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Anabolic Effects of 1,25-Dihydroxyvitamin D_3 on Osteoblasts Are Enhanced by Vascular Endothelial Growth Factor Produced by Osteoblasts and by Growth Factors Produced by Endothelial Cells*

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ABSTRACT

Human osteoblast-like cells (HOB) produce vascular endothelial growth factor (VEGF), the steady state level of which is stimulated by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. As osteoblasts and endothelial cells are proximally located in skeletal tissue, we investigated the anabolic effects of 1,25-$(OH)_2D_3$ and VEGF on HOB cocultured with endothelial cells.

When HOB with high alkaline phosphatase (Al-P) activity and human umbilical vein endothelial cells (HUVEC) with little activity were cultured together, Al-P activity increased, accompanied by an increase in cell number. When HOB and HUVEC were cultured separately, 1,25-$(OH)_2D_3$ did not directly stimulate [^{3}H]thymidine incorporation into HUVEC, but stimulated it in the presence of HOB. VEGF did not directly stimulate the Al-P activity of HOB but stimulated it in the presence of HUVEC. The conditioned medium of HOB stimulated the proliferation of HUVEC, and this was partially blocked by anti-VEGF antibody. Conversely, the conditioned medium of HUVEC increased Al-P activity and [^{3}H]thymidine incorporation into HOB, and this was partially blocked by antiinsulin-like growth factor I antibody and BQ-123, a specific antagonist of the endothelin-1 (ET-1) receptor. 1,25-$(OH)_2D_3$ stimulated the release of VEGF and ET-1 from HOB and HUVEC, respectively. Furthermore, the 1,25-$(OH)_2D_3$-induced release of VEGF was enhanced in HOB cocultured with HUVEC. A quantitative reverse transcription-PCR study revealed that genes for VEGF receptors (Flt-1 and KDR) were expressed in HUVEC, but not in HOB, and that 1,25-$(OH)_2D_3$ increased the levels of expression of VEGF receptor genes in endothelial cells only when cocultured with HOB.

In summary, we demonstrated that 1,25-$(OH)_2D_3$ exerts an anabolic effect on osteoblasts by enhancing their production of VEGF, which stimulates its receptors on endothelial cells, followed by increased production of osteotropic growth factors, such as insulin-like growth factor I and ET-1. These *in vitro* findings suggest that the VEGF/VEGF receptor system may be involved in both bone formation and bone remodeling *in vivo*. (*Endocrinology* **138:** 2953–2962, 1997)

VITAMIN D and its analogs are currently being used in many countries, particularly Japan, for the treatment of osteoporosis (1, 2). However, the cellular and molecular mechanisms underlying the induction of osteogenesis by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] are still poorly understood (3, 4). Recently, we demonstrated that vascular endothelial growth factor (VEGF) messenger RNA (mRNA) is expressed by human osteoblast-like cells and that its steady state level is stimulated by 1,25-$(OH)_2D_3$ (5), suggesting that VEGF synthesized by osteoblasts in response to 1,25-$(OH)_2D_3$ is involved in bone formation, presumably via a VEGF receptor-mediated process (6). This hypothesis is supported by recent *in vitro* findings that the levels of expression of VEGF mRNA are also enhanced by PGE, insulin-like growth factor I (IGF-I), and PTH (5, 7, 8), which are capable of stimulating bone formation when administered continuously or intermittently *in vivo* (9, 10).

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VEGF, a homodimeric protein with a signal peptide, stimulates specifically endothelial cell proliferation by binding to VEGF receptors (Flt-1 and KDR) that are expressed exclusively on the cells (11, 12). In view of the recent findings that endothelial cells produce growth factors for osteoblasts, such as endothelin-1 (ET-1) and IGFs (13, 14), and that osteoblasts express receptors for ET-1 and IGF-I (15, 16), it is highly likely that a mutual communication system exists between them, as demonstrated in liver and thyroid (17, 18).

This hypothesis is substantiated by histological findings indicating that osteoblasts and osteoprogenitor cells are always located adjacent to endothelial cells in blood vessels at sites of new bone formation (19–21). In embryonic skeletal tissue, osteogenesis and angiogenesis are temporally related (22). Furthermore, older subjects and patients with osteoporosis have decreased blood vessels in their skeletal tissue, accompanied by a parallel decrease in osteoblasts (23–25). These *in vivo* findings also suggest that angiogenesis and osteogenesis are mutually interdependent (26, 27), and that endothelial cells may accelerate bone formation through angiogenesis as well as in bone remodeling.

Therefore, we employed a coculture system of human osteoblast-like cells (HOB) and human umbilical venous endothelial cells (HUVEC), and investigated 1) whether osteoblast function is enhanced by coculture with endothelial cells; 2) if so, what osteotropic growth factors are involved in

the interaction between osteoblasts and endothelial cells; and 3) whether the anabolic effects of 1,25-$(OH)_2D_3$ on HOB are enhanced by coculture with HUVEC. Furthermore, using quantitative reverse transcription-PCR (RT-PCR) (28), we investigated the effects of 1,25-$(OH)_2D_3$ on expression of VEGF receptor genes on endothelial cells.

Materials and Methods

Materials

Tissue culture plates were purchased from Nunc (Roskilde, Denmark). Cell culture medium (αMEM) and reagents were supplied by Life Technologies (Grand Island, NY). FCS was purchased from Filtron (Brooklyn, Australia). 1,25-$(OH)_2D_3$ was obtained from Wako Pure Chemical Industries (Tokyo, Japan). IGF-I was purchased from Becton Dickinson Labware (Bedford, MA). Recombinant human VEGF was obtained from Pepro Tech (Rocky Hill, NJ). Human ET-1, ET-2, and ET-3 were obtained from Cosmo Bio Co. (Tokyo, Japan). IGF-I and ET-1 were dissolved in PBS containing 0.2% BSA and stored in aliquots at −80 as stock solution. Dilutions of the stock solution were prepared immediately before use. ET-A receptor antagonist (BQ-123) (26) was purchased from Research Biochemical International (Natick, MA) (29). Anti-IGF-I monoclonal and anti-VEGF polyclonal antibodies were obtained from Oncogene Science (Uniondale, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. [*Methyl*-^{3}H]thymidine was obtained from Amersham Corp. (Arlington Heights, IL). [γ-^{32}P]ATP was purchased from Amersham (Downers Grove, IL). Nylon filters were purchased from Schleicher and Schuell (Tokyo, Japan). Reagents for DNA synthesis and AmpliTaq DNA polymerase were obtained from Life Technologies (Gaithersburg, MD). Reverse transcriptase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). All chemicals were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell cultures

HOB were cultured from trabecular bone explants obtained at the time of orthopedic procedures performed on patients who had no evidence of metabolic bone disease. The bone fragments were washed extensively and repeatedly with culture medium to remove adherent marrow cells and to expose the trabecular surface of the bone. Small bone chips (1 × 1 × 1 mm) were then placed in culture flasks (75 cm^2), each containing 15 ml αMEM supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (50 μg/ml; αMEM-10% FCS), and cultured at 37 C in a humidified atmosphere with 5% CO_2. Cell outgrowth from the trabecular bone surfaces was apparent after 5 days, and the osteoblast-like cells became confluent after 10–14 days of culture. Cell passages were performed by incubating confluent cells in 0.25% trypsin diluted in calcium- and magnesium-free PBS and replating the cells at a density of 1:3. Experiments were usually performed with HOB subcultured at the fourth to eighth passage. Under the culture conditions employed, HOB produced alkaline phosphatase (Al-P) activity for more than 10 passages (30).

