C-Terminal Parathyroid Hormone-Related Protein Increases Vascular Endothelial Growth Factor in Human Osteoblastic Cells

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Abstract. The N-terminal region of parathyroid hormone (PTH) and PTH-related protein (PTHrP) interacts with a common PTH/PTHrP receptor in osteoblasts. These cells synthesize PTHrP, but its role in bone turnover is unclear. Intermittent treatment with N-terminal PTHrP or PTH stimulates bone growth in vivo, possibly by increasing local bone factors. In addition, C-terminal PTHrP (107–139), which does not bind to the PTH/PTHrP receptor, appears to affect bone resorption in vivo and in vitro, although its effect on bone formation in vivo remains controversial. Bone angiogenesis is an often overlooked but critical event in the process of bone remodeling. Recently, PTH (1–34) has been shown to induce gene expression of vascular endothelial growth factor (VEGF), a potent angiogenic factor, by osteoblastic cells. However, no data are available on the effect of PTHrP (107–139) on VEGF expression in these cells. Using semiquantitative reverse transcription followed by PCR, we found that PTHrP (107–139), between 10 nM and 1 µM, increased VEGF mRNA in human osteoblastic (hOB) cells from trabecular bone. This effect of this agonist, at 10 nM, was maximal (fivefold for VEGF165, and twofold for VEGF121, compared to control) within 1 to 4 h. This effect was similar to that induced by PTHrP (1–34) in these cells, as well as in human osteosarcoma MG-63 cells, using Northern blot analysis. Moreover, the effect of both peptides, added together at 100 pM, was not higher than that observed with each peptide alone in hOB cells. The effects of PTHrP (107–139) and that of PTHrP (1–34) were abolished by actinomycin D in hOB cells. In these cells, the protein kinase C inhibitor staurosporine, but not the protein kinase A inhibitor H89, inhibited the increase in VEGF mRNA induced by 10 nM PTHrP (107–139). PTHrP (107–139), at 10 nM, also stimulated cytosolic VEGF immunostaining in hOB cells, and VEGF secretion into the medium conditioned by hOB or MG-63 cells for 24 h, which was (ng/mg protein): 10 ± 1 or 5 ± 3 (control), respectively, and 21 ± 1 or 11 ± 2 (PTHRP [107–139]-stimulated), respectively. Furthermore, medium conditioned by these cells for 24 h in the presence of 10 nM PTHrP (107–139), with or without 10 nM PTHrP (1–34), increased about 30% bovine aortic endothelial cell (BAEC) growth at 48 h. This effect was inhibited by adding a specific anti-VEGF antibody to the BAEC incubation medium. These findings demonstrate that the C-terminal domain of PTHrP induces expression and secretion of VEGF, a main angiogenic factor, in hOB cells and MG-63 cells. This relationship between PTHrP and VEGF has potential implications for both bone vascularization and bone formation, and neangiogenesis in PTHrP-producing tumors.

The important role of bone microvasculature in osteogenesis was postulated decades ago (1). In fact, electrical stimulation induces an increase of bone formation associated with a parallel increase of blood capillaries (2). On the other hand, a reduction in blood flow and number of sinusoids is a well known feature of osteoporotic bone, and impairment of vascular supply leads to bone necrosis (3–5). It is now known that vascularization occurs before osteogenesis during both intramembranous and endochondral bone formation. During the latter process, bone-forming osteoblasts line the terminal capillary wall of the sprouting capillaries invading the epiphysis plate, so that cartilage matrix in this area is degraded by osteoclasts, and then replaced by new bone after vascular invasion (6,7). In fracture repair, callus formation depends on the restoration of blood supply in the fracture cavity. Endothelial cells in the vicinity of the fracture gap become transformed, associated with the appearance of dedifferentiated mesenchymal cells and new osteoblasts (8). Moreover, endothelial cells are known to synthesize various bone formation inducers (9,10). Taken together, these findings support the point of view that bone growth and angiogenesis occur in a coordinate manner. However, the mechanisms of interaction between bone endothelium and osteoblastic cells to promote an adequate angiogenesis to encompass bone formation are still unclear.

