Induction of Vascular Endothelial Growth Factor Expression by Prostaglandin E_2 and E_1 in Osteoblasts

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Abstract

PGE_1 and PGE_2 are potent stimulators of bone formation. Osteogenesis is strongly dependent on angiogenesis. Vascular endothelial growth factor (VEGF), a secreted endothelial cell–specific mitogen, has been implicated in physiological and pathological angiogenesis. The aim of this study was to examine the possible role of VEGF in PG stimulation of bone formation. We found that in rat calvaria-derived osteoblast-enriched cells and in the osteoblastic RCT-3 cell line PGE_2 and E_1 increased VEGF mRNA and protein levels. The increased expression of VEGF mRNA produced by PGE_2 was rapid (maximal at 1 h), transient (declined by 3 h), potentiated by cycloheximide, and abolished by actinomycin D. PGE_2 had no effect on VEGF mRNA stability, suggesting transcriptional regulation of VEGF expression by PGE_2. Rp-cAMP, a cAMP antagonist, suppressed VEGF mRNA induced by PGE_2, indicating cAMP mediation. The upregulation of VEGF expression by PGE_2 in the preosteoblastic RCT-1 cells was potentiated by treatment with retinoic acid, which induces the differentiation of these cells. The upregulation of VEGF mRNA by PGE_2 was inhibited by dexamethasone treatment. In addition, Northern blot analysis showed that VEGF mRNA is expressed in adult rat tibia. In summary, we documented, for the first time, the expression of VEGF in osteoblasts and in bone tissue. Stimulation of VEGF expression by PGs and its suppression by glucocorticoids, which, respectively, stimulate and suppress bone formation, strongly implicate the involvement of VEGF in bone metabolism. (*J. Clin. Invest.* 1994. 93:2490–2496.) Key words: angiogenesis • bone formation • glucocorticoids • cyclic AMP • differentiation

Introduction

Angiogenesis, the development of a microvascular network for blood supply, is critical for the development, remodeling, and healing (1, 2) of most tissues, including bone (3). In endochondral bone formation, the avascular cartilage which precedes bone may produce antiangiogenic factor(s) (4), while the appearance of osteoblasts coincides with blood vessel invasion (5, 6). Furthermore, surgical vascularized bone grafts are better retained than nonvascularized tissue (7). A reduction in bone blood flow and microvascular defects in the sinusoidal compartment have been reported in osteoporotic bone as well as in aplastic anemia (8). Moreover, impairment of vascular supply results in avascular necrosis of bone (9), indicating the importance of angiogenesis for the development and maintenance of bone tissue.

Prostaglandins (PGs) are produced in bone in response to inflammation, injury, and mechanical stress (10) and have been implicated in the local regulation of bone metabolism (11). Increasing evidence indicates that PGs stimulate bone formation in vivo. Infusion of PGE_1 in infants with cyanotic heart disease produces periosteal new bone formation (12), and systemic and local administration of PGE_1 or PGE_2 increase periosteal and/or trabecular bone formation in dogs and rats (13–15). Although it has been shown that PGE_2 stimulates proliferation and other osteoblast functions in organ and cell culture (16, 17), the mechanism of PG stimulation of bone formation in vivo has not yet been clarified.

One of the most pronounced effects of PGs in vivo is on the vascular system. PGE_2 and PGE_1 can cause vasodilation and stimulate angiogenesis (18–20). However, PGs do not stimulate endothelial cell growth, suggesting that the angiogenic effect may be mediated by the paracrine action of angiogenic factors released from other cells.

Vascular endothelial growth factor (VEGF)[1] is a heparin-binding dimeric glycoprotein that induces endothelial cell proliferation, angiogenesis, and capillary permeability (21–24). Cloning of VEGF cDNAs from several sources demonstrated that four different molecular species of VEGF are generated by alternative splicing of mRNA ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) (25). VEGF is distinct from other identified endothelial cell mitogens such as fibroblast growth factors, in that VEGF is a secreted protein (25), an endothelial cell–specific mitogen in vitro, and the only known growth factor with vascular permeability–inducing activity (26). VEGF mRNA is expressed in a variety of highly vascularized tissues such as pituitary glands, brain, lung, heart, and kidney (27, 28). In the ventricular neuroectoderm of the mouse embryo and in the rat ovary, the expression of VEGF mRNA is temporally and spatially related to the development of capillaries (29, 30), suggesting that VEGF is a physiological mediator of angiogenesis.

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1. *Abbreviations used in this paper:* Dex, dexamethasone; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; HUVE, human umbilical endothelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor.

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Furthermore, recent findings in the rat female reproductive system suggest that VEGF mRNA expression is hormonally regulated in steroid-producing and steroid-responsive cells (31).

We therefore examined the role of VEGF in bone metabolism especially in the context of PG-induced osteogenesis. We show that VEGF is expressed in osteoblastic cells and in bone tissue and that PGE_2 and PGE_1 rapidly increase the levels of VEGF in osteoblastic cells.

Methods

Reagents. Tissue culture plates were purchased from Costar Corp. (Cambridge, MA) or Nunc (Kamstrup, Denmark). Rp-cAMP was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). H7 was from Seikagaku America, Inc. (Rockville, MD). Staurosporine was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Human PTH-(1-34) was from Bachem California (Torrance, CA). PGE_2, PGE_1, dibutyryl cAMP, forskolin, all-*trans*-retinoic acid, dexamethasone, 17β-estradiol, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), actinomycin D, cycloheximide, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Primary cultures of osteoblast-enriched cells were prepared by collagenase/hyaluronidase digestion of fetal rat calvaria from 19-d-old pregnant Sprague-Dawley rats as described previously (32). The cells released during 30–50 min of digestion (fraction 3) were plated in F-12 medium containing 5% FBS (JRH Biosciences, Lenexa, KS). RCT-1 and RCT-3 cells are clonal cells immortalized by infection with a recombinant retrovirus containing the cDNA for SV-40 large T antigen and the bacterial neomycin resistance gene produced by ψ-tex cells (33). RCT-1 cells, derived from cells released during the first 10 min of enzyme digestion from fetal rat calvaria (fraction 1), were cultured in RPMI 1640 medium containing 10% FBS and 400 μg/ml of G418 (GIBCO BRL, Gaithersburg, MD). RCT-3 cells, derived from fraction 3 calvaria cells, were cultured in F-12 medium containing 5% FBS and 400 μg/ml of G418. Human umbilical vein endothelial (HUVE) cells were cultured in MCDB 107 medium (Sigma Chemical Co.) containing 15% FBS, 90 μg/ml heparin, and 25 μg/ml endothelial mitogen (Biochemical Technologies Inc., Stoughton, MA).