HUVEC were obtained from Kurabo (Osaka, Japan). The cells were cultured in the manufacturer's recommended medium (E-BM) supplemented with 2% FBS, recombinant human EGF (10 ng/ml), hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), and amphotericin-*b* (50 ng/ml) (supplemented E-BM). When cells reached subconfluence or confluence, they were cultured in αMEM-10% FCS containing 1 nM VEGF at 37 C in 95% air-5% CO_2. Under the present culture conditions, the cells continued to proliferate for more than 5 days. Preliminary experiments revealed that HUVEC, when cultured in αMEM-10% FCS without VEGF, continued to incorporate [^{3}H]thymidine for 24–36 h and showed rapidly decreased uptake by 48 h. Experiments were performed using endothelial cells at the third to sixth passage from different donors.

Coculture of HOB and HUVEC with direct contact

HOB were plated in a 24-multiwell dishes at 2–4 10^4 × cells/well in 1 ml αMEM-10% FCS. After 24 h, when the cell reached 50% confluence, HUVEC were added to each well at 2–4 × 10^4 cells in 200 μl αMEM-10% FCS without VEGF. In control culture, the same volume of αMEM-10% FCS was added. On the following day, 1,25-$(OH)_2D_3$ (dissolved in 10% ethanol) or VEGF was added to HUVEC, HOB, and HOB cultured with HUVEC. The final ethanol concentration in the culture medium was below 0.1%. After 2–4 days of culture, the cell number and Al-P activity in HUVEC, HOB, and HOB cocultured with HUVEC were determined as described previously (30).

In the same experiments, [^{3}H]thymidine (2 μCi/ml) was added to each culture well, and the cells were cultured for an additional 5 h. Then, the cell monolayer was washed with Hanks' solution (pH 7.4) and extracted with cold 5% trichloroacetic acid. The resulting precipitates were washed with ethanol-ether (volume ratio, 4:1) and solubilized with 1 N sodium hydroxide. The radioactivity was determined with a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). All determinations were performed in quadruplicate.

Coculture of HOB and HUVEC without direct contact

In several experiments, HUVEC and HOB were cultured in the same well, but separately, by placing a 0.4-μm filter insert (12 mm in diameter; Millipore, Nunclon). In this coculture system, each well was composed of double chambers, consisting of an outer chamber (24-multiwell plate) and an inner Millcell-CM chamber. In the inner chamber, HUVEC (2–4 × 10^4) were seeded in 0.5 ml αMEM-10% FCS, and 1 ml αMEM-10% FCS containing HOB was poured into the outer chamber. At 50% confluence of HOB, the inserts containing HUVEC were placed in wells of HOB. In control cultures, the cell inserts without HOB were also placed in the control wells. After an additional 2–4 days of culture, Al-P activity and cell number were measured.

Effects of conditioned medium of HOB (HOB-CM) on [^{3}H]thymidine incorporation into HUVEC

HOB were cultured in αMEM-10% FCS until they reached confluence. Then, the medium was changed to fresh αMEM-10% FCS, and the cells were cultured for an additional 3 days. At the end of the incubation period, the HOB-CM was centrifuged at 200 × *g* for 5 min at room temperature, followed by filtering through a 0.45-μm Millipore filter. The supernatants were used immediately or frozen at −20 C until further assay. In a few experiments, HOB-CM was obtained by culturing HOB in αMEM-10% FCS supplemented with 10 nM 1,25-$(OH)_2D_3$ for 3 days.

HUVEC were grown in 24-multiwell dishes containing 1 ml supplemented E-BM until 70–80% confluence. Then, the medium was changed to αMEM-10% FCS containing various concentrations of HOB-CM. After an additional 1–2 days of culture, [^{3}H]thymidine incorporation into HUVEC was determined.

To investigate which growth factors are involved in the anabolic effects of HOB-CM on HUVEC, HUVEC were cultured in αMEM-10% FCS supplemented with 50% HOB-CM and various concentrations of anti-VEGF-antibody (31). After 2 days of culture, [^{3}H]thymidine was added, and after an additional 5 h of culture, [^{3}H]thymidine incorporation was determined.

Effects of HUVEC-conditioned medium on HOB

HUVEC were grown in the supplemented E-BM on 75-cm^2 plastic dishes (Nunclon) until they reached confluence. The confluent monolayers (~4 days after the dishes had been seeded) were washed twice with Hanks' solution (pH 7.4), and the medium was replaced with αMEM-10% FCS supplemented with 1 nM VEGF. The conditioned medium (HUVEC-CM) was obtained as described above.

HOB were plated at a density of 4 × 10^4 cells/well in 24-multiwell dishes. After the cells had reached 50% confluence, the medium was changed to αMEM-10% FCS supplemented with various concentrations of HUVEC-CM. Cultures were carried out for 1–4 days, and then Al-P activity and cell number were determined.

To investigate which growth factors are involved in the anabolic effects of HUVEC-CM on osteoblast-like cells, HOB were cultured in αMEM-10% FCS supplemented with 50% HUVEC-CM and various concentrations of anti-IGF-I antibody and/or BQ-123, a specific inhibitor of the ET-1 receptor. After 2 days of culture, [^{3}H]thymidine was added, and

after an additional 5 h of culture, [^{3}H]thymidine incorporation was determined.

Measurement of VEGF and ET-1 in conditioned medium

The VEGF concentration in HOB-CM was measured at Mitsubishi Kagaku BCL (Tokyo, Japan) using a solid phase enzyme-linked immunosorbent assay designed to measure levels in cell culture supernatants, serum, and plasma (R&D Systems, Minneapolis, MN). This assay contains insect cell *Sf21*-expressed recombinant human $VEGF_{165}$ and antibodies raised against the recombinant protein, and its sensitivity is less than 15 pg/ml. The ET-1 concentration in HUVEC-CM was also measured by enzyme-linked immunosorbent assay (Wako Jun-yaku, Tokyo, Japan). The minimal sensitivity of the assay was less than 0.5 pg/ml.

Isolation of total RNA

HUVEC and HOB were cultured alone or cocultured in 6-cm diameter dishes and then treated with hormones or growth factors as described above. Total RNA was extracted by the method of Chomczynski and Sacchi (32). Isolated RNAs were stored at −20 C until assayed.

Primers and probes

Oligodeoxyribonucleotide primers and probes for quantitative RT-PCR were synthesized by Kurabo (Osaka, Japan). The primer sequences were 5′-ACTATGGAAGATCTGATTTCTTACAGT-3′ (nucleotides 3232–3258) and 5′-GGTATAAATACACATGTGCTTCTAG-3′ (complement of nucleotides 4289–4314) (33) for detecting *fms*-like tyrosine kinase 1 (*flt-1*) mRNA, and 5′-TATAGATGGTGTAACCCGGA-3′ (nucleotides 873–892) and 5′-TTTGTCACTGAGACAGCTTGG-3′ (complement of nucleotides 1406–1427) (34) for kinase insert domain-containing receptor (*kdr*) mRNA.