Vascular endothelial growth factor (VEGF) is a potent and specific mitogen for endothelial cells, which has a key role in
normal and pathologic angiogenesis (11). Alternative mRNA splicing yields four different VEGF molecular species of 206, 189, 165, and 121 amino acids (12). The latter two isoforms are diffusible proteins detected in the majority of cells expressing the VEGF gene (12). A recent study has demonstrated that VEGF mRNA is present in hypertrophic chondrocytes in the mouse epiphyseal growth plate, where VEGF-dependent blood vessel invasion appears to be essential for coupling cartilage resorption with bone formation (13). Recently, mRNA for VEGF and its receptors, flt-1 and KDR, have been detected in preosteoblasts and osteoblasts in the human fracture callus (14). VEGF mRNA is rapidly induced by prostaglandin E₁ (PGE₁) and PGE₂, potent stimulators of bone formation in vivo, in rat osteoblastic cells (15). Moreover, insulin growth factor I and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), two bone anabolic factors, stimulate VEGF expression in human osteoblastic (hOB) cells (10,16,17).

Parathyroid hormone (PTH)-related protein (PTHrP) and the common type 1 PTH/PTHrP receptor are present in a variety of normal and fetal tissues and cell types, including chondrocytes and osteoblasts in skeletal tissue (18). Both PTH and PTHrP, when administered in an intermittent manner in vivo, are anabolic in bone (19,20). This effect is likely to be accounted for by their action on the synthesis of various growth factors in bone cells (21,22). In this regard, PTH (1–34) has recently been shown to increase VEGF gene expression or protein secretion, alone or in the presence of 1,25(OH)₂D₃, respectively, in human osteosarcoma SaOS-2 cells (16,17). Furthermore, the bone anabolic effects of PTHrP might not be restricted to its N-terminal domain. Thus, PTHrP peptides containing the 107–111 epitope, so called osteostatin, have been shown to inhibit bone resorption in rodents both in vitro and in vivo (20,23–25). In addition, local injection of low doses of one of these peptides to mouse calvaria decreases the number of osteoblasts (23), while intermittent administration of high doses of PTHrP (107–111) into ovariectomized rats decreases trabecular bone formation, but restores cortical bone mass (20). In these in vivo models, however, a putative positive effect of C-terminal PTHrP on bone mass might be difficult to assess in the presence of the marked inhibition of bone resorption induced by this PTHrP domain (20,23,25). Recent in vitro studies from our group and from other investigators (26–28) have found either inhibitory or stimulatory effects of PTHrP (107–139), a putative PTHrP fragment (29), on osteoblastic proliferation and/or differentiation, depending on the type of osteoblastic cells studied. Thus, the true effect of this functional domain of PTHrP on bone formation is unclear.

In the present study, we specifically investigated the effect of the C-terminal PTHrP peptide PTHrP (107–139) on VEGF expression at the transcriptional and protein levels in hOB cells. We have also assessed the putative intracellular mechanism involved in the effect of this PTHrP peptide on VEGF expression in these cells. The effect of PTHrP (107–139) on VEGF expression was compared with that of the PTH-like peptide PTHrP (1–34) in hOB cells.

Materials and Methods

Materials

Human PTHrP (1–34) amide (PTHrP [1–34]), human PTHrP (107–139), actinomycin D, and staurosporine were from Sigma (St. Louis, MO). N-[2-[p-(bromocinnamyl)amino]-ethyl]-5-isouquinolinesulfonamid dihydrochloride (H89) was from Calbiochem (San Diego, CA). VEGF cDNA probe was kindly donated by Dr. B. Williams (Leicester University, United Kingdom). This probe corresponds to a domain within exon 3 in the VEGF gene, a coding region common to the four VEGF isoforms.

Cell Cultures

hOB cells were cultured from trabecular bone explants obtained at the time of surgery on osteoarthritic patients. The patients (six women and three men, ages 56 to 81 yr) had no evidence of metabolic bone disorders. The bone fragments were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ at 37°C, as described (26). Experiments were performed with these cells subcultured at first passage and grown to confluence, which display features of functional osteoblasts (26). The hOB cells were preincubated for 48 h in phenol red-free DMEM (1 g/L glucose) supplemented with 10 nM vitamin K, 50 µg/ml ascorbic acid, 0.1% bovine serum albumin (BSA), and antibiotics (differentiation medium), and then the test agents, dissolved in the same medium, were added for various time periods.