RNA isolation and Northern blot analysis. Total cellular RNA was isolated by guanidium isothiocyanate and phenol extraction as previously described (34). Polyadenylated RNA from cultured cells or tibia of 4–5-wk-old Sprague-Dawley rats was obtained by oligo(dT)-cellulose using Quick-Prep mRNA isolation kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Total RNA (30 μg) or polyadenylated RNA (5 μg) was electrophoresed through 1% agarose-formaldehyde (0.22 M) and electroblotted to nylon filters (Hybond N; Amersham Corp., Arlington Heights, IL). The filters were prehybridized in buffer containing 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), 5× Denhardt's solution, and sonicated salmon sperm DNA (100 μg/ml) and hybridized at 42°C in fresh buffer containing rat VEGF cDNA (35) labeled by a random primer DNA labeling kit (Pharmacia LKB Biotechnology Inc.) using [α-^{32}P]deoxy-CTP (Amersham Corp.). Filters were washed at 60°C in 0.1× SSC/0.1% SDS (at 50°C in 0.5× SSC/0.1% SDS for the filters with HUVE cell RNA). For comparison of RNA loading, filters were rehybridized with synthetic oligonucleotide corresponding to antisense mouse β-actin cDNA (5'-AGCCGTTGTCGACGACCAGCGCAGCGATATCGT-CATCCAT-3') labeled by deoxynucleotide terminal transferase (Boehringer-Mannheim, Indianapolis, IN) using [α-^{32}P]deoxy-ATP (Amersham Corp.) or human glyceraldehyde 3-phosphate dehydrogenase cDNA.

Immunoblot analysis. Immunoblot analysis of VEGF in culture media was carried out by Prot Blot AP system (Promega Corp., Madison, WI) using affinity-purified polyclonal antibody against NH_2-terminal peptide of rat VEGF (Nagy, J. A., E. M. Masse, K. T. Herzberg, M. S. Meyers, K.-T. Yeo, T. K. Yeo, T. M. Sioussat, and H. F. Dvorak, manuscript in preparation). Conditioned media from fetal rat–derived osteoblast-enriched cells or RCT-3 cells were centrifuged at 1,000 *g* for 5 min to remove cellular debris. AEBSF (4-[2-aminoethyl]-benzene sulfonyl fluoride, HCl) (1 mM) (Calbiochem-Novabiochem Corp.) was added to the supernatant. The media were concentrated 40-fold by Centricon-10 (Amicon Corp., Danvers, MA) at 4°C, and protein levels were measured by BCA-protein assay reagent (Pierce Chemical Co., Rockville, IL). Equal amounts of samples (~ 150 μg) were analyzed by 4–20% gradient SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (GIBCO BRL). The membranes were blocked in Blotto (5% nonfat dry milk, 2 mM $CaCl_2$, 0.05% Tween 20, 0.01% Antifoam A, 50 mM Tris, pH 8.0) for 1 h at room temperature and subsequently incubated with anti-rat VEGF polyclonal antibody in Blotto at 4°C overnight. The membranes were washed with Blotto for 20 min three times and incubated with anti-rabbit IgG–AP conjugate for 1 h at room temperature. After washing with Blotto, TBST (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween 20), TBS (150 mM NaCl, 10 mM Tris-HCl, pH 8.0), and AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) for 10 min each, the membranes were incubated with substrate solution containing 66 μl nitroblue tetrazolium (Promega Corp.), 33 μl BCIP (5-bromo-4-chloro-3-indolyl phosphatase) (Promega Corp.), and 10 ml AP buffer.

Results

Induction of VEGF by PGE_2 in osteoblastic cells. RCT-3 cells express osteoblastic features, such as alkaline phosphatase, type I collagen, osteopontin, parathyroid hormone, and PGE_2-responsive adenylate cyclase (33). Since PGE_2 was shown to increase bone formation, we evaluated the effects of PGE_2 on VEGF mRNA in these osteoblastic cells. RNA prepared from untreated cultures of RCT-3 cells contains a single VEGF transcript of ~ 3.8 kb. Treatment of RCT-3 cells with 10^{-6} M PGE_2 resulted in a rapid and transient increase in the level of VEGF mRNA which peaked at 60 min and declined to basal levels within 3 h (Fig. 1 *A*). The increased expression of VEGF mRNA induced by PGE_2 was dose dependent as shown in Fig. 1 *B* (10^{-8}–10^{-6} M). A similar rapid increase in VEGF mRNA levels by PGE_2 was also seen in rat calvaria-derived osteoblast-enriched cells (Fig. 2). PGE_2 did not increase the level of other angiogenic factors such as TGF-β1 or basic fibroblast growth factor mRNA in these cells (data not shown).

To confirm that PGE_2 increased the expression of VEGF protein as well as VEGF mRNA, conditioned medium was collected from rat calvaria-derived osteoblast-enriched cells after treatment with 10^{-6} M PGE_2 and was analyzed by immunoblotting using affinity-purified polyclonal antibody against the NH_2-terminal peptide of rat VEGF. Conditioned media from control cultures contained immunoreactive polypeptides of 24 and 18 kD (Fig. 3), similar in size to the murine VEGF (36). Treatment with 10^{-6} M PGE_2 for 3–6 h increased VEGF protein levels in conditioned media threefold (Fig. 3). Similar increases in VEGF protein levels by PGE_2 were observed in conditioned media of RCT-3 cells (data not shown).