The internal oligodeoxyribonucleotide probes were 5′-GAGCTGGAAAGGAAAATCGCGTGCTGCTCC-3′ for detecting *flt-1* complementary DNA (cDNA) and 5′-ATCCAGTGGGCTGATGACCAAGAAGAACAG-3′ for *kdr* cDNA, corresponding to nucleotides 4186–4215 (33) and 921–950 (34), respectively. The sequences of the primers and probe for detecting β-actin mRNA have been reported previously (35).

RT-PCR

PCR was performed in 25 μl reaction solution containing cDNA derived from 1 μg total RNA, 1.25 UAmpliTaq polymerase (Life Technologies), 200 μmol/liter of each deoxy-NTP, and 0.5 μmol/liter sense and antisense primers. The reaction mixture was overlaid with 15 μl mineral oil and heated at 94 C for 3 min. Each PCR cycle included 1 min of denaturation at 94 C, 1 min of primer annealing at 55 C, and 1.5 min of extension/synthesis at 72 C. PCR was performed with a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). After the last cycle, all samples were incubated for an additional 5 min at 72 C.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously (28). A 10-μl aliquot of each RT-PCR reaction mixture was electrophoresed on 2% agarose gel and transferred to nylon membranes (Nytran, Keene, NH). The membranes were UV autocross-linked, prehybridized at 42 C for 4 h, and hybridized with specific oligodeoxyribonucleotide probes that had been ^{32}P end labeled with [γ-^{32}P]ATP and polynucleotide kinase, using a DNA 5′- end labeling kit (Takara Shuzo Co., Shiga, Japan). The filters were washed once for 30 min in 2 × SSC (standard saline citrate)-0.1% Denhardt's solution at 42 C, once for 30 min in 0.1 × SSC-1% SDS at 42 C, and then twice for 30 min in 0.1 × SSC at room temperature. Autoradiographic exposure of the washed membranes was performed at −80 C for various time periods, and the radioactivities of the hybridization bands were measured with a BioImagin analyzer (Fuji Photo Film Co., Hamamatsu, Japan).

Statistical analysis

All values are expressed as the mean ± SD. Means were compared by Student's *t* test. Most experiments were repeated at least three times. ANOVA with Bonferroni's test was employed to determine the significance of differences in multiple comparisons. Differences at $P < 0.05$ were considered statistically significant.

Results

Effect of coculture of HOB with HUVEC on cell growth and Al-P activity

After HOB reached confluence, they showed high Al-P activity (138 ± 89 mU/mg protein; mean ± SD of 12 experiments), whereas the enzyme activity was negligible in HUVEC (<0.1 ± 0.01). When Al-P-positive HOB and Al-P-negative HUVEC were cultured together for 4 days, Al-P activity increased more than additively to 252 ± 168 mU/mg protein ($P < 0.001$). The increase in enzyme activity was more remarkable when expressed in terms of milliunits per well (data not shown).

The number of HUVEC increased continuously when cultured in the supplemented E-BM. However, the cells ceased to grow in αMEM-10% FCS within 1 day and started to degenerate after 2 days of culture. After 4 days of culture, the number of HUVEC, which had been growing to more than 10,000/well, decreased to 4,818 ± 2,933 cells/well (mean ± SD of eight experiments). The number of HOB more than doubled during 4 days of culture in αMEM-10% FCS and increased to 103,431 ± 48,866 cells/well. When both cell types were cultured together, total cell number increased synergistically to 172,003 ± 76,023 cells/well (mean ± SD of eight experiments; $P < 0.01$).

Effects of VEGF and 1,25-$(OH)_2D_3$ on HUVEC, HOB, and HOB cocultured with HUVEC

As expected, VEGF significantly stimulated the proliferation of HUVEC (Fig. 1A), but had no effect on HOB (Fig. 1A). VEGF did not stimulate Al-P activity in HOB *per se*, but stimulated the enzyme activity significantly at 10 nM in HOB cocultured with HUVEC (Fig. 1B).

As is well known, 1,25-$(OH)_2D_3$ stimulated Al-P activity in HOB in a concentration-dependent manner, whereas it had no effect on HUVEC (Fig. 1B). At 10 nM, active vitamin D stimulated enzyme activity by 116 ± 34% (mean ± SD of eight experiments). When HOB and HUVEC were cultured together, 1,25-$(OH)_2D_3$ stimulated Al-P activity by 127 ± 24% (mean ± SD of eight experiments). Although there was no significant difference between the increased ratio of the enzyme activity (expressed in milliunits per mg protein), the enzyme activity per Al-P-positive cell was increased to a greater extent in HOB cocultured with HUVEC than in HOB alone. Consistent with previous reports, 1,25-$(OH)_2D_3$ decreased the number of HOB, and this was also the case in HOB cocultured with HUVEC (Fig. 1A).

Effects of VEGF and 1,25-$(OH)_2D_3$ on HOB or HUVEC cultured in nondirect contact with each other

To investigate whether the increase in Al-P activity is mediated by cell to cell contact, HUVEC and HOB were cultured separately in the same well using filter inserts. In the presence of HUVEC that had been cultured on the inserts, the Al-P activity of HOB grown on the bottom of culture flask increased significantly compared with that of HOB cultured