Human osteosarcoma cells MG-63 (American Type Culture Collection CRL 1427, Manassas, VA) were grown in DMEM with 10% FBS and antibiotics. Confluent cells were FBS-depleted for 24 h before stimulation with the agonists for various time periods.

Bovine endothelial cells (BAEC), obtained by digestion with type II collagenase, were cultured in minimum essential medium with D-valine, supplemented with 20% FBS, iron, nonessential amino acids, and antibiotics, as described (30). Cells were used between two to four passages.

Assay of BAEC Proliferation

BAEC grown to 60 to 70% confluence in the aforementioned medium were incubated for 24 h in hOB differentiation medium, replacing 0.1% BSA by 1% FBS. Then, the conditioned medium from hOB cells was added for 48 h. Cell number was counted using a Neubauer chamber (Afora, Madrid, Spain). In some experiments, a neutralizing concentration (2 µg/ml) of a specific anti-human VEGF monoclonal antibody (Sigma) (30), or the same concentration of nonimmunogenic IgG, was added to BAEC.

Isolation of Total RNA and Reverse Transcription-PCR

VEGF mRNA levels in hOB cells were assessed by reverse transcription followed by PCR (RT-PCR). Total RNA was extracted by guanidinium thiocyanate-phenol-chloroform extraction (31). Total RNA aliquots were added to a reaction mixture (10 µl) with 1 mM MgSO₄, 0.2 mM of each deoxynucleotide triphosphate, 1 U of avian myeloblastosis virus reverse transcriptase, 1 U of thermostable DNA polymerase from Thermus flavus (Access RT-PCR System; Promega, Madison, WI), and 0.5 µM of the specific primers for the human VEGF gene (32): 5'-CTAGAATTCTTCTGCTCTGTTG-3' (sense), and 5'-CTACCGCCCTGCTTGTGTCAC-3' (antisense).

RT-PCR of mRNA encoding the 121, 165, 189, and 206 amino acid VEGF isoforms, which arise by alternative splicing, yields 459-, 591-, and 599-nt products, respectively. The restriction enzyme sites for EcoRI and BamHI were introduced in the 5’ position of the primer.
sequence to facilitate sequencing of PCR products. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was co-amplified, using specific primers for the human gene (33) as a constitutive control.

Total RNA and the primers were preincubated for 5 min at 65°C. Then, the reaction mixture was incubated for 45 min at 48°C, and 2 min at 95°C, followed by 30 to 35 cycles of 1 min at 95°C, 1 min at 60°C, and 2 min at 68°C, with a final extension of 7 min at 68°C. The PCR products were separated on 2% agarose gels, and bands were visualized by ethidium bromide staining and quantified by densitometric scanning (ImageQuant; Molecular Dynamics, Sunnyvale, CA). Densitometric values for VEGF PCR product were normalized by comparison with the GAPDH product signal. The identity of the PCR products were confirmed by automatic sequencing in a 373 DNA Sequencer (Applied Biosystems, United Kingdom).

**Northern Blot Analysis**

Because availability of hOB cell cultures was limited, and their RNA yield was poor, we further analyzed the response of VEGF mRNA to PTHrP fragments in MG-63 cells, which express both VEGF and the PTH/PTHrP receptor (16,34). Total RNA was isolated from MG-63 cells as described above. Twenty micrograms of total RNA were size-fractionated on 1% agarose gel containing 1.2 M formaldehyde and transferred to nylon membranes (Hybond-N+, Amersham, Buckinghamshire, United Kingdom). The membranes were prehybridized at 42°C for 8 h, and hybridized overnight at 42°C with 10^6 cpm/ml 32P-labeled human VEGF cDNA probe. This probe was labeled with [α-32P]dCTP using a random-primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). Filters were washed for 30 min at 42°C in 1× saline-sodium phosphate-ethylene-diaminotetra-acetic acid, 0.3% sodium dodecyl sulfate. Filters were then exposed on Kodak X-Omat film at −70°C for 4 to 6 d. The filters were reprobed with a 28S cDNA probe.

