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Effect of Human Endothelial Cells on Human Bone Marrow Stromal Cell Phenotype: Role of VEGF?

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Abstract Angiogenesis is a tightly regulated process involved in growth, repair, and bone remodeling. Several studies have shown that there is a reciprocal regulation and functional relationship between endothelial cells and osteoblast-like cells during osteogenesis, where systemic hormones and paracrine growth factors play an active role. Angiogenesis is induced by a variety of growth factors; among them vascular endothelial growth factor (VEGF) may be an important mediator for the angiogenic process involved in bone physiology. We studied the VEGF effect on osteoblast progenitor cells (Human Bone Marrow Stromal Cells: HBMSE) cultured alone or associated with endothelial cells (Human Umbilical Vein Endothelial Cells: HUVEC) in different co-culture models (co-culture with or without direct contact, conditioned medium), to determine the influence of VEGF on these cells and on their relationship. In agreement with other studies, we show that HBMSC express and synthesize VEGF, HUVEC conditioned medium has a proliferative effect on them, and early osteoblastic marker (Alkaline phosphatase activity) levels increase when these cells are co-cultured with HUVEC only in direct contact. However, unlike previous studies, we did not find that VEGF increased these processes. These results suggest that the intercommunication between endothelial cells and osteoblastic-like cells requires not only diffusible factors, but also involving cell membrane proteins. J. Cell. Biochem. 79:672–685, 2000.†

Key words: vascular endothelial growth factor; endothelial; osteoblast; co-culture

Angiogenesis, the development of a microvascular network for blood supply, is a tightly regulated process, essential in development and tissue repair including bone [Winet, 1996]. The importance of the bone vasculature in osteogenesis is well recognized. During endochondral bone formation, capillary sprouts of the metaphyseal vessels invade the growth plate before the cartilage matrix becomes mineralized [Brown et al., 1990; Ganey et al., 1992; Bittner et al., 1998; Gerber et al., 1999]. Defects in bone vasculature have been reported in pathophysiological conditions such as osteoporosis and rickets [Burkhardt et al., 1987; Hunter et al., 1991].

The atrophic changes in bone and marrow of osteoporotic patients are related with a decrease in the number of bone marrow sinusoids and arterial capillaries [Burkhardt et al., 1987; Shapiro et al., 1987]. In rickets, the overall architecture of the metaphysal vessels is significantly altered, and rachitic reversal by treatment is associated with angiogenesis [Shapiro et al., 1987; Hunter et al., 1991].

An intimate functional relationship exists between bone vascular endothelium and osteoblastic cells during osteogenesis, in which complex hormones and growth factors mediate signals and play an active role [Guenther et al., 1986; Villanueva et al., 1990; Collin-Osdoby et al., 1994; Decker et al., 1995, Formigli et al., 1997; Matusuno et al., 1998]. Angiogenesis is induced by a variety of growth factors, but vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), appears to be a unique potent endothelial cell-specific cytokine, inducing both vascular permeability and angiogenesis in vivo [Dvorak et al., 1995; Ferrara et al., 1997]. It is now well known that VEGF may be an important mediator of the angiogenic process required for bone formation and repair [Goad et al., 1996; Schlaeppi et al., 1997; Wang et al., 1997; Gerber et al., 1999].

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The up-regulation of VEGF by calcitropic hormones (1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$]), and parathyroid hormone [PTH 1-34] in osteoblast-like cells points to the role of VEGF in bone physiology [Wang et al., 1996, 1997; Schlaeppi et al., 1997]. Several in vitro studies have shown that there is a reciprocal regulation between endothelial cells and osteoblast-like cells (such as primary osteoblasts and osteoblastic-cell lines) [Bianchi et al., 1985; Guenther et al., 1986; Villanueva et al., 1990; Jones et al., 1995; Formigli et al., 1997], and that VEGF appears to be a main signal between osteoblast cells and endothelial cells.

However, the involvement of others factors in the communication pathways between bone cells and endothelial cells, particularly those which are known to be active in these two cell lines, such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), and insulin-like growth factors (IGF-I and IGF-II) [Cullinan-Bove et al., 1993] are well studied. Among the soluble mediators, growth factors and cytokines secreted by endothelial cells such as IL-1, IL-6, prostacyclin, endothelin-1, gaseous, and free radical messengers like nitric oxide and superoxide anions [Collin-Osdoby 1994], could be also involved in this endothelial and bone cells interaction.