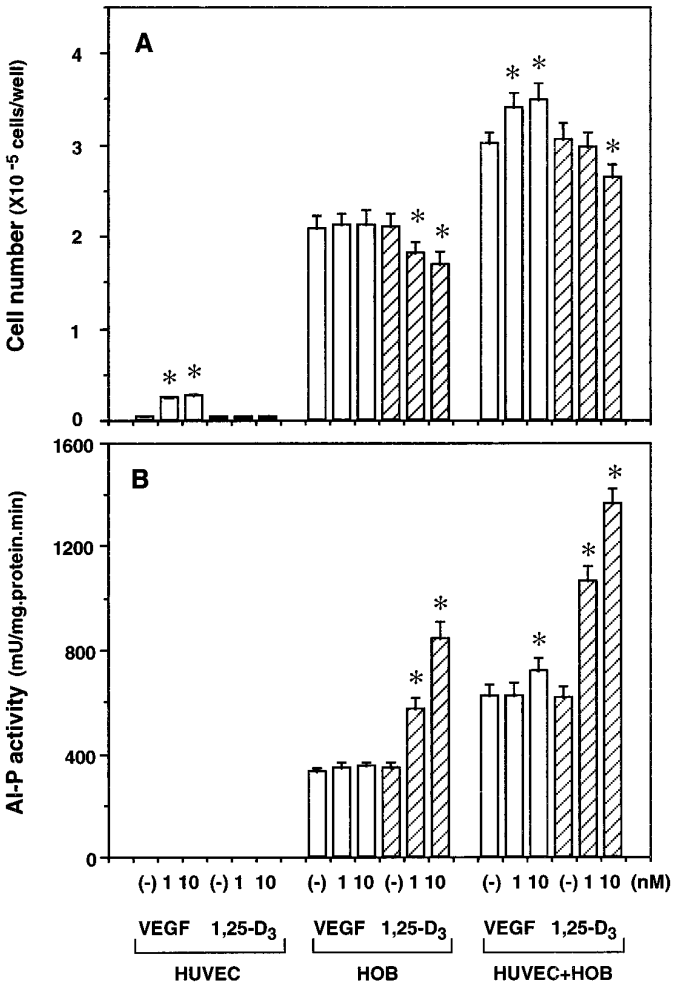

FIG. 1. Effects of VEGF and 1,25-$(OH)_2D_3$ on cell growth and Al-P activity on HUVEC, HOB, and HOB cocultured with HUVEC. HUVEC (2×10^4 cells/well) and HOB (4×10^4 cell/well) were cultured in 1 ml supplemented E-BM and αMEM-10% FCS, respectively. On the following day, HUVEC (2×10^4 cells/well) were overlaid onto HOB. Then, the cells were cultured with αMEM-10% FCS containing various concentrations of VEGF or 1,25-$(OH)_2D_3$. After 4 days of culture, the number of cells (A; *upper panel*) and Al-P activity (B; *lower panel*) were determined as described in *Materials and Methods*. Data are shown as the mean ± SD of quadruplicate samples. Representative data from four experiments are shown. *, $P < 0.05$; **, $P < 0.01$ [with *vs.* without VEGF or 1,25-$(OH)_2D_3$].

alone (without HUVEC, 235 ± 110; with HUVEC, 304 ± 132 mU/mg protein; mean ± SD of quadruplicate samples; $P < 0.01$). The number of HOB also significantly increased in the presence of HUVEC (91,910 ± 5,852 *vs.* 130,635 ± 25,688 cells/well; mean ± SD of quadruplicate samples; $P < 0.01$). 1,25-$(OH)_2D_3$ significantly increased the Al-P activity of HOB cultured alone (292 ± 20 mU/mg protein; $P < 0.01$), and this was further enhanced in the presence of HUVEC on the insert (564 ± 25 mU/mg protein; $P < 0.01$). In contrast, VEGF elicited no effect on Al-P activity of HOB alone, whereas the angiogenic factor slightly, but significantly, increased the Al-P activity of HOB in the presence of HUVEC on the insert (without VEGF, 279 ± 14 mU/mg protein; with 10^{-8} M VEGF, 347 ± 30 mU/mg protein; $P < 0.05$).

Conversely, when HUVEC were cultured on the bottom of culture flask in the presence of HOB on the cell inserts, the number of HUVEC was greater than that of HUVEC cultured alone (without HOB, 8,344 ± 3,890; with HOB, 10,086 ± 4,203 cells/well; mean ± SD of quadruplicate samples; $P < 0.01$). 1,25-$(OH)_2D_3$ did not affect the growth of HUVEC directly (data not shown), but it increased the number of HUVEC slightly, but significantly, in the presence of HOB [without 1,25-$(OH)_2D_3$, 10,086 ± 4,203; with 1,25-$(OH)_2D_3$ (10^{-8} M), 11,846 ± 5,721 cells/well; $P < 0.05$].

Anabolic effects of HOB-CM on HUVEC

As reported previously (5), 1,25-$(OH)_2D_3$ stimulated the release of VEGF from HOB in a concentration-dependent manner (Table 1). Consistent with this finding, HOB-CM increased the number of HUVEC significantly, accompanied by a dose-dependent increase in [^{3}H]thymidine incorporation into the cells (Fig. 2). The proliferative effect of HOB-CM on endothelial cells was potentiated when HOB was cultured with 1,25-$(OH)_2D_3$ (Table 2D), whereas the active vitamin D had no direct proliferative effect on HUVEC (Table 2B).

Consistent with these findings, anti-VEGF antibody inhibited HOB-CM-induced [^{3}H]thymidine incorporation in a concentration-dependent manner, whereas the antibody *per se* had no effect on the proliferation of HUVEC alone (Fig. 3). However, anti-VEGF antibody could not completely inhibit HOB-CM-induced [^{3}H]thymidine incorporation, even at a final concentration of 1.6×10^{-8} M, which nearly completely inhibited the [^{3}H]thymidine incorporation induced by 10^{-10} M VEGF (data not shown), suggesting that an angiogenic factor(s) other than VEGF is present in HOB-CM.

Anabolic effects of HUVEC-CM on HOB

HUVEC-CM significantly increased the number of HOB in a concentration-dependent manner, accompanied by a significant increase in [^{3}H]thymidine incorporation (Fig. 4). Furthermore, HUVEC-CM significantly increased the Al-P activity of HOB in a dose-dependent manner.

HUVEC constitutively produced ET-1 (Table 1). Interestingly, 1,25-$(OH)_2D_3$ significantly increased the level of ET-1 in HUVEC-CM (Table 1B). This was also observed when HUVEC were cultured in αMEM-10% FCS supplemented with 1 nM VEGF (data not shown). In contrast to HUVEC, a trace amount of ET-1 was detected in HOB-CM.

Consistent with the above findings, 10^{-6} M BQ-123 and 1.5×10^{-8} M anti-IGF-I antibody inhibited HOB-CM-induced [^{3}H]thymidine incorporation by 24% and 35%, respectively (Fig. 5). However, the simultaneous addition of BQ-123 and anti-IGF-I inhibited it additively, but not completely (by 58%), suggesting that HUVEC produce an angiogenic factor(s) other than VEGF.

Effects of ETs on growth and Al-P activity of HOB

As the effects of ETs on osteoblast-like cells are controversial (13), we investigated the effects of ET-1, ET-2, and ET-3 on HOB. As shown in Fig. 6, ET-1 stimulated Al-P activity and [^{3}H]thymidine incorporation in a concentration-dependent manner. However, ET-2 and ET-3 elicited no significant effect on HOB.

TABLE 1. Effects of 1,25-$(OH)_2D_3$ on release of VEGF and ET-1 from HUVEC, HOB, and HOB cocultured with HUVEC

1,25-$(OH)_2D_3$ (M)	HUVEC	HOB		HOB + HUVEC	
		pg/ml	pg/10^4 cells	pg/ml	pg/10^4 cells
A) VEGF					
0	ND	954 ± 112	233 ± 12	1715 ± 242[a]	308 ± 42[a]
10^{-10}	ND	1580 ± 713[b]	402 ± 39[b]	2295 ± 182[a,b]	429 ± 37[b]
10^{-8}	ND	1767 ± 886[b]	442 ± 26[b]	2400 ± 237[a,b]	467 ± 45[b]
		HUVEC		HOB	
		pg/ml	pg/10^4 cells	pg/ml	pg/10^4 cells
B) ET-1					
0		153 ± 90	204 ± 121	2.0 ± 0.5	0.5 ± 0.1
10^{-10}		419 ± 289[b]	461 ± 263[b]	5.7 ± 3.7	1.5 ± 0.9
10^{-8}		364 ± 169[b]	436 ± 240[b]	4.7 ± 2.2	1.2 ± 0.6

HUVEC, HOB, and HOB cocultured with HUVEC were cultured in αMEM-10% FCS containing various concentrations of 1,25-$(OH)_2D_3$ without VEGF. After 24 h of culture, the conditioned medium was taken and assayed for VEGF and ET-1. Data are presented as picograms per ml or picograms per 10^4 cells and are shown as the mean ± SD of quadruplicate cultures. Representative data from two experiments are shown. ND, Not detectable (<15 pg/ml).