**VEGF Enzyme-Linked Immunoassay**

hOB cells and MG-63 cells were incubated in phenol red-free medium with supplements and FBS-depleted DMEM, respectively, in the presence or absence of the test agents for 24 h. The conditioned medium was collected, and kept at −20°C for up to 3 wk before assay. VEGF was determined by a sandwich-type enzyme-linked immunoassay (ELISA) (CYTELisaTM VEGF assay; CYTImmune Sciences, Inc., College Park, MD) combining a capture monoclonal antibody precoated onto the microplate, and a signal biotinylated polyclonal antibody. The assay sensitivity is 5 pg/ml, and detects both secreted and/or cell-associated VEGF. Cell protein was assayed in 0.1N NaOH-solubilized cell extracts, using the Bradford method (35). Data were expressed as the amount of VEGF secreted per milligram of cell protein.

**Immunocytochemistry**

hOB cells were incubated with the agonists in phenol red-free medium with supplements on chamber slides (Nalge Nunc International, Naperville, IL) for 24 h. The cells were then fixed in ethanol. Immunocytochemistry was performed using an affinity-purified anti-VEGF IgG. This antibody was raised by rabbit immunization with a peptide corresponding to a common region for all human VEGF isoforms, coupled to keyhole limpet hemocyanin (36). The cells were incubated with this affinity-purified antibody (10 μg/ml) for 2 h at room temperature, followed by sequential addition of avidin-biotin-peroxidase complex and 3,3′-diaminobenzidine (Sigma) (27). As negative controls, some cell preparations were incubated with 10 μg/ml nonimmunogenic rabbit IgG.

**Statistical Analyses**

Results are expressed as means ± SD. Statistical analyses were performed by unpaired t test or one-way ANOVA, when appropriate. P < 0.05 was considered significant.

**Results**

Agonist-induced changes of VEGF expression were evaluated by using semiquantitative RT-PCR. We carried out preliminary titration experiments to determine the suitable amount of total RNA and the number of cycles that provide submaximal amplification of VEGF in our RT-PCR system. We found that 30 to 35 cycles and 10 ng of total RNA from either PTHrP-stimulated or nonstimulated hOB cells fit this requirement. Using these conditions, PTHrP (107–139), at 10 nM, was found to induce an approximately fivefold increase of VEGF165 mRNA within 1 to 4 h in these cells, declining thereafter (Figure 1, A and C). Moreover, this PTHrP peptide, at 10 nM, also significantly increased by approximately twofold VEGF121 mRNA in hOB cells within the same time period (Figure 1, A and C). Because a recent study in human osteosarcoma SaOS-2 cells has shown that PTH (1–34) stimulates VEGF gene expression at 48 h (16), we then sought to examine the effect of PTHrP (1–34), interacting with a common PTH/PTHrP receptor in osteoblasts (37), on VEGF mRNA in hOB cells. We found that this peptide, similar to PTHrP (107–139), at 10 nM increased both VEGF165 and VEGF121 mRNA in these cells (Figure 1, A and C). This effect of either PTHrP (107–139) or PTHrP (1–34), both at 10 nM, on VEGF mRNA was of a range similar to that induced by 10% FBS, a positive control (11), in these cells (Figure 1A). The stimulatory effect of PTHrP (107–139) or PTHrP (1–34) on the expression of each VEGF isoform in hOB cells was already detectable with 1 pM of these peptides (not shown), and it was neither additive nor synergistic when both peptides were added together at a submaximal concentration (100 pM) (Figure 1B). Using Northern blot analysis, we have also found that each PTHrP peptide stimulated VEGF mRNA in MG-63 cells (Figure 2).