Effects of protein synthesis and RNA synthesis inhibitors on VEGF mRNA. The rapid increase of VEGF mRNA induced by PGE_2 and the subsequent rapid decline in osteoblastic cells (Fig. 1 *A*) suggest rapid turnover of VEGF mRNA similar to that of early genes, such as c-*fos* and c-*myc,* and low stability of VEGF mRNA. To characterize the mechanism for PGE_2 upregulation of VEGF mRNA, we examined the effects of inhibitors of protein synthesis and RNA polymerase. In RCT-3 cells, the increase in VEGF mRNA after 60 min of treatment with PGE_2 was potentiated by cycloheximide (5 μg/ml), which alone had

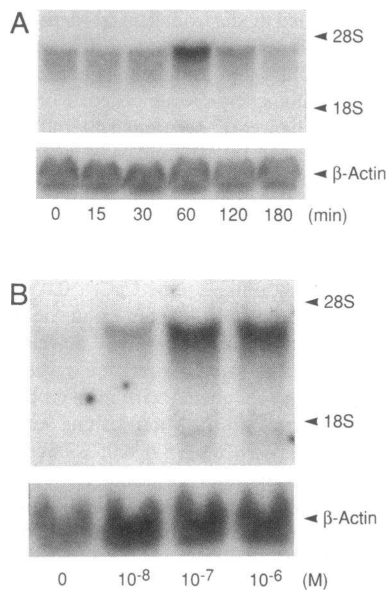

Figure 1. The effect of PGE_2 on VEGF mRNA in RCT-3 cells. Total RNA (30 μg/lane) was subjected to RNA blot analysis using a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. (*A*) Time course of PGE_2 induction of VEGF mRNA. RCT-3 cells were treated with 10^{-6} M PGE_2 for the indicated times, from 0 to 180 min. The figure represents the results from one of three similar experiments. (*B*) Dose-dependent induction of VEGF mRNA by PGE_2. RCT-3 cells were treated with increasing concentrations of PGE_2 (10^{-8}–10^{-6} M) for 60 min. The figure represents the results from one of three similar experiments.

no effect on VEGF mRNA at that time point. Furthermore, at 3 h, when VEGF mRNA declined to basal levels after PGE_2 treatment, cycloheximide maintained increased VEGF mRNA levels, suggesting that cycloheximide prevented the degradation of VEGF mRNA (Fig. 4 *A*). On the other hand, the effect of PGE_2 on VEGF mRNA was abolished completely by the addition of actinomycin D (2 μg/ml) or DRB (25 μg/ml) (Fig. 4 *B*), suggesting transcriptional regulation of VEGF expression by PGE_2.

To further study the mechanism of VEGF mRNA upregulation by PGE_2, we examined the effect of PGE_2 on the stability of VEGF mRNA. RCT-3 cells, pretreated with TPA (150 nM), which was shown to increase steady state levels of VEGF mRNA (37), were subsequently treated with DRB (25 μg/ml) in the presence or absence of PGE_2 (10^{-6} M). PGE_2 further increased VEGF mRNA pretreated by TPA in the absence of DRB (Fig. 5 *A*), but had no effect on the half-life of VEGF mRNA (Fig. 5 *B*). These results are consistent with PGE_2 stimulation of VEGF mRNA transcription in osteoblastic cells.

Role of cAMP in PGE_2 induction of VEGF mRNA. It has been shown that PGE_2 increases intracellular cAMP levels as well as phosphatidyl inositol turnover in cultured osteoblastic cells (38, 39). As shown in Fig. 6, PGE_1, which increases intracellular cAMP levels but does not induce phosphatidyl inositol turnover in osteoblastic cells (38, 39), was as potent as PGE_2 in increasing VEGF mRNA levels, suggesting cAMP mediation.

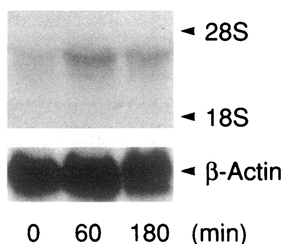

Figure 2. The effect of PGE_2 on VEGF mRNA in fetal rat calvaria-derived osteoblast-enriched cells. Fetal rat calvaria-derived osteoblastic cells were treated with PGE_2 for the indicated times, from 0 to 180 min. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of two similar experiments.

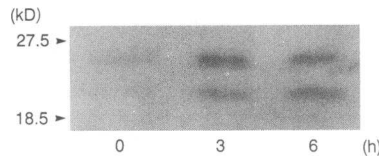

Figure 3. The effect of PGE_2 on VEGF production in culture media of fetal rat calvaria-derived osteoblast-enriched cells. Fetal rat calvaria-derived osteoblast-enriched cells were cultured in serum-free media for 6 h and were treated with PGE_2 for the indicated times. Media were concentrated by a Centricon-10 (Amicon Corp.), and aliquots (150 μg of protein) were examined by immunoblot analysis using affinity-purified polyclonal antibody against NH_2-terminal polypeptide of rat VEGF as described in Methods. Molecular markers in kilodaltons are indicated by arrowheads. The figure represents the results from one of three similar experiments.

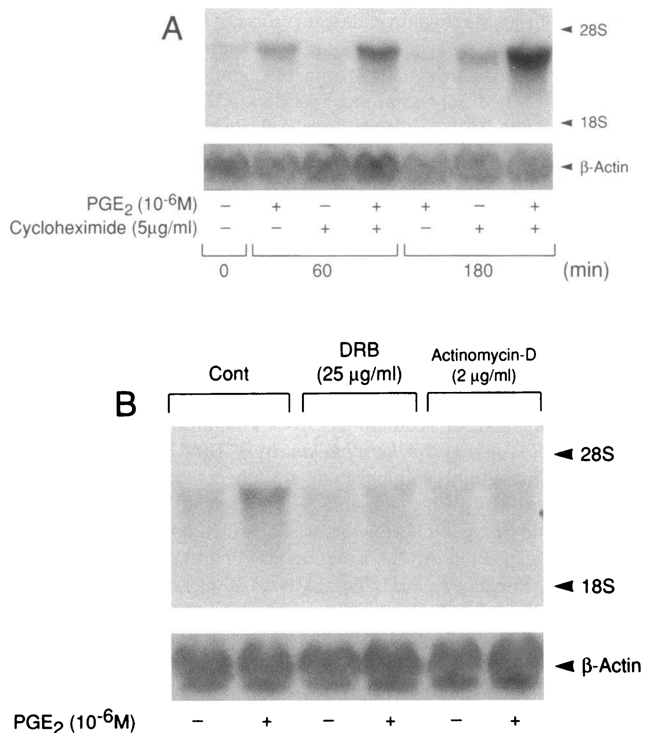

Figure 4. (*A*) The effect of cycloheximide on PGE_2 induction of VEGF mRNA. RCT-3 cells were treated with 10^{-6} M PGE_2 in the presence or absence of cycloheximide (5 μg/ml) for the indicated times, from 0 to 180 min. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of three similar experiments. (*B*) The effect of actinomycin D or DRB on PGE_2 induction of VEGF mRNA. RCT-3 cells were treated with 10^{-6} M PGE_2 for 60 min in the presence or absence of actinomycin D (5 μg/ml) or DRB (25 μg/ml). Total RNA (30 μg/lane) was subjected to RNA blot analysis using a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of two similar experiments.