[a] $P < 0.05$, without *vs.* with HUVEC.

[b] $P < 0.05$, without *vs.* with 1,25-$(OH)_2D_3$.

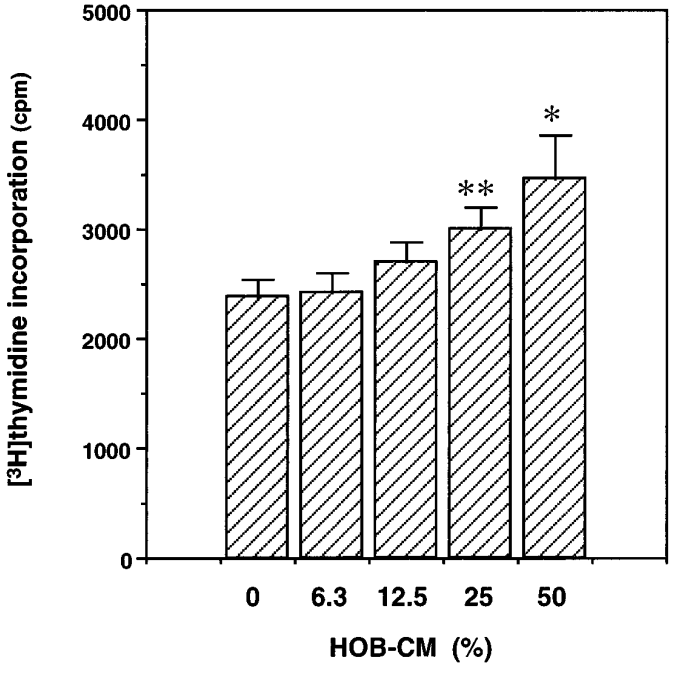

FIG. 2. Effect of HOB-CM on [^{3}H]thymidine incorporation into HUVEC. HUVEC were cultured in supplemented E-BM until subconfluent. Then, the medium was changed to fresh αMEM-10% FCS supplemented with various concentrations of HOB-CM, which had been prepared by culturing confluent HOB in αMEM-10% FCS for 48 h. [^{3}H]thymidine incorporation was determined as described in *Materials and Methods*. Results represent the mean ± SD for four samples. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Effects of 1,25-$(OH)_2D_3$ on VEGF receptor expression in HUVEC cultured with HOB

The quantitative RT-PCR analysis showed that *kdr* and *flt-1* were expressed constitutively in HUVEC, but not in HOB (Fig. 7). VEGF (1 nM) increased the levels of *kdr* and *flt-1* gene expression in HUVEC (Fig. 7, lanes 3 and 4). In contrast, 1,25-$(OH)_2D_3$ elicited no significant effect on VEGF receptor expression in HUVEC alone (data not shown) or in HUVEC in the presence of VEGF. However, when HUVEC were cultured together with HOB, 1,25-$(OH)_2D_3$ increased the levels of VEGF receptor gene expression (Fig. 7, lane 6).

HUVEC cultured in supplemented E-BM expressed VEGF receptors constitutively (Fig. 8, lanes 1, 5, and 9), whereas HUVEC cultured in αMEM-10% FCS without VEGF showed a time-dependent decrease in the level of VEGF receptor gene expression (Fig. 8, lanes 2, 6, and 10); the levels of KDR expression in HUVEC cultured for 24, 36, and 48 h without VEGF decreased to 90%, 56%, and 21% of that in the control culture, respectively. However, HUVEC cocultured with HOB maintained their level of VEGF receptor gene expression, which was enhanced by 48% (*kdr*) and 60% (*flt-1*) by 1,25-$(OH)_2D_3$ (Fig. 8, lane 12).

Discussion

Previously, we reported that HOB express VEGF mRNA constitutively, and that this is enhanced by 1,25-$(OH)_2D_3$, leading to increased secretion of VEGF into the conditioned medium (5). In the present system where Al-P-positive HOB were cocultured with Al-P-negative HUVEC in either direct or indirect contact, we clearly demonstrated that osteoblast-like cells stimulated the proliferation of endothelial cells by producing VEGF, and that the activated endothelial cells then enhanced the proliferation and differentiation of osteoblast-like cells by producing osteotropic growth factors such as IGF-I and ET-1 (Fig. 9). Furthermore, 1,25-$(OH)_2D_3$ enhanced all of these phenomena by stimulating the level of VEGF receptor gene expression on endothelial cells. Therefore, there is a mutual communication system between osteoblasts and endothelial cells that leads to enhancement of each cellular function by 1,25-$(OH)_2D_3$ (Fig. 9). A similar mutual communicating system between endothelial cells and parenchymal cells has been demonstrated in the liver involving hepatocyte growth factor and in the thyroid involving TSH (17, 18).

Among a number of angiogenesis factors (36), VEGF with a hydrophobic signal peptide is regarded as the most im-

TABLE 2. Effects of 1,25-$(OH)_2D_3$ on HOB-induced cell growth of HUVEC

	Cell no. (cells/well)	[^{3}H]Thymidine (cpm)
A) αMEM/10% FCS	19,740 ± 1,090	5,768 ± 461
B) + 1,25-$(OH)_2D_3$	20,013 ± 1,569	5,686 ± 628
C) HOB-CM	30,026 ± 473[a]	13,585 ± 1,226[a]
D) HOB-CM + 1,25-$(OH)_2D_3$	31,973 ± 793[a]	16,286 ± 1,187[a,b]

HUVEC were grown in a 24-multiwell dish containing supplemented E-BM until 80% confluency. Then, the medium was changed to 1 ml fresh αMEM-10% FCS (A), αMEM-10% FCS supplemented with 1,25-$(OH)_2D_3$ (10^{-8} M); B), αMEM-10% FCS (50%) and HOB-CM (50%) that had been prepared by culturing HOB for 2 days (C), or αMEM-10% FCS (50%) and HOB-CM (50%) that had been prepared by culturing HOB with 1,25-$(OH)_2D_3$ (10^{-8} M) for the same period (D). Data are shown as the mean ± SD of quadruplicate cultures. Representative data from three experiments are shown.

[a] $P < 0.05$, A *vs.* C or D.

[b] $P < 0.05$, C *vs.* D.