Additional studies were carried out to further characterize the mechanism involved in the upregulation of VEGF mRNA by both PTHrP (107–139) and PTHrP (1–34) in hOB cells. To examine whether the increased VEGF mRNA induced by both PTHrP peptides was the result of an increased transcription, we incubated the hOB cells with the RNA polymerase inhibitor actinomycin D (38) at 10 μg/ml in the presence and absence of these PTHrP peptides. We found that actinomycin D inhibited VEGF mRNA in nonstimulated hOB cells, and abolished the stimulatory effect of 10 nM PTHrP (107–139) or PTHrP (1–34) on VEGF mRNA in these cells (Figure 3). Meanwhile, actinomycin D did not affect the mRNA of the housekeeping gene GAPDH in these cells (Figure 3).

Previous studies have suggested the involvement of protein kinase C (PKC) in various effects of PTHrP (107–139) on cell proliferation and/or differentiation in a variety of cell types, including hOB cells (24,26,27,38–41). In the present study, 100 nM staurosporine, a PKC inhibitor (42), in contrast to 100 nM H89, a PKA inhibitor (43), abrogated the effect of 10 nM PTHrP (107–139) on VEGF mRNA in hOB cells (Figure 3).
Conversely, 100 nM H89 abolished the effect of 10 nM PTHrP (1–34) on VEGF expression in these cells (Figure 3).

We next assessed whether PTHrP (107–139) increased VEGF protein in hOB cells. A weak VEGF immunostaining was found in the cytoplasm of nonstimulated hOB cells (Figure 4A). Treatment with 10 nM PTHrP (107–139) for 24 h increased VEGF positivity in these cells (Figure 4B). In contrast, no cell staining was observed after incubation with nonimmunogenic IgG (Figure 4C). PTHrP (107–139), at 10 nM, was also found to increase VEGF levels in hOB cell-conditioned medium. Thus, at 24 h, immunoreactive VEGF in this medium was (ng/mg protein): $10 \pm 1$ (nonstimulated hOB cells) and $21 \pm 1$ (PTHrP [107–139]-treated hOB cells) ($n = 3$; $P < 0.01$). At this time period, 10 nM PTHrP (107–139) also induced an increase of VEGF secretion in MG-63 cells, which was (ng/mg protein): $5 \pm 3$ and $11 \pm 2$ in cells untreated or treated with 10 nM PTHrP (107–139), respectively ($n = 3$;
Furthermore, the medium conditioned by hOB cells for 24 h in the presence of 10 nM PTHrP (107–139) induced a significant increase in BAEC growth, compared to that of these cells incubated in hOB cell-conditioned medium without this peptide (Figure 5). This effect was abolished by the simultaneous addition of an anti-VEGF antibody, but not by nonimmunogenic IgG (Figure 5). This effect of the conditioned medium from PTHrP (107–139)-treated hOB cells was not further increased by the simultaneous presence of various concentrations (100 pM to 10 nM) of PTHrP (1–34) (not shown).

**Discussion**

Angiogenesis is known to play a significant role in bone formation. Current data support the existence of a finely regulated cross-talk between endothelium and bone cells during bone growth and repair (6–8). However, the interactions between endothelial cells and other cells in the bone microenvironment are poorly understood. A variety of agents that stimulate bone formation, including PTH (1–34), induce expression and secretion of VEGF, a potent angiogenic factor, in osteoblastic cells (10,15–17). On the other hand, dexamethasone, which induces avascular bone necrosis (44), inhibits VEGF expression in rat calvaria-derived osteoblastic cells and human osteosarcoma SaOS-2 cells (15,17). Recent findings indicate that suppression of blood vessel invasion by systemic administration of a VEGF receptor chimeric protein to mice leads to an inhibition of cartilage resorption and an impaired bone formation (13). These data point to VEGF as a critical coordinator of cartilage remodeling and ossification in endochondral bone formation.