Moreover, the common mesenchymal origin of endothelial cells and bone cells, and their close contact within the bone marrow, lead us to suppose that the communication between endothelial cells and stromal cells is highly complex.

In this paper, we studied the effect of VEGF on human osteoblast progenitor cell (Human Bone Marrow Stromal Cells: HBMSC) proliferation and differentiation cultured alone or associated with endothelial cells (Human Umbilical Vein Endothelial Cells: HUVEC) in different co-culture models. Through these cell co-culture models we investigated the relationship between these two human primary cultures of osteoblast and endothelial cells.

MATERIALS AND METHODS

Materials

Tissue culture plates Nunclon™ were purchased from Nunc (Denmark), culture flasks from Corning (Corning, NY), and Insert porous membrane (Cyclopore™, Falcon®) from Becton-Dickinson (Franklin Lakes, NJ). Cell culture medium (IMDM: Iscove's Modified Dulbecco's Medium) was supplied by GibcoBRL® Life Technologies (Gaithersburg, MD). Foetal calf serum (FCS) and trypsin were purchased from Boehringer Mannheim (Mannheim, Germany). Antibiotics (Penicillin 10.000 UI-Streptomycin 10 mg), DMSO (dimethylsulfoxide), MTT (3-[4,5-dimethylthyazol-2-yl]-2-5-diphenyl-tetrazolium bromide) were obtained from Sigma Aldrich Chemical (St. Louis, MO), and Sigma 104 phosphatase substrate with alkaline buffer solution, Fast Blue PR Salt, Naphtol AS-MX Phosphate with alkaline solution (diagnostic kit 85L-2), were purchased from Sigma Aldrich Diagnostics (St. Louis, MO). Recombinant human VEGF165 was obtained from TEBU PeproTechnic (Le Perray-en-Yvelines, France), and anti-human VEGF-kit was purchased from R&D Systems (Abingdon, UK). EPOS anti-human von Willebrand Factor, DAKO DAB chromogene tablets were purchased from DAKO A/S (Glostrup, Denmark), and Dakopatts DAKO PAP kit System 40, were obtained from DAKO Corporation (Carpinteria, CA). Acetylated LDL labeled with (1,1'-dioctadecyl-3,3,3',3'-tetrametyl-indo-carbocyanine perchlorate) were purchased from Biomedical Technologies Inc. (Stoughton, UK). OSTK-PR used for radioimmuno assay of human osteocalcin quantitative assay of osteocalcin was obtained from Cis Bio International (Gif-sur-Yvette, France), and [Methyl-^{3}H]Thymidine Radiochemical batch analysis from Amersham International (Buckinghamshire, UK). Reagents for molecular biology (SuperScript™ preamplification system, agarose electrophoresis grade, Taq DNA polymerase) were purchased from GibcoBRL® Life Technologies, and RNeasy Mini Kit was from QIAGEN (Hilden, Germany).

Cell Culture

Endothelial cell culture. Endothelial cells were extracted from human umbilical veins essentially as described by Bordenave et al. [1993], according to the procedure of Jaffe et al. [1980]. Endothelial cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) with 20% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 90 μg/ml heparin, 20 μg/ml Endothelial Cell Growth Supplement (ECGS).

Cell characterization was evaluated either by the assessment of von Willebrand factor expression, detected by immunocytochemical reaction using DAKO-EPOS anti-von Willebrand factor/HRP coupled with peroxydase (DAKO DAB chromogene tablets), or by uptake of fluorescent acetylated low-density lipoprotein (DiI-Ac-LDL) according to the operative conditions of Voyta et al. [1984], which demonstrated the "scavenger cell pathway" of endothelial cells, and finally by their typical cobblestone morphology on reaching confluence. Cells were used for the experiments after the first or the second passage.

Human bone marrow stromal cell culture. Human bone marrow was obtained by aspiration from the femoral diaphysis or iliac bone from patients (aged 20–70 years) undergoing hip prosthesis surgery after trauma. According to Vilamitjana-Amédée et al. [1993] and others publications [Richard et al., 1996; Faucheux et al., 1997] cells were separated into a single suspension by sequential passage through syringes fitted with 16-, 18-, and 21-gauge needles. Cells were then counted and plated at 10^5 cells/cm^2 into 75 cm^2 flasks in IMDM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 mg/ml streptomycin, 10^{-8} M dexamethasone, and incubated in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2 at 37°C.