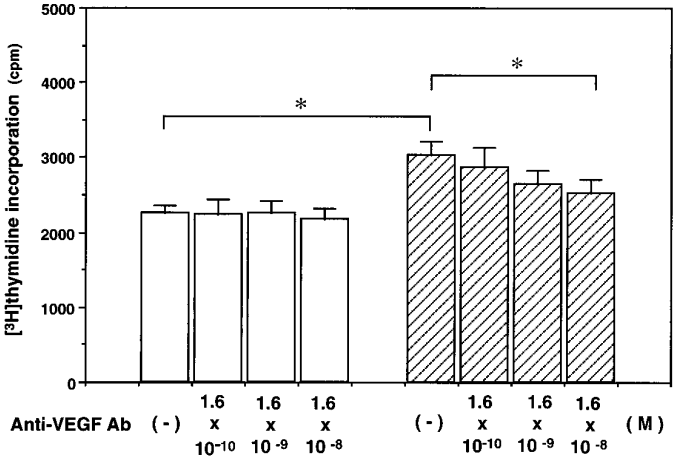

FIG. 3. Effect of anti-VEGF antibody on HOB-CM-induced [^{3}H]thymidine incorporation into HUVEC. HUVEC were cultured in E-BM until subconfluent. Then, the medium was changed to αMEM-10% FCS containing 50% HOB-CM, which had been prepared by culturing confluent HOB for 2 days. In some cultures, polyclonal anti-VEGF antibody was added as depicted. After an additional 24 h of culture, [^{3}H]thymidine incorporated into HUVEC was determined as described in *Materials and Methods*. Data are the mean ± SD of quadruplicate samples. □, Without HOB-CM; ▨; with HOB-CM. *, $P < 0.05$, with *vs.* without the antibody.

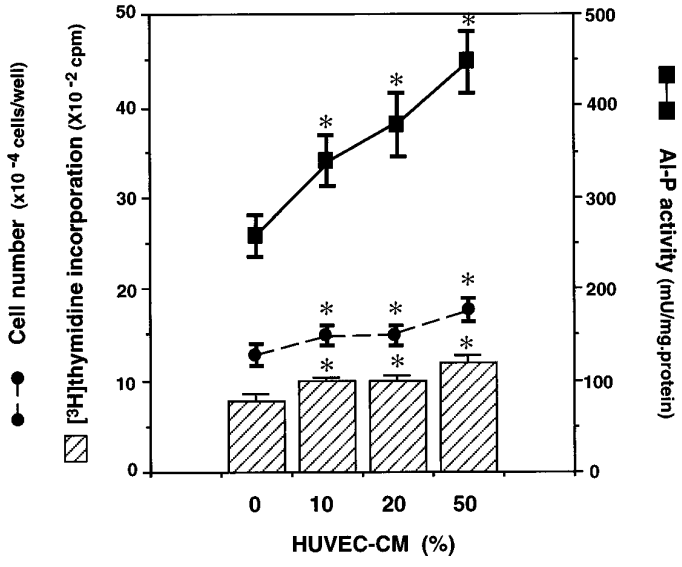

FIG. 4. Effect of HUVEC-CM on cell growth and Al-P activity of HOB. HOB were cultured in αMEM-10% FCS supplemented with various concentrations of HUVEC-CM. After 96 h of culture, cell number, [^{3}H]thymidine incorporation, and Al-P activity were determined as described in *Materials and Methods*. Data are the mean ± SD of quadruplicate samples. Representative data from three experiments are shown. ▨, [^{3}H]Thymidine incorporation; ●, cell number; ■, Al-P activity. *, $P < 0.05$; **, $P < 0.01$ (with *vs.* without HUVEC-CM).

portant, because it specifically stimulates endothelial cell proliferation not only *in vitro* but also *in vivo* by stimulating VEGF receptors. As the anti-VEGF antibody partially inhibited the proliferative effect of HOB-CM on HUVEC, VEGF was at least partly involved as one of the angiogenic factors produced by osteoblasts. However, the incomplete inhibition by the antibody indicated that additional angiogenic factors would also play a role. As IGF-I and basic fibroblast growth factor are produced by osteoblast-like cells and stimulate the proliferation of endothelial cells (37–41), both would certainly be candidates for these angiogenic factors. It was noteworthy that VEGF did not increase the Al-P activity of HOB directly, but stimulated it only upon coculture with HUVEC. Recently, Midy and Plouet reported that VEGF stimulates Al-P activity and migration of bovine osteoblasts isolated from fetal calvaria (42). However, it is likely that the fetal osteoblasts they prepared were contaminated with endothelial cells. Slight, but significant, detection of ET-1 in the HOB-CM supports the idea that osteoblast-like cells under our present culture conditions also contain some endothelial cells. It is also possible that fetal osteoblast-like cells express VEGF receptors. However, VEGF can act only in a paracrine manner, except in certain malignant tumor cell lines, where it stimulates cell growth in an autocrine manner (43).

As reported by Guenther *et al.* (44), endothelial cells synthesize potent growth factors for osteoblasts, and these have recently been identified as IGF-I, ET-1, basic fibroblast growth factor, *etc.* (13–14, 37–41). We also confirmed that HUVEC under our present experimental conditions produce IGF-I and ET-1. Receptors for 1,25-$(OH)_2D_3$ and IGF-I are present not only on osteoblasts, but also on endothelial cells (45). Kurose *et al.* (46) reported that 1,25-$(OH)_2D_3$ and IGF-I synergistically stimulate Al-P activity on osteoblast-like cells. As 1,25-$(OH)_2D_3$ is capable of increasing the number of IGF-I receptors on osteoblast-like cells (47), it is reasonable that HOB cultured in direct or indirect contact with HUVEC increased Al-P activity to a greater extent than in HOB cultured alone, and that the effect was further potentiated by 1,25-$(OH)_2D_3$.

The effects of ET-1 on bone metabolism are a matter of debate (13). The vasoactive peptide stimulates proliferation of osteoblast-like cells (MC3T3-E1) (48) and increases the steady state levels of expression of osteopontin and osteocalcin mRNA in rat osteosarcoma cells (49). We demonstrated that 1,25-$(OH)_2D_3$ increased ET-1 concentration in the HUVEC-CM. To the best of our knowledge, this is the first report of 1,25-$(OH)_2D_3$ on the vasoactive peptides produced by HUVEC. Furthermore, we demonstrated that ET-1 stimulated Al-P activity in human osteoblast-like cells, although the opposite effect was reported in neonatal osteoblasts or murine cell lines (13). ET-1 can also affect osteoclasts, either inhibiting osteoclastic bone resorption or stimulating bone resorption depending on the organ culture system employed. As endothelial cells are abundant in bone marrow and lie in close proximity to osteoblasts and osteoclasts (50), ETs may be added to the list of potential modulating factors in bone remodeling (51). The histochemical localization of ET-1 in endothelial cells, osteoblasts, and osteoclasts supports this hypothesis (52).