In the present study, PTHrP (107–139) was found to increase VEGF mRNA in hOB cells and MG-63 cells. This induction was rapid and transient in hOB cells and conditioned medium without this peptide (Figure 5). This effect was abolished by the simultaneous addition of an anti-VEGF antibody, but not by nonimmunogenic IgG (Figure 5). This effect of the conditioned medium from PTHrP (107–139)-treated hOB cells was not further increased by the simultaneous presence of various concentrations (100 pM to 10 nM) of PTHrP (1–34) (not shown).
induced by 1,25(OH)2D3 or 10% FBS in hOB cells (reference 16 and present results, respectively), suggesting its putative physiologic importance. The enhanced VEGF mRNA after stimulation with PTHrP (107–139) was accompanied by an increase in VEGF protein in these cells, as shown by an increased VEGF immunostaining in hOB cells and a stimulated immunoreactive VEGF in the conditioned medium from PTHrP (107–139)-treated hOB and MG-63 cells. In addition, stimulation of hOB cells with PTHrP (107–139) also increased biologically active VEGF in these cells’ conditioned medium, as demonstrated by its induction of an anti-VEGF antibody-inhibitable BAEC growth stimulation.

The effects of 1,25(OH)2D3 and PGE2 on VEGF expression in osteoblastic cells appear to depend on PKC and PKA activation, respectively, consistent with the hypothesis that both kinases are involved in the induction of VEGF increase (45). In the present study, using specific inhibitors, we found that VEGF mRNA induction by PTHrP (107–139) in hOB cells is likely to occur by activation of PKC. This is consistent with our previous observation that PTHrP (107–139) fails to stimulate PKA activity in rat osteoblastic osteosarcoma UMR 106 and hOB cells (26,27). On the other hand, this PTHrP peptide has been shown to increase PKC activity in rat osteoblastic osteosarcoma cells ROS 17/2.8 (39), and also in rat spleen lymphocytes and human keratinocytes (40,41).

A previous report, using Northern blot analysis, has shown a stimulatory effect of PTH (1–34) on VEGF mRNA in human osteosarcoma SaOS-2 cells (16). We found herein that PTHrP (1–34), similar to PTHrP (107–139), increased VEGF mRNA in hOB and MG-63 cells. In addition, our results suggest that this effect is cAMP-dependent, supporting the hypothesis that cAMP stimulation is the major pathway involved in the bone anabolic effects of the N-terminal region of PTH and PTHrP (46,47).

Both PTHrP (107–139) and PTHrP (1–34), added together at a concentration that triggers a submaximal effect on VEGF expression, failed to induce a greater increase of VEGF mRNA compared to that triggered by each peptide alone in hOB cells. This suggests the existence of a common final effector pathway affecting VEGF expression, and supports that a cross-talk in the signal transduction pathways associated with the response to each PTHrP peptide occurs in these cells, as noted previously (38,48).

The physiologic relevance of our findings is supported by the fact that several PTHrP fragments, including the domains 1–36 and 107–139, are secreted by a variety of cells (29,49). In addition, C-terminal PTHrP fragments containing its 107–111 region accumulate in plasma of uremic patients, associated with the decrease of renal function (50). The latter PTHrP domain has been shown to induce either a somewhat inhibitory or a stimulatory effect on bone formation in mouse calvaria or ovariecotomized rats, respectively, associated with a dramatic inhibition of bone resorption in both in vivo models (20,23). Therefore, these studies do not rule out a possible direct effect of this PTHrP region on bone formation. In this regard, we recently found that PTHrP (107–139) increases the expression of interleukin-6, a putative osteoblast differentiation factor, in hOB cells (38,51). Our present findings support a role for PTHrP (107–139) as a bone formationpromoter. In addition, considering the aforementioned inhibitory effect of this peptide on bone resorption, our data are also consistent with the hypothesis that bone angiogenesis can occur independently of bone resorption, as recently suggested (52).

In summary, the results herein support that the C-terminal domain of PTHrP, in a manner similar to that of its PTH-like region, could promote bone angiogenesis by inducing VEGF in normal and transformed human osteoblastic cells. Additional studies are required to clarify the functional significance of the cross-talk between the different intracellular pathways that appeared to be linked to the effect of both PTHrP regions on VEGF expression in hOB cells. However, it is reasonable to speculate that this might provide alternative pathways to ensure bone vascularization. Our findings also suggest a putative mechanism that might be responsible, at least in part, for neoangiogenesis in PTHrP-producing tumors.

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