different concentrations of the anti-VEGF-D MAb 3.11A25. E,
nodule formation in osteoblast cultures treated for 14 days with 25 ng/ml VEGF-D in the presence of different amounts of the MAb 3.11A25 able to inhibit the binding of VEGF-D to its receptors. NC, not correlated purified antibodies (40 µg/ml). F, cell viability assay using MTT as metabolic activity indicator. Osteoblast cells were grown in 24-well plates and treated for 14 days with 25 ng/ml VEGF-D and 36 µg/ml MAb 3.11A25. Data represent the ± SEM of 6 independent experiments each in triplicate.

Fig. 4. VEGF-D treatment stimulates osteocalcin production, a gene related to osteoblast differentiation. A, analysis of the osteocalcin and RUNX2 mRNA levels by quantitative real-time RT-PCR. RNA was extracted from primary human osteoblasts treated for 14 days with increasing amounts of VEGF-D. B, analysis of the osteocalcin transcripts by quantitative real-time RT-PCR. RNA was collected from osteoblasts treated for 14 days with 25 ng/ml VEGF-D and different amounts of a monoclonal antibody anti-VEGF-D which inhibits the binding of VEGF-D to its receptors. NC, not correlated purified antibodies (40 µg/ml). C-D, human osteoblasts untreated (control) or treated for 12 days with 25 ng/ml VEGF-D and analyzed by immunofluorescence staining using anti-osteocalcin antibodies. E and F, TL interference contrast of the images shown in C and D. Scale bars, 16 µm. Data represent the ± SEM of 3 independent experiments each in triplicate.

Fig. 5. VEGFR-3 silencing reduces nodule formation and osteocalcin expression in primary human osteoblasts. A, Western blot analysis of cell extracts from cells infected with a lentiviral vector expressing unrelated (unr) or VEGFR-3 shRNA (D and F) grown for 14 days in complete medium. The membranes were probed with anti-VEGFR-3, anti-P-ERK1/2, anti-β-tubulin or anti-ERK1/2 antibodies as indicated. B, nodule formation in control (unr) and VEGFR-3 silenced osteoblast cultures treated for 14 days with 25 ng/ml VEGF-D. C, analysis of the osteocalcin transcripts by quantitative real-time RT-PCR, using RNA collected from osteoblasts treated as in B. Data represent the ± SEM of 3 independent experiments each in triplicate.

Fig. 6. In primary human osteoblasts VEGF induces nodule formation via VEGF-D. A, analysis of VEGF-D mRNA levels by quantitative real-time RT-PCR. RNA was extracted from primary human osteoblasts treated (VEGF) or not (NT) with 10 ng/ml VEGF (Sigma Chemical) at the times indicated. B, nodule formation in osteoblast cultures treated for 14 days with 10 ng/ml VEGF and in the presence of different amounts of the anti-VEGF-D monoclonal antibody that inhibits the binding of VEGF-D to its receptors. NC, not correlated purified antibodies (40 µg/ml). C, nodule formation in unrelated (unr) and VEGFR-3 silenced osteoblast cultures untreated (NT) or treated with 10 ng/ml VEGF-A for 14 days. D, mineralized nodules in osteoblasts silenced for unrelated (unr) or silenced for VEGFR-3, untreated (NT) or treated with 10 ng/ml VEGF for 14 days. The mineralized nodules were stained with Alizarin Red. Quantitative real-time RT-PCR data represent the ± SEM of 3 independent experiments and nodule formation data represent the ± SEM of 6 independent experiments each in triplicate. Scale bar, 100 µm.
Table I

Oligonucleotide sequences used in real-time quantitative RT-PCR and VEGF-D cloning in pET22b.

F, forward primer; R, reverse primer.

<table>
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<th>Gene</th>
<th>Sequence</th>
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<td>VEGFR-2</td>
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<tr>
<td></td>
<td>R 5’-CACAACCAGAGAGACCACA-3’</td>
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<td>VEGFR-3</td>
<td>F 5’-CAACAGACCCACACAGAAT-3’</td>
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<td></td>
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<tr>
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<td></td>
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<td>R 5’-GAGAGAGTCGACTCAGTGATGATGGTGATGGTGATGGA TAATTGAGTATGGATGGCG-3’</td>
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FIG. 1
FIG. 2

A. VEGFR-3

B. osteocalcin

C. Image of cells

D. Western blot analysis of IP VEGFR-3 and P-Tyr

E. Western blot analysis of P-ERK

Legend:
- NT: Negative Control
- VEGF-D: Vascular Endothelial Growth Factor-D

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FIG. 3

A

B

C

D

E

F
FIG. 4

A

![mRNA levels graph](image)

B

![Osteocalcin mRNA levels graph](image)

C

![Control image](image)

D

![VEGF-D image](image)

E

![αD MAb image](image)

F

![VEGF-D control image](image)
FIG. 5
Fig. 6

A. VEGF-D mRNA levels (arbitrary units) over 15 days of culture for NT and VEGF.

B. A bar chart showing nodules/well in response to different concentrations of αD MAb. VEGF-A was either present (+) or absent (-) in the culture medium.

C. A bar chart showing nodules/well for unr shRNA and shRNA R-3 (D) conditions.

D. Images showing the effect of VEGF on nodules formation compared to NT conditions. The images are labeled as NT and VEGF.
Vascular endothelial growth factor-D activates VEGFR-3 expressed in osteoblasts inducing their differentiation

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