Cells were tested for their intracellular alkaline phosphatase (Al-P) activity as described previously [Ackerman, 1962] with the SIGMA diagnostic kit (85L-2). HBMSC were also characterized by immunocytochemical detection of intracytoplasmic type I collagen and osteocalcin synthesis (OC) by radioimmunoassay as described by Vilamitjana-Amédée et al. [1993]. HBMSC were used after the second and until the sixth passage.

Co-culture of HBMSC and HUVEC with direct contact. HBMSC were plated in 24-multiwell dishes at 10^4 cells/well in 0.5 ml IMDM, containing 10% (v/v) FCS and antibiotics as described above. After 3 or 4 days, when the cells reached 50% of confluence, HUVEC were added to each well at 3×10^4 cells/well in 0.7 ml IMDM, containing 10% (v/v) FCS without growth factors. Thereafter, the medium was changed every day, and supplemented or not with VEGF (10 ng/ml) for 3, 6, or 9 days.

Co-culture of HBMSC and HUVEC without direct contact. HUVEC and HBSMC were cultured in the same well, but separately, by using a 0.45 μm filter insert (6.25 mm diameter; CYCLOPORE™). In this model, each well was composed of a double chamber, consisting of an outer chamber (24-multiwell plate of 2 cm^2) and the insert cyclopore chamber (0.31 cm^2). In the insert chamber, HUVEC were seeded at 4×10^4 cells/insert (12×10^4 cells/cm^2) in 0.2 ml IMDM, supplemented with 10% (v/v) FCS and antibiotics. Inserts were placed in these wells containing HBSMC only when they reached 50% of confluence.

Effects of HUVEC-conditioned medium on HBMSC and HBMSC-conditioned medium on HUVEC. HUVEC were grown in IMDM with 20% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 90 μg/ml heparin, 20 μg/ml Endothelial Cell Growth Supplement (ECGS), in 75 cm^2 flasks (Falcon®) until they reached confluence. The confluent monolayers were washed twice with Hank's buffer (pH 7.4), and the medium was replaced with IMDM, 10% (v/v) FCS. Then the cells were cultured for an additional 2 days. At the end of the incubation period, the conditioned medium (HUVEC-CM) was obtained by centrifugation at 300*g* for 10 min at room temperature, and the supernatants were immediately used or frozen at −20°C. For HBMSC the process was the same at the difference that they were grown in IMDM containing 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, onto 75 cm^2 plastic dishes (Falcon®) until they reached confluence.

For the cell proliferation assays, HBMSC were plated at 2×10^3 cells/well and HUVEC were plated at 1.5×10^4 cells/well in 24-multiwell plates containing 0.5 ml IMDM supplemented with 10% (v/v) FCS. One day later, and then every 2 days, the medium was changed with IMDM containing 10% (v/v) FCS supplemented with 50% (v/v) HUVEC-CM or with 50% (v/v) HBMSC-CM, for HBMSC or HUVEC proliferation assays, respectively. Cultures were carried out for 1, 3, 6, 9, 12, and 15 days and cell proliferation was assessed by [^{3}H]thymidine incorporation as described by Hauschka et al. [1986].

To investigate the effect of HUVEC-CM on HBMSC differentiation, HBMSC were plated at 10^4 cells/well in 24-multiwell plates containing 0.5 ml IMDM supplemented with 10% (v/v) FCS. One day later, and then every 2 days, the medium was changed with IMDM containing

10% (v/v) FCS and supplemented with 50% (v/v) HUVEC-CM. Cultures were carried out for 3, 6, and 9 days and then Al-P activity was determined as described previously [Majeska, 1962].