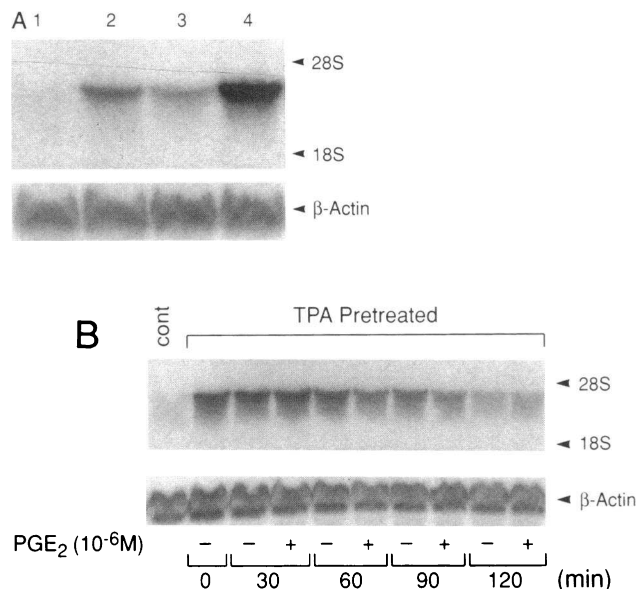

Figure 5. (*A*) The effect of PGE_2 on VEGF mRNA in RCT-3 cells pretreated with TPA. RCT-3 cells were pretreated with 150 nM TPA for 1 h and subsequently treated with 10^{-6} M PGE_2 for 1 h. Lane *1*, control; lane *2*, TPA (1 h); lane *3*, TPA (1 h) → none (1 h); lane *4*, TPA (1 h) → PGE_2 (1 h). Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of two similar experiments. (*B*) The effect of PGE_2 on the half-life of VEGF mRNA in RCT-3 cells. RCT-3 cells were treated with 150 nM TPA for 60 min and subsequently treated with DRB (25 μg/ml) in the presence or absence of 10^{-6} M PGE_2 for the indicated times, from 0 to 120 min. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of two similar experiments.

VEGF mRNA was also induced by other agents which increase cAMP, forskolin, PTH, and dibutyryl cAMP, however, less than by PGE_2 or PGE_1. Although the effect of PGE_2 on VEGF mRNA was suppressed by H7, a general inhibitor of protein kinase, staurosporine, a more specific inhibitor of protein kinase C, did not suppress VEGF mRNA upregulation by PGE_2 (Fig. 7 *A*), suggesting the involvement of other protein kinases, such as protein kinase A. Furthermore, Rp-cAMP, a cAMP antagonist (40), inhibited the effect of PGE_2 on VEGF mRNA (Fig. 7 *B*), pointing to possible mediation by intracellular cAMP in PGE_2 induction of VEGF mRNA in osteoblastic cells.

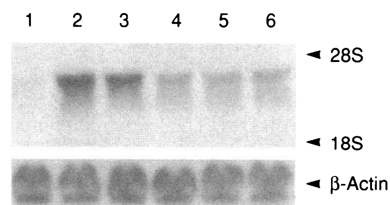

Figure 6. The effects of cAMP stimulating agents on VEGF mRNA. RCT-3 cells were treated with 10^{-6} M PGE_2 (lane *2*), 10^{-6} M PGE_1 (lane *3*), 10^{-7} M human PTH (1–34) (lane *4*), 10^{-5} M forskolin (lane *5*), or 10^{-4} M dibutyryl cAMP (lane *6*) for 60 min. Lane *1* shows untreated control. Total RNA (30 μg/lane) was subjected to RNA blot analysis using a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. The figure represents the results from one of two similar experiments.

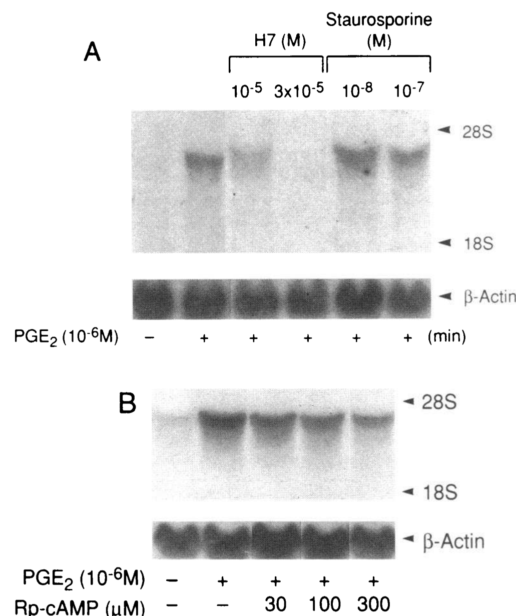

Figure 7. The effect of protein kinase inhibitors (*A*) or Rp-cAMP (*B*) on PGE_2 induction of VEGF mRNA. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. (*A*) RCT-3 cells were treated with 10^{-6} M PGE_2 in the presence or absence of indicated concentrations of H7 or staurosporine. The figure represents the results from one of two similar experiments. (*B*) RCT-3 cells were treated with 10^{-6} M PGE_2 in the presence or absence of indicated concentrations of Rp-cAMP. The figure represents the results from one of two similar experiments.

Differentiation-related upregulation of VEGF expression by PGE_2 in preosteoblastic cells. RCT-1 cells are clonal simian virus 40 immortalized preosteoblastic cells, in which retinoic acid induces differentiation (33). Pretreatment of these cells with retinoic acid (10^{-6} M) for 7 d strongly potentiated the effect of PGE_2 (10^{-6} M) on VEGF mRNA (Fig. 8 *A*). As shown in Fig. 8 *B*, pretreatment with retinoic acid for 1 or 4 d was insufficient, suggesting that the effect of retinoic acid on VEGF mRNA was indirect and may relate to effects on the differentiation of these cells. Interestingly, in HUVE cells which proliferate in response to VEGF (22), VEGF mRNA was barely detectable by polyadenylated RNA preparation and was not regulated by PGE_2 (Fig. 9).