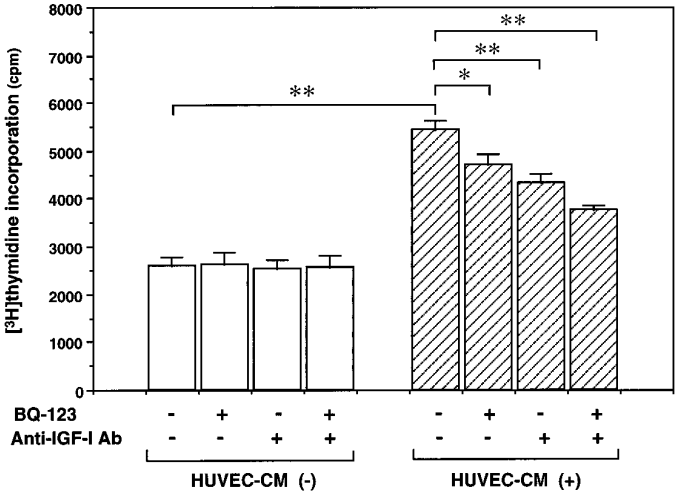

FIG. 5. Effects of BQ-123 and anti-IGF-I antibody on HUVEC-CM-induced [^{3}H]thymidine incorporation by HOB. HOB were cultured in αMEM-10% FCS until subconfluent, when the medium was changed to fresh αMEM-10% FCS supplemented with 50% HUVEC-CM containing various concentrations of BQ-123 and anti-IGF-I antibody alone or in combination. After culture for 48 h, [^{3}H]thymidine incorporation was determined as described in *Materials and Methods*. Data are the mean ± SD of quadruplicate samples. □, Without HUVEC-CM; ▨, with HUVEC-CM. Representative data from three experiments are shown. *, $P < 0.05$; **, $P < 0.01$ (with *vs.* without BQ-123 and/or anti-IGF-I antibody).

Under our present experimental conditions, endothelial cells expressed the VEGF receptor genes (*flt-1* and *kdr*) constitutively. In addition, when HUVEC were cultured with HOB, the level of VEGF receptor expression was maintained for a longer period and was further augmented by 1,25-$(OH)_2D_3$. However, 1,25-$(OH)_2D_3$ had no apparent direct effect on VEGF receptors in the absence of HOB. These results raise the possibility that VEGF produced by HOB may act on adjacent endothelial cells as an angiogenic factor in a paracrine manner. These *in vitro* findings are compatible with the *in vivo* observation that bone formation was stimulated in a diffusion chamber containing both endothelial cells and osteoblasts, which were implanted into rats (53). Furthermore, this hypothesis is substantiated by the adjacent histological localization of endothelial cells and osteoblast-like cells in skeletal tissue, suggesting that VEGF produced by osteoblast-like cells and VEGF receptors expressed on endothelial cells are involved in both bone formation and bone remodeling.

There is increasing evidence to suggest that vitamin D is effective for the treatment of patients with osteoporosis, mainly by stimulating the absorption of calcium and phosphate in the intestine and maintaining the serum levels of calcium and phosphate, so that bone mineralization proceeds efficiently (54, 55). Furthermore, active vitamin D elicits a number of anabolic effects on osteoblasts, such as stimulation of the production of osteocalcin, osteopontin, and bone matrix protein; stimulation of Al-P activity; and increase in the number of IGF-I receptors and the release of IGF-binding proteins (3, 4, 47, 48) (Fig. 9). Our present

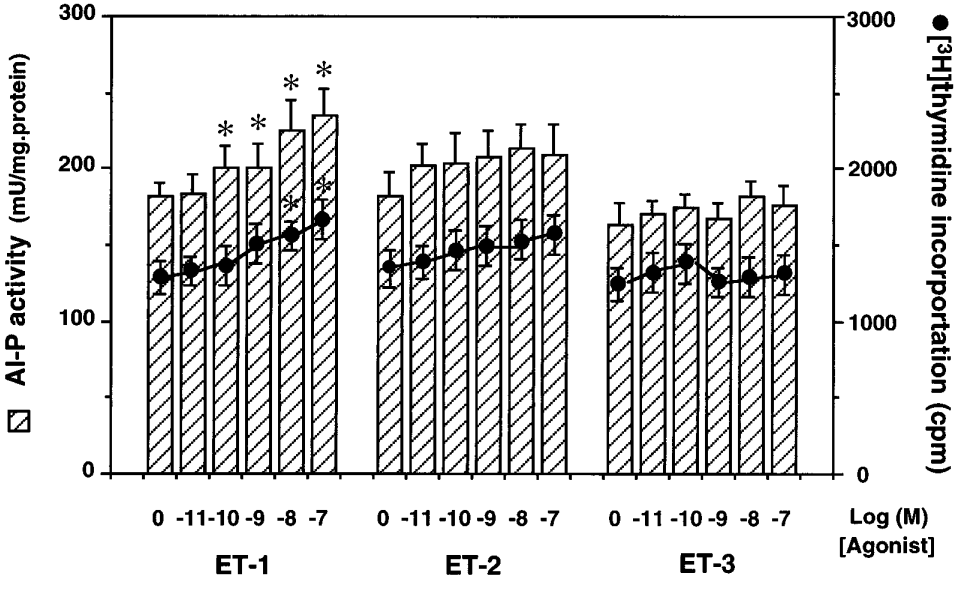

FIG. 6. Effects of ET on Al-P activity and [^{3}H]thymidine incorporation into HOB. HOB were cultured in αMEM-10% FCS until confluent. Then, the medium was changed to αMEM containing 1% BSA and various concentrations of ETs. After 4 days of culture, Al-P activity was determined as described in *Materials and Methods*. To investigate [^{3}H]thymidine incorporation, HOB were cultured in αMEM-10% FCS until subconfluent. Then, the medium was changed to αMEM supplemented with 0.5% FCS. On the following day, the medium was replaced with αMEM containing 1% BSA and various concentrations of ETs. After an additional 48 h of culture, [^{3}H]thymidine incorporation was determined as described in *Materials and Methods*. Data are shown as the mean ± SD of quadruplicate samples. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

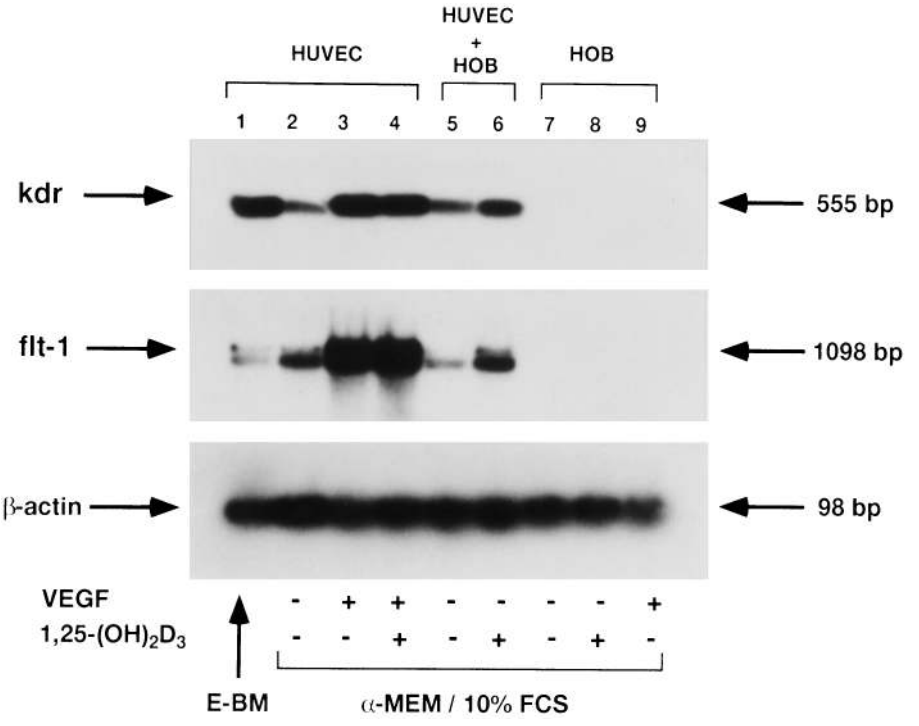

FIG. 7. Effects of VEGF and 1,25-$(OH)_2D_3$ on *kdr* and *flt-1* gene expression in HUVEC, HUVEC cocultured with HOB, and HOB cultured alone. Total RNA was prepared from HUVEC, HUVEC cultured with HOB, and HOB cultured in αMEM-10% FCS containing VEGF (10^{-9} M) and/or 1,25-$(OH)_2D_3$ (10^{-8} M; lanes 2–9). As a positive control, HUVEC were cultured in supplemented E-BM (lane 1). After reverse transcription, cDNAs were amplified by RT-PCR as described in *Materials and Methods*. The products were electrophoresed on 2% agarose gel, transferred to nylon membranes, and hybridized with ^{32}P end-labeled probes specific to *kdr* (*upper panel*), *flt-1* (*middle panel*), and β-actin (*lower panel*) mRNA. PCR amplification for the latter was performed for 27 cycles. The *kdr* blot was exposed for 7 h, and that for *flt-1* was exposed for 24 h. Radioactivities of hybridization bands were measured with a BioImagin analyzer.

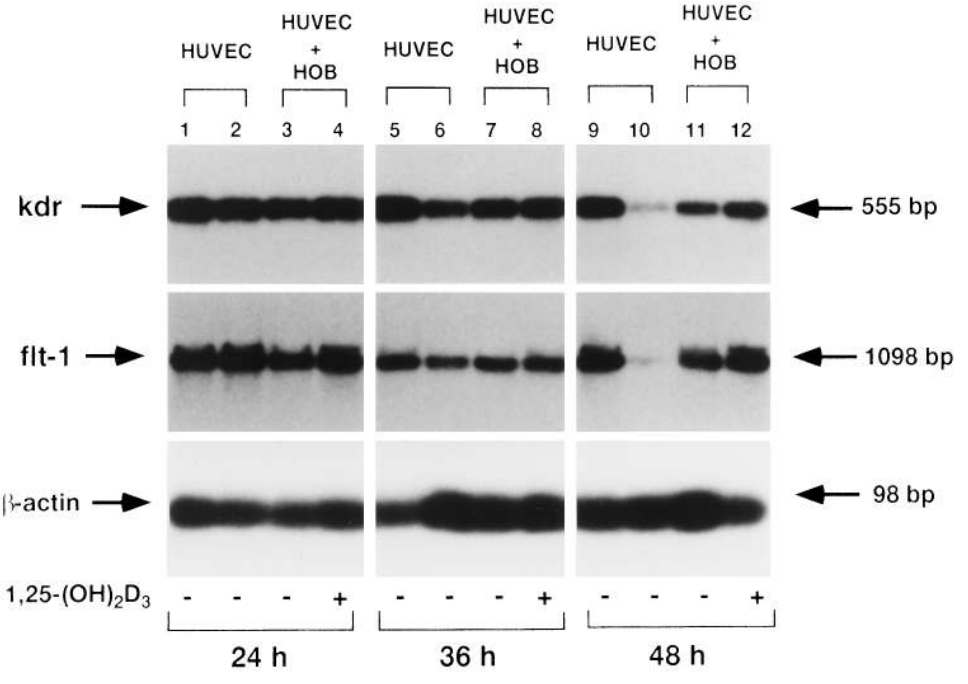

FIG. 8. Time-course effect of 1,25-$(OH)_2D_3$ on *kdr* and *flt-1* gene expression in HUVEC cocultured with HOB. HUVEC were cultured in 6-cm dishes with the supplemented E-BM until subconfluent. Then, the medium was changed to αMEM-10% FCS (lanes 2, 6, and 10) or the supplemented E-BM (lanes 1, 5, and 9) and cultured for an additional 24–48 h. In several dishes, approximately the same number of HOB was added, and both types of cell were cultured in αMEM-10% FCS in the presence or absence of 1,25-$(OH)_2D_3$ (10^{-8} M; lanes 3 and 4, 7 and 8, and 11 and 12). After culture for 24–48 h, total RNA was prepared. After reverse transcription, cDNAs were amplified by PCR as described in *Materials and Methods*. The products were electrophoresed on a 2% agarose gel, transferred to nylon membranes, and hybridized with ^{32}P end-labeled probes specific to *kdr*, *flt-1*, and β-actin mRNA. PCR amplification for the latter was performed for 27 cycles. The *kdr* blot was exposed for 7 h, and that for *flt-1* was exposed for 24 h. Radioactivities of hybridization bands were measured with a BioImagin analyzer.

in vitro findings suggest that these anabolic effects of 1,25-$(OH)_2D_3$ are intensified by endothelial cells, which are abundant in the vicinity of osteoblasts in bone marrow.

In summary, we demonstrated that 1,25-$(OH)_2D_3$ stimulates Al-P activity in osteoblasts and increases the steady state level of VEGF mRNA, followed by increased secretion of VEGF. The secretable angiogenesis factor acts on KDR and Flt-1 receptors on endothelial cells in a paracrine manner, thereby causing the proliferation of endothelial cells.

The activated endothelial cells expressing a greater amount of VEGF receptor genes, in turn, produce osteotropic growth factors, such as ET-1 and IGF-I, which synergistically stimulate the proliferation of HOB accompanied by an increase in Al-P activity (Fig. 9). Therefore, osteogenesis and angiogenesis may be mutually dependent, and it is reasonable to assume that the anabolic effects of 1,25-$(OH)_2D_3$ on skeletal tissue are mediated in a paracrine manner and enhanced by the VEGF/VEGF receptor system between osteoblasts and endothelial cells.

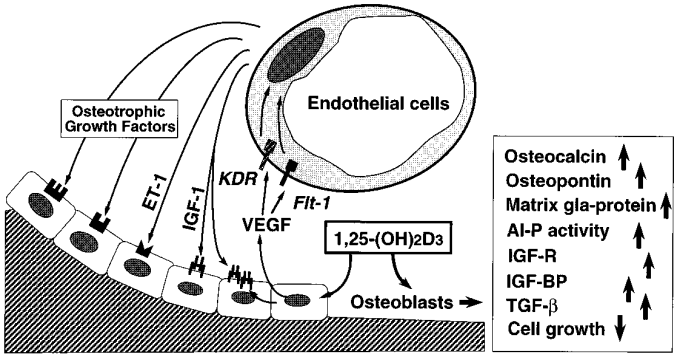

FIG. 9. Hypothesis of anabolic effects of 1,25-$(OH)_2D_3$ on osteoblasts in the presence of endothelial cells.

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