Effects of HUVEC-extracellular matrix on HBMSC. HUVEC were grown in IMDM containing 20% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 90 μg/ml heparin, 20 μg/ml Endothelial Cell Growth Supplement (ECGS) onto 25 cm^2 plastic dishes (Falcon®) until they reached confluence. Extracellular matrix (ECM) of HUVEC was obtained by stimulating the cells every day for 10 days after they had reached confluence, with D-glucose (30 mM) and ascorbic acid (50 μg/ml) added to IMDM containing 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and without heparin or ECGS. The cells were removed from their ECM material by rinsing with 10 ml of 5% glycerol solution (v/v in water) for 15 min, and then with 10 ml of water alone [Buján et al., 1995]. There after the ECM was scraped in 2 ml of PBS 0.1 M pH 7.4 solution with protease inhibitors (EDTA 5 mM, Benzaminidiumchlorid 0.3 M, Iodoacetamide 0.1 mM, Phenyl-meyhyl-Sulfonyl Fluorid 4.5 mM, 6-amino hexanoic acid 0.1 M) and sonicated for 30 s at 20 kHz and 8% amplitude (Vibra-Cell™; Bioblock-Scientific). Then ECM solution was dialysed 24 H at 4°C against PBS 0,1 M (100 volumes for 1 volume). One-hundred μl of the dialysate were used for quantitative measurement of protein content of ECM, and 10 $\mu g/cm^2$ were used for an homogeneous coating. There after the ECM was sterilised by gamma rays (25 kGy/8 h).

For proliferation assays, HBMSC cultures were carried out for 1 , 3, 6, 9, 12, and 15 days and cell proliferation was assessed by MTT test [Mosmann, 1983]. To investigate the anabolic effect of HUVEC-ECM on HBMSC, HBMSC were cultured in the same conditions used for proliferation assay, and cultures were carried out for 3, 6, and 9 days. Then Al-P activity was determined.

Quantitative measurement of alkaline phosphatase activity. Intracellular alkaline phosphatase activity (Al-P) was determined at confluence, as described by Majeska and Rodan [1982], arising from the different culture conditions. Data are expressed as a ratio of nanomole inorganic phosphate (Pi) cleaved by the enzyme in 30 min per μg protein. The quantitative measurements of cellular proteins were done using Lowry's method [Lowry et al., 1951].

Quantitative measurement of osteocalcin synthesis. Osteocalcin synthesis was determined at confluence arising from the different culture conditions as described previously [Faucheux et al., 1997]. Osteocalcin synthesis was measured by a specific radioimmunoassay (OSTK-PR; Cis Bio International, France) with an antibody raised in rabbit against bovine osteocalcin and which cross-links with human osteocalcin [Vilamitjana-Amédée et al., 1993]. The limit detection for the assay was 1 ng/ml.

Quantitative measurement of VEGF in the medium. The VEGF concentration in HBMSC conditioned medium was measured using a solid phase enzyme-linked immunosorbent assay designed to measure VEGF levels in supernatants, serum, and plasma (R&D systems, Abingdon, UK), for which the sensitivity is less than 15 pg/ml. To measure the VEGF concentration in the culture supernatants, HBMSC were exposed or not to 1,25-$(OH)_2D_3$ at 10^{-8} M as described previously [Wang et al., 1996].

RT-PCR Analysis

HBMSC were cultured alone and treated with 1,25-$(OH)_2D_3$ at 10^{-8} M for 24 h as previously described [Harada et al., 1994]. Total RNA was extracted using the RNeasy™ Total RNA kit (QIAGEN) and 3 μg were used as template for single strand cDNA synthesis with the Superscript preamplification system (Gibco, BRL) in 20 μl final volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM DTT, 0.5 mM of each dATP, dCTP, dGTP, dTTP, 0.5 μg oligo$(dT)^{12-18}$, and 200 U of reverse transcriptase. After incubation of 50 min at 42°C, the reaction was stopped at 70°C for 15 min and kept on ice. Synthesized cDNA were treated with 2 U of RNase H (*Escherichia coli*) at 37°C for 20 min and samples were mixed with PCR cocktail (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 μg/μl BSA, 10 mM DTT, 0.5 mM of each dATP, dCTP, dGTP, dTTP, 2.5 U of Taq polymerase), and 0.5 μM each of forward and reverse primers (Table I) in 50 μl final volume. Amplification was performed in a HYBAID thermocycler using the following conditions: a denaturation at 94°C for 5 min, then 30 cycles of 94°C for 10 s, hybridisation at Tm (indicated in Table I) for 30 s, followed by a

TABLE I. Sequences and Tm of Primers Used for RT-PCR Analysis and Corresponding Size of Attempted PCR Products

Transcript	TM °C	Sequence	Expected size for PCR products
β-actin	64°C	forward: GTG GGG CGC CCC AGG CAC CA	0.548 Kb
		reverse: CTC CTT AAT GTC ACG CAC GAT	
Al-P	51°C	forward: TTA GTG CCA GAG AAA GAG	0.987 Kb
		reverse: CTT GGC TTT TCC TTC ATG GTG	
OC	64°C	forward: ATG AGA GCC CTC ACA CTC CTC	0.302 Kb
		reverse: CTA GAC CGG GCC GTA GAA GCG	
VEGF	64°C	forward: TCG GGC CTC CGA AAC CAT GA	0.520 Kb
		reverse: CCT GGT GAG AGA TCT GGT TC	0.650 Kb
			0.730 Kb
flt-1	51°C	forward: ACT ATG GAA GAT CTG ATT TCT TAC	1.098 Kb
		reverse: GGT ATA AAT ACA CAT GGT CTT CTA	
KDR	51°C	forward: TAT AGA TGG TGT AAC CCG GA	0.555 Kb
		reverse: TTT GTC ACT GAG ACA GCT TGG	

final extension step at 72°C for 1 min. To control for the integrity of the various RNA preparations, the expression of β-actin was also assessed. PCR products were electrophoretically separated on a 1% (w/v) agarose gel, and visualized by ethidium bromide staining.

For VEGF mRNA, specific oligonucleotide primer pairs were selected in order to detect four possible splicing variants [Leung et al., 1989] (forward primer at position 41 bp to 60 bp, and reverse primer at position 670 bp to 690 bp).

Statistical Analysis

The data are expressed as the mean ± SD, and analysed using the non-parametric U-test of Mann and Whitney (used to compare means of few independent samples).

RESULTS

VEGF mRNA and VEGF-Receptor mRNA Expression Analysis in HBMSC by RT-PCR

As shown in Figure 1a, and in accordance with the literature, OC and Al-P mRNA expression in our culture model (i.e., HBMSC) appeared to be stimulated by a treatment with 1,25-$(OH)_2D_3$ for 24 H at 10^{-8} M, which is well known to stimulate the expression of osteoblastic markers [Vilamitjana-Amédée et al., 1993]. VEGF mRNA expression was also detected in HBMSC [Wang et al., 1996, 1997; Schlaeppi et al., 1997] as shown in Figure 1b. This experiment was realized with specific oligonucleotide primer pairs selected in order to detect four possible splicing variants, and showed that HBMSC expressed two of the four forms of VEGF corresponding to VEGF121 (520 bp) and VEGF165 (650 bp) which are the soluble forms of VEGF. Moreover, the VEGF-receptor mRNA, flt-1 and KDR, were also expressed in HBMSC (Fig. 1b).

VEGF Secretion in HBMSC- and HUVEC-Conditioned Medium

Basal VEGF level in the medium conditioned by HBMSC at confluence was 893 ± 89 pg/ml (Fig. 2). Consistent with literature, HBMSC treatment with 1,25-$(OH)_2D_3$ at 10^{-8} M for 2 days produced a 2.4-fold (2.132 ± 145) increase in immunoreactive VEGF levels in the conditioned medium, suggesting that VEGF was synthesized and secreted into the medium, and that this secretion was increased in response to 1,25-$(OH)_2D_3$. These amounts of VEGF correspond to the mitogenic activity of this cytokine, i.e., 1 to 4 ng/ml (22 to 88 pM) [Ferrara et al., 1997]. In the cell culture conditions described above, no VEGF was detected in the HUVEC-conditioned medium (data not shown).

Effects of VEGF on HUVEC and HBMSC Proliferation

VEGF at 10 ng/ml significantly stimulated the proliferation of HUVEC (Fig. 3a), but had no effect on HBMSC (Fig. 3b). Although HUVEC were cultured in the presence of only 10% FCS which is not the best concentration normally used for this cell culture model, VEGF alone at 10 ng/ml had a direct proliferative effect on HUVEC.

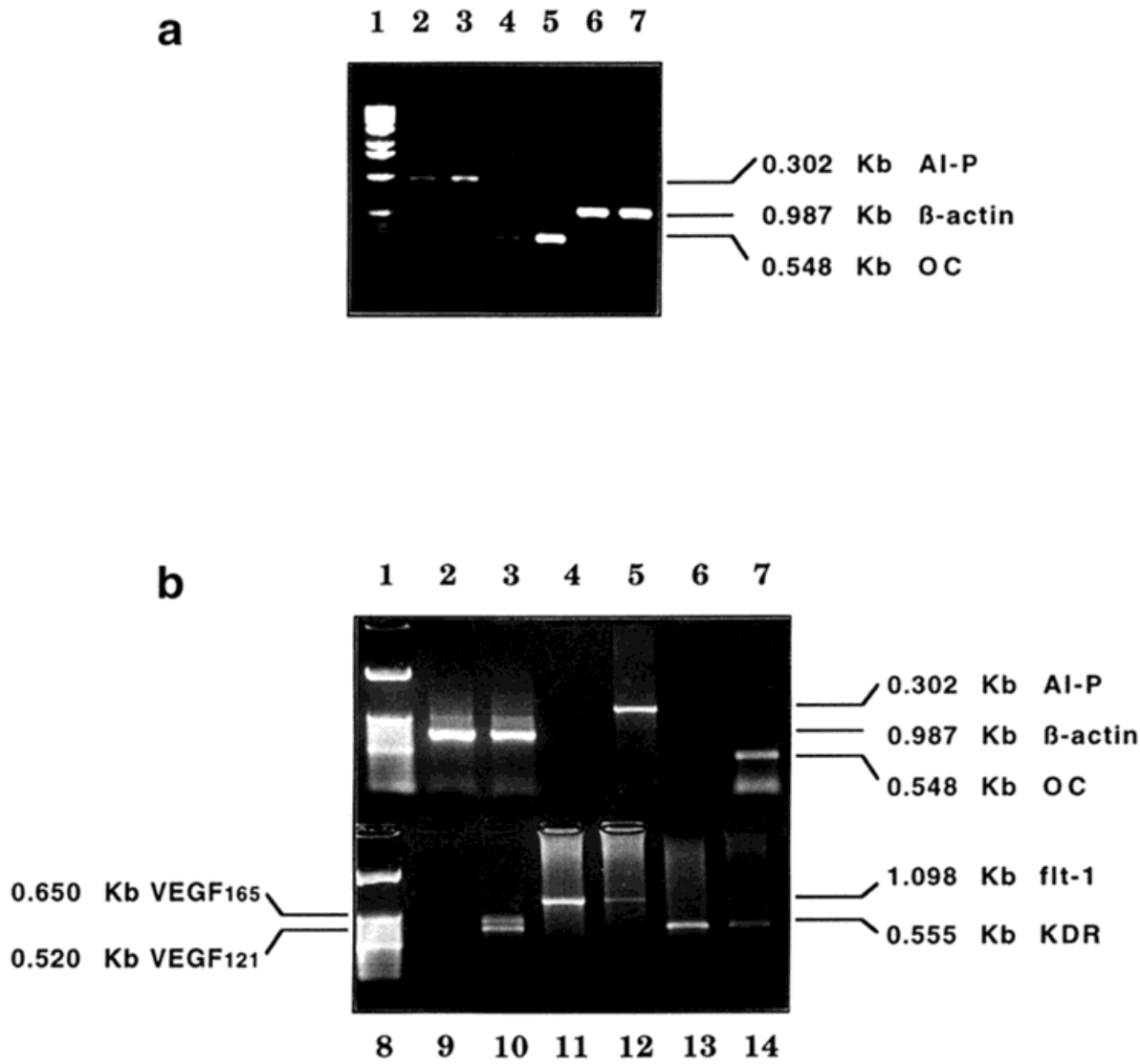


Fig. 1. **a**: RT-PCR of osteoblastic markers mRNA expression in HBMSC grown in presence or not of 1,25-$(OH)_2D_3$ at 10^{-8} M. HBMSC express Al-P (lane 2) and OC (lane 4). mRNA expression of osteoblastic markers are enhanced with 1,25-$(OH)_2D_3$ at 10^{-8} M (Al-P lane 3, and OC lane 5). β-actin (lane 6: HBMSC control, and lane 7: HBMSC treated with 1,25-$(OH)_2D_3$ at 10^{-8} M) is used as housekeeping gene. Lane 1: 1Kb DNA ladder (GIBCO). **b**: RT-PCR of osteoblastic markers, VEGF and VEGF receptors mRNA expression in HUVEC and HBMSC. HUVEC don't express Al-P (lane 4) or OC (lane 6) contrary to HBMSC (respectively, lanes 5 and 7). HBMSC express two of the four forms of VEGF (obtained by alternative splicing of a unique gene, lane 10) whereas no form of VEGF is expressed by HUVEC (lane 9). Moreover, HBMSC express VEGF receptors, flt-1 and KDR (respectively, lanes 12 and 14), but this expression seems to be less than that obtained with HUVEC (lanes 11 and 13, respectively). β-actin (lane 2 for HBMSC, and lane 9 for HUVEC) is used as housekeeping gene. Lanes 1 and 8: 1Kb DNA ladder (GIBCO).

Conditioned medium arising from HBMSC cultured in the presence of 10% FCS, also significantly increased cell proliferation of HUVEC (Fig. 3a). The proliferative effect of HBMSC-CM on endothelial cells was similar of that obtained in the presence of VEGF 10 ng/ml. In the same manner, HUVEC-CM significantly increased [^{3}H]-thymidine incorporation into HBMSC (Fig. 3b).

Effects of VEGF on Al-P Activity of HBMSC, Co-Cultured or not With HUVEC, or Treated With HUVEC-CM

When Al-P positive HBMSC were cultured with Al-P negative HUVEC, whatever the osteogenic potential of the bone marrow samples, co-culture induced an increase (2.4-fold) of the enzymatic activity up to 6 days of di-

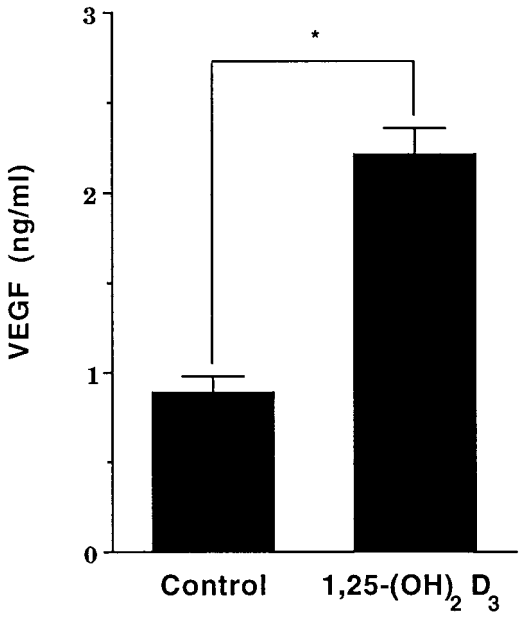


Fig. 2. Quantitative measurement of VEGF synthesized and secreted in supernatant of osteoblast-like cell cultures (HBMSC) with or without stimulation by 1,25-$(OH)_2D_3$ at 10_{-8} M for 48 h. The amount of VEGF was determined as described in Materials and Methods and expressed in pg/ml. Data are expressed as mean ± standard deviation (n = 4; $*P < 0.05$).

rect contact (Fig. 4), while this enzymatic activity was undetectable in HUVEC (data not shown). Moreover, in some assays, an effect of direct co-culture can be detectable as soon as 3 days of contact between HBMSC and HUVEC (Fig. 5).

In this experiment no effect was detectable after 3 days of co-culture. Surprisingly, when HUVEC and HBMSC were co-cultured without direct contact, Al-P activity of the HBMSC decreased significantly, even at day 3 when compared to HBMSC cultured alone (three-fold less; Fig. 4 with all data summarized in corresponding table).

Treatment of these different culture models with VEGF at 10 ng/ml had no positive effect on Al-P enzymatic activity compared with untreated cells, even with the direct co-culture HBMSC/HUVEC. In contrast, a striking negative effect of VEGF on Al-P activity of HBMSC cultured alone was obtained.

While HUVEC-CM increased DNA synthesis into HBMSC, it did not acts on the enzymatic activity when compared to direct co-culture of HBMSC and HUVEC (Figs. 3b, 5a, 5b).

Effects of VEGF on Osteocalcin Synthesized by HBMSC Cultured Alone or Co-Cultured With HUVEC in Direct Contact

Osteocalcin (OC) or Gla Bone Protein is a specific marker of bone cell lineage. The level of OC synthesis in HBMSC cultured alone in IMDM supplemented with 10% FCS increased with time of culture as shown in Figure 6, and reached a plateau after 6 days of culture.

Surprisingly, when OC-positive HBMSC and HUVEC were cultured with direct contact for 3, 6, and 9 days, the level of OC synthesis in HBMSC was lower than that obtained with HBMSC cultured alone (Fig. 6). The effect of VEGF at 10 ng/ml on co-cultured HBMSC with HUVEC with direct contact treated for 3, 6, and 9 days emphasizes this decrease in OC synthesis (Fig. 6 with all data summarized in corresponding table).

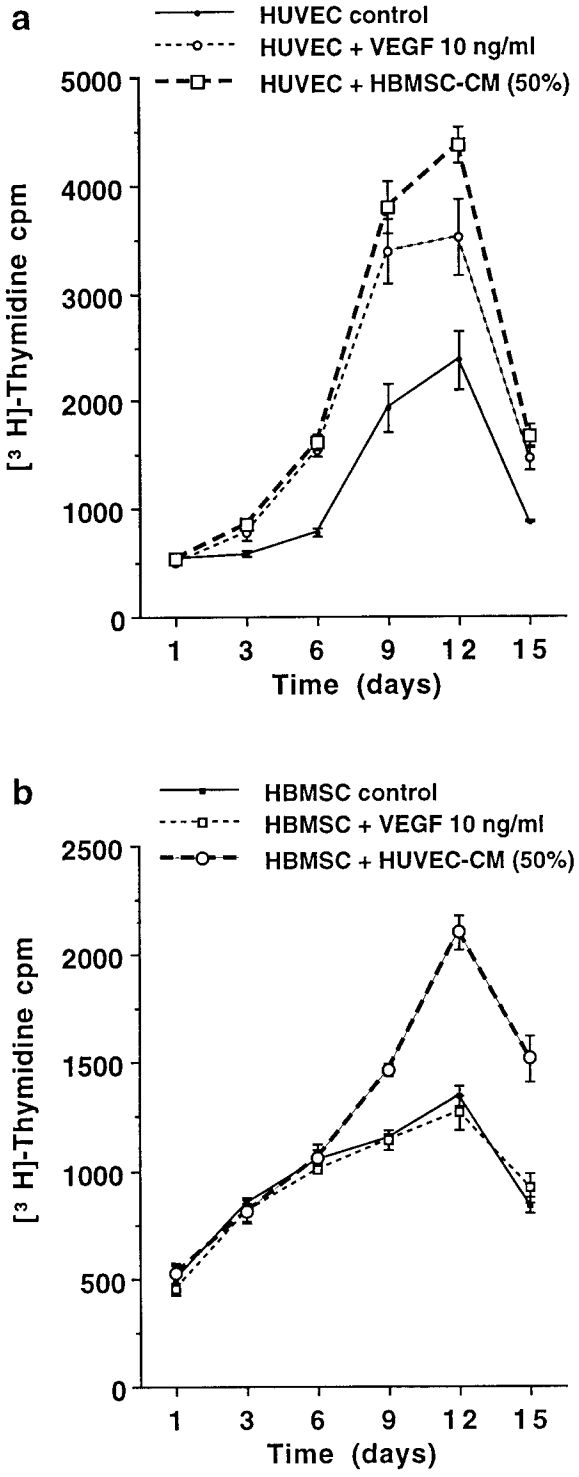
Effects of HUVEC Extracellular Matrix on HBMSC Proliferation and Differentiation

It is well known that ECM play a fundamental role in cell phenotype, proliferation, migration, and physiology [Hauschka et al., 1986]. Then, the interactive relationship between endothelial cells and osteoprogenitor cell could be mediated through ECM components. HBMSC seeded onto HUVEC-ECM have a proliferative effect in comparison to HBMSC control seeded onto plastic or onto their own ECM (Fig. 5c), but HUVEC-ECM seemed to have no effect on Al-P activity of HBMSC, in comparison to HBMSC cultured on plastic control, and to HBMSC co-cultured with HUVEC with direct contact (Fig. 5d).

DISCUSSION

Bone is a complex tissue that contains several cell types and an extracellular matrix characterized by a heavy mineralized type I collagen fiber network. It is well known that vasculature plays an important role in bone generation [Trueta, 1963; Villanueva et al., 1990; Collin-Osdoby, 1994]. Bone development and its remodelling is highlighted by the prerequisite for vascularization that precedes osteogenesis and by the special intimate physical relationship which exists both during embryogenesis and growth between the vasculature and osteoblasts that form bone, as well as the osteoclasts that resorb it [Collin-Osdoby, 1994;

Decker et al., 1995]. However, both intramembranous and endochondral ossification occur in association with blood capillaries [Shapiro et al., 1987; Brown et al