Suppression of PGE_2 upregulation of VEGF mRNA by glucocorticoids. In RCT-3 cells, the increase in VEGF mRNA after 60 min of treatment with PGE_2 (10^{-6} M) was strongly attenuated by dexamethasone (Dex) (10^{-7} M), which alone also decreased the levels of VEGF mRNA (Fig. 10 *A*). PGE_2 upregulation of VEGF mRNA was also suppressed after pretreatment with Dex (10^{-7} M) for periods as short as 1 h, while pretreatment with either $1,25(OH)_2D_3$ (10^{-8} M) or estradiol (10^{-7} M) had no effect on VEGF mRNA (Fig. 10 *B*). Pretreatment with Dex also suppressed the increase in VEGF mRNA caused by TPA (150 nM) (Fig. 10 *C*), suggesting that Dex may modulate directly the level of VEGF mRNA.

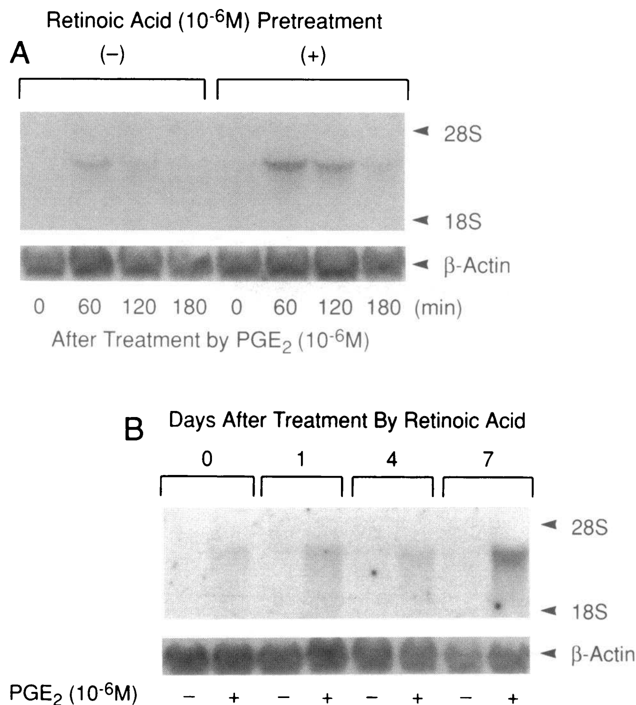

Figure 8. The effect of retinoic acid pretreatment on PGE_2 upregulation of VEGF mRNA in RCT-1 cells. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. (*A*) Time course of PGE_2. RCT-1 cells cultured in the presence or absence of 10^{-6} M retinoic acid for 7 d were treated with 10^{-6} M PGE_2 for the indicated times, from 0 to 180 min. The figure represents the results from one of three similar experiments. (*B*) Time course of retinoic acid pretreatment. RCT-1 cells were treated with 10^{-6} M retinoic acid for the indicated times, from 0 to 7 d and were subsequently cultured in the presence or absence of 10^{-6} M PGE_2 for 1 h. The figure represents the results from one of two similar experiments.

Expression of VEGF mRNA in adult rat tibia. To examine if VEGF is expressed in bone tissue in vivo, polyadenylated RNA was isolated from 4–5-wk-old rat tibia and analyzed by RNA blotting with the VEGF cDNA probe. A single transcript of ~ 3.8 kb was observed in this RNA preparation, indicating VEGF expression in bone tissue (Fig. 11).

Discussion

Formation of new blood vessels under tight, spatial, and temporal control is required for the development and repair of all tissues, except cartilage, cornea, and lens, and is particularly important for bone formation and remodeling. Angiogenesis involves the proliferation of endothelial cells likely to be regulated by polypeptide growth factors. Among the known angiogenic polypeptides, which stimulate the proliferation of vascular endothelial cells in vitro and induce angiogenesis in vivo, VEGF is the only well characterized secreted mitogenic factor

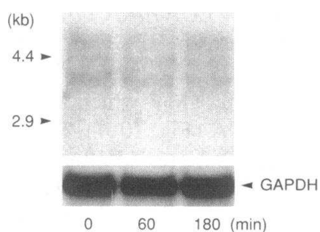

Figure 9. The effect of PGE_2 on VEGF mRNA in HUVE cells. Polyadenylated RNA (4 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe in a low stringent condition as described in Methods. The locations of RNA markers are indicated as arrowheads. For comparison of RNA loading, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) probe was hybridized to the same filter. The figure represents the results from one of two similar experiments.

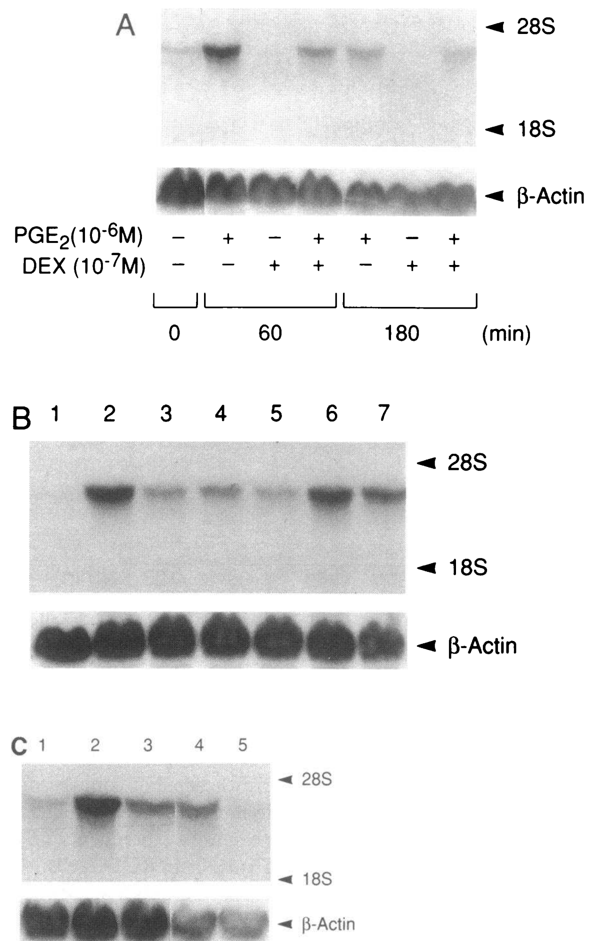

Figure 10. The effect of Dex on PGE_2 upregulation of VEGF mRNA. Total RNA (30 μg/lane) was subjected to RNA blot analysis with a rat VEGF cDNA probe. The locations of 28S and 18S rRNAs are indicated by arrowheads. For comparison of RNA loading, β-actin probe was hybridized to the same filter. (*A*) The effect of Dex on PGE_2 upregulation of VEGF mRNA. RCT-3 cells were treated with 10^{-6} M PGE_2 in the presence or absence of 10^{-7} M Dex for the indicated times, from 0 to 180 min. The figure represents the results from one of two similar experiments. (*B*) The effect of Dex pretreatment on PGE_2 induction of VEGF mRNA. RCT-3 cells were pretreated with 10^{-7} M Dex, 10^{-8} M $1,25(OH)_2D_3$, or 10^{-7} M 17β-estradiol and subsequently treated with 10^{-6} M PGE_2 for 1 h. Lane *1*, control; lane *2*, PGE_2 without pretreatment; lanes *3–7*, PGE_2 after pretreatment with Dex for 1 h (lane *3*), 2 h (lane *4*), or 3 h (lane *5*), with $1,25(OH)_2D_3$ for 3 h (lane *6*), or with 17β-estradiol for 3 h (lane *7*). The figure represents the results from one of two similar experiments. (*C*) The effect of Dex pretreatment on TPA induction of VEGF mRNA. RCT-3 cells were cultured in the presence or absence of 10^{-7} M Dex for 3 h and subsequently treated with 10^{-6} M PGE_2 or 150 nM TPA. Lane *1*, control; lane *2*, PGE_2 without pretreatment; lane *3*, PGE_2 after pretreatment with Dex; lane *4*, TPA without pretreatment; lane *5*, TPA after pretreatment with Dex. The figure represents the results from one of two similar experiments.

(kb)
9.5
7.5
4.4
2.4

Figure 11. Expression of VEGF mRNA in adult rat tibia. Polyadenylated RNA (5 μg/lane) prepared from adult rat tibia was subjected to RNA blot analysis using a rat VEGF cDNA probe. RNA size markers are indicated by arrowheads.

that acts exclusively on endothelial cells (27). The specific association of VEGF mRNA and the expression of one of its receptors, *flk-1,* with vascular endothelial cells at all stages of mouse development suggest that VEGF plays a physiological role in development (27–30, 41, 42). Moreover, vascularization of the cartilage anlage is an early event in bone development, and VEGF as well as *flk-1* mRNA expression is prominent in the intersegmental regions of the vertebrae in rat or mouse embryos (41, 42).

In this study, we show that VEGF mRNA is expressed in rat calvaria-derived osteoblast-enriched cells, in clonal osteoblastic cells, and in bone tissue, and that PGE_2, which is a potent stimulator of osteogenesis in vivo, causes rapid induction of VEGF mRNA and VEGF production in osteoblastic cells. The rapid short-lived induction of VEGF mRNA in osteoblastic cells by PGE_2 is consistent with a tight regulation of angiogenesis both in time and space. VEGF mRNA was barely detectable and was not regulated by PGE_2 in HUVE cells, which proliferate in response to VEGF, suggesting that VEGF may act as a paracrine factor rather than an autocrine factor.

PGs are among the most potent inducers of bone formation in vivo. Systemically and locally administered PGE_2 and PGE_1 were shown to stimulate bone formation in rat, dog, and humans (12–15). PGs stimulate proliferation of osteoblastic cells (38, 39) and increase steady state levels of IGF-I mRNA (43) and the binding of IGF-I to its receptor (44) and its mRNA level (45) in vitro. However, the lack of osteogenic activity by $PGF_{2\alpha}$ (Balena, R., S. Harada, S. B. Rodan, and G. A. Rodan, manuscript in preparation), a potent mitogen for osteoblasts in vitro (38, 39, 45), and the lack of mitogenic activity of PGE_1 in vitro (38, 39, 45) suggest that in vitro mitogenic activity does not explain the in vivo osteogenic effects (45). Furthermore, the lack of strong osteogenic activity of IGF-I in vivo and the late expression of IGF-I mRNA in ablation-induced osteogenesis (46) suggest that IGF-I mediation can not fully explain the osteogenic effect of PGE. Recent observations in mice carrying null mutations of *igf-1* and *igf1r* showed delayed but grossly normal bone development (47). Thus, IGF-I may play a role in the maintenance of bone rather than de novo bone induction. Since PGE_2 and PGE_1 but not $PGF_{2\alpha}$ (data not shown) strongly stimulate the expression of VEGF and since angiogenesis is associated with the early phase of bone formation (5, 6), the induction of VEGF may play a role in PGE stimulation of bone formation in vivo. However, further studies are required to evaluate the role of VEGF in osteogenesis in vivo.

Regarding the mechanism for PG induction of VEGF, PGs can increase intracellular cAMP levels and phosphatidyl inositol turnover in osteoblastic cells (38, 39). Although stimulation of both protein kinase C and protein kinase A were shown to increase VEGF mRNA expression in other cell types (36, 37), the suppression of the PGE_2 effect by H7 and by the cAMP antagonist, Rp-cAMP, but not by staurosporine implicates mediation by cAMP. However, the involvement of other second messengers cannot be excluded, since other stimulators of cAMP accumulation, forskolin, dibutyryl cAMP, and PTH did not increase VEGF mRNA levels as much as PGE_1 and PGE_2. Recently, cDNAs for three different PGE_2 receptors were identified (48–50). Examination of the expressions and functions of these PGE_2 receptors in osteoblasts may further elucidate the mechanisms for PGE_2 upregulation of VEGF as well as for PGE_2 induction of osteogenesis.

Tissue development requires the formation of new blood vessels to support proper function. Stimulation of VEGF expression by PGE_2 in the preosteoblastic RCT-1 cells was strongly potentiated by pretreatment with retinoic acid, which induces differentiation in these cells (33). Osteoblasts originate from pluripotent mesenchymal progenitor stem cells, which also give rise to muscle cells, adipocytes, and other cell types (51, 52). It has been shown that VEGF mRNA expression is also upregulated during the differentiation of adipocytes and myoblasts (36). Our observations, in addition to the previous findings, suggest that VEGF expression is linked to the differentiation of the cells originating from mesenchymal stem cells. Interestingly, pretreatment of RCT-1 cells with retinoic acid also potentiated PGE_2 induction of IGF-I mRNA (data not shown), which was shown to be associated with newly developed bone (53). The osteogenic effects of PGE_2 may thus involve the production of a series of autocrine and paracrine peptide factors.

A role for VEGF in bone metabolism is further supported by the suppression of PGE_2-induced VEGF mRNA by glucocorticoids. Long-term glucocorticoid treatment inhibits bone formation and causes bone loss (54). Furthermore, glucocorticoid treatment can induce avascular necrosis of bone (55). Suppression of VEGF expression by glucocorticoid could thus be involved in the pathogenesis of glucocorticoid-induced bone disorders.

In summary, we documented, for the first time, the expression of VEGF in osteoblasts and in bone tissue. Stimulation of VEGF expression by PGs and its suppression by glucocorticoid which, respectively, stimulate and suppress bone formation strongly implicate the involvement of VEGF in the physiology and pathological metabolism of bone tissue.

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References

1. Klagsbrun, M., and P. A. D'Amore. 1991. Regulators of angiogenesis. *Annu. Rev. Physiol.* 53:217–239.
2. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931–10934.
3. Streeten, E. A., and M. L. Brandi. 1990. Biology of bone endothelial cells. *Bone Miner.* 10:85–94.
4. Sorgente, N., K. E. Kuettner, L. W. Soble, and R. Eisenstein. 1975. The resistance of certain tissues to invasion. *Lab. Invest.* 32:217–222.

5. Holder, N. 1978. The onset of osteogenesis in the developing chick limb. *J. Embryol. Exp. Morphol.* 44:15–29.

6. Carrington, J. L., and A. H. Reddi. 1991. Parallels between development of embryonic and matrix-induced endochondral bone. *Bioessays.* 13:403–408.

7. Cutting, C. B., and J. G. McCarthy. 1983. Comparison of residual osseous mass between vascularized and nonvascularized onlay bone transfers. *Plast. Reconstr. Surg.* 72:672–675.

8. Burkhardt, B., G. Kettner, W. Bohm, M. Schmidmeier, R. Schlag, B. Frisch, B. Mallmann, W. Eisenmenger, and T. H. Gilg. 1987. Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. *Bone (Elmsford).* 8:157–164.

9. Cruess, R. L. 1986. Osteonecrosis of bone. *Clin. Orthop. Relat. Res.* 208:30–39.

10. Murray, D. W., and N. Rushton. 1990. The effect of strain on bone cell prostaglandin E_2 release: a new experimental method. *Calcif. Tissue Int.* 47:35–39.

11. Raisz, L. G., and T. J. Martin. 1984. Prostaglandins in bone and mineral metabolism. *In* Bone and Mineral Research. vol 3. W. A. Peck, editor. Elsevier, Amsterdam. 286–310.

12. Ueda, K., A. Saito, H. Nakano, M. Aoshima, M. Yokota, R. Muraoka, and T. Iwaya. 1980. Cortical hyperostosis following long-term administration of prostaglandin E_1 in infants with cyanotic congenital heart disease. *J. Pediatr.* 97:834–836.

13. Marks, S. C., and S. Miller. 1988. Local infusion of prostaglandin-E_1 stimulates mandibular bone formation in vivo. *J. Oral Pathol.* 17:500–505.

14. Mori, S., W. S. S. Jee, X. J. Li, S. Chan, and D. B. Kimmel. 1990. Effects of prostaglandin E_2 on production of new cancellous bone in the axial skeleton of ovariectomized rats. *Bone (Elmsford).* 11:103–113.

15. Li, X. J., W. S. S. Jee, Y. L. Li, and P. Patterson-Buckendahl. 1990. Transient effects of subcutaneously administered prostaglandin E_2 on cancellous and cortical bone in young adult dogs. *Bone (Elmsford).* 11:353–364.

16. Chyun, Y. S., and L. G. Raisz. 1984. Stimulation of bone formation by prostaglandin E_2. *Prostaglandins.* 27:97–103.

17. Flanagan, A. M., and T. J. Chambers. 1992. Stimulation of bone nodule formation in vitro by prostaglandin E_1 and E_2. *Endocrinology.* 130:443–448.

18. Ziche, M., J. Jones, and P. Gullino. 1982. Role of prostaglandin E_1 and copper in angiogenesis. *J. Natl. Cancer Inst.* 69:475–482.

19. Form, D. M., and R. Auerbach. 1983. PGE_2 and angiogenesis. *Proc. Soc. Exp. Biol. Med.* 172:214–218.

20. Spisni, E., F. Monica, and V. Tomasi. 1992. Involvement of prostanoids in the regulation of angiogenesis by polypeptide growth factors. *Prostaglandins Leukotrienes Essenti. Fatty Acids.* 47:111–115.

21. Leung, D. W., G. Cachianes, W.-J. Kuang, D. W. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Wash. DC).* 246:1306–1309.

22. Conn, G., D. D. Soderman, M. Schaeffer, M. Wile, V. B. Hatcher, and K. A. Thomas. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc. Natl. Acad. Sci. USA.* 87:1323–1327.

23. Gospodarowicz, L. E., J. A. Abraham, and J. Schilling. 1989. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc. Natl. Acad. USA.* 86:7311–7315.

24. Senger, D., C. A. Perruzzi, J. Feder, and H. F. Dvorak. 1986. A highly conserved vascular permeability factor secreted by a variety of human and rodent cell lines. *Cancer Res.* 46:5629–5632.

25. Houck, K. A., N. Ferrara, J. Winer, G. Cachianes, B. Li, and D. W. Leung. 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* 5:1806–1814.

26. Connolly, D. T., D. M. Heuvelman, R. Nelson, J. V. Olander, B. L. Eppley, J. J. Delfino, N. R. Siegel, R. M. Leimgruber, and J. Feder. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84:1470–1478.

27. Ferrara, N., K. Houck, L. Jakeman, and D. W. Leung. 1992. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr. Rev.* 13:18–32.

28. Berse, B., L. F. Brown, L. Van De Vater, H. F. Dvorak, and D. R. Senger. 1992. Vascular permeability factor (vascular endothelial growth factor) gene is differentially expressed in normal tissues, macrophages and tumors. *Mol. Biol. Cell.* 3:211–220.

29. Breier, G., S. Albrecht, S. Sterrer, and W. Risau. 1992. Expression of vascular endothelial growth factor during embryogenic angiogenesis and endothelial cell differentiation. *Development (Camb.).* 114:521–532.

30. Phillips, H. S., J. Hains, D. W. Leung, and N. Ferrara. 1990. Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology.* 127:965–968.

31. Shweiki, D., A. Itin, G. Neufeld, H. Gitay-Goren, and E. Keshet. 1993. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J. Clin. Invest.* 91:2235–2243.

32. Rodan, S. B., G. Wesolowski, K. A. Thomas, and G. A. Rodan. 1987. Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor. *Endocrinology.* 121:1917–1923.

33. Heath, J. K., S. B. Rodan, K. Yoon, and G. A. Rodan. 1989. Rat calvaria cell line immortalized with SV-40 large T antigen: constitutive and retinoic acid-inducible expression of osteoblastic features. *Endocrinology.* 124:3060–3068.

34. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

35. Conn, G., M. L. Bayne, D. D. Soderman, P. W. Kwok, K. A. Sullivan, T. M. Palisi, D. A. Hope, and K. A. Thomas. 1990. Amino acid and cDNA sequences for a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 87:2628–2632.

36. Claffey, K. P., W. O. Vilkison, and B. M. Spiegelman. 1992. Vascular endothelial growth factor: regulation by cell differentiation and activated second messenger pathways. *J. Biol. Chem.* 23:16317–16322.

37. Tischer, E., R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. C. Fiddes, and J. A. Abraham. 1991. The human gene for vascular endothelial growth factor. *J. Biol. Chem.* 266:11947–11954.

38. Hakeda, Y., T. Hotta, N. Kurihara, E. Ikeda, N. Maeda, Y. Yagyu, and M. Kumegawa. 1987. Prostaglandin E_1 and $F_{2\alpha}$ stimulate differentiation and proliferation, respectively, of clonal osteoblastic MC3T3-E1 cells by different second messengers *in vitro. Endocrinology.* 121:1966–1974.

39. Yamaguchi, D. T., T. J. Hahn, T. G. Beeker, C. R. Kleeman, and S. Muallem. 1988. Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR-106 cells. *J. Biol. Chem.* 263:10745–10753.

40. Pereira, M. E., D. L. Segaloff, M. Ascoli, and F. Eckstein. 1987. Inhibition of choriogonadotropin-activated steroidogenesis in cultured Leydig tumor cells by the Rp diastereomer of adenosine 3′, 5′-cyclic phosphorothioate. *J. Biol. Chem.* 262:6093–6100.

41. Jakeman, L. B., M. Armanini, H. S. Phillips, and N. Ferrara. 1993. Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology.* 133:848–859.

42. Millauer, B., S. Wizigmann-Voos, H. Schnürch, R. Martinez, N. P. H. Møller, W. Risau, and A. Ullich. 1993. High affinity VEGF binding and developmental expression suggest *flk-1* as a major regulator of vasculogenesis and angiogenesis. *Cell.* 72:835–846.

43. McCarthy, T. L., M. Centrella, L. G. Raisz, and E. Canalis. 1991. Prostaglandin E_2 stimulates insulin-like growth factor-I expression in osteoblast-enriched culture from fetal rat bone. *Endocrinology.* 128:2895–2900.

44. Hakeda, Y., S. Harada, T. Matsumoto, K. Tezuka, T. Hashimoto-Goto, E. Ogata, and M. Kumegawa. 1991. Prostaglandin $F_{2\alpha}$ stimulates proliferation of clonal osteoblastic MC3T3-E1 cells by up-regulation of insulin-like growth factor I receptors. *J. Biol. Chem.* 266:21044–21050.

45. Harada, S., R. Balena, G. A. Rodan, and S. B. Rodan. 1994. The role of prostaglandins in bone formation. *Connect. Tissue Res.* In press.

46. Suva, L. J., G. J. Seedor, N. Endo, H. A. Quartuccico, D. D. Thompson, I. Bab, and G. A. Rodan. 1993. Pattern of gene expression following tibial marrow ablation. *J. Bone Miner. Res.* 8:379–388.

47. Liu, J.-P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutation of the genes encoding insulin-like growth factor (*igf-1*) and type 1 IGF receptor (*igf 1r*). *Cell.* 75:59–72.

48. Sugimoto, Y., T. Namba, A. Honda, Y. Hayashi, M. Negishi, A. Ichikawa, and S. Narumiya. 1992. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_3 subtype. *J. Biol. Chem.* 267:6463–6466.

49. Honda, A., Y. Sugimoto, T. Namba, A. Watabe, A. Irie, M. Negishi, S. Narumiya, and A. Ichikawa. 1993. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_2 subtype. *J. Biol. Chem.* 268:7759–7762.

50. Watabe, A., Y. Sugimoto, A. Honda, A. Irie, T. Namba, M. Negishi, S. Ito, S. Narumiya, and A. Ichikawa. 1993. Cloning and expression of cDNA for a mouse EP_1 subtype of prostaglandin E receptor. *J. Biol. Chem.* 268:20175–20178.

51. Rodan, G. A., and M. Noda. 1991. Gene expression in osteoblastic cells. *Crit. Rev. Eukaryotic Gene Expression.* 1:85–98.

52. Owen, M. 1985. Lineage of osteoblastic cells and their relationship to the stromal system. *In* Bone and Mineral Research. vol 3. W. A. Peck, editor. Elsevier, Amsterdam. 1–26.

53. Shinar, D. M., N. Endo, D. Halperin, G. A. Rodan, and M. Weinreb. 1993. Differentiation expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology.* 132:1158–1167.

54. Baylink, D. J. 1983. Glucocorticoid induced osteoporosis. *N. Engl. J. Med.* 309:306–308.

55. Cruess, R. L. 1981. Steroid-induced osteonecrosis: a review. *Can. J. Surg.* 24:567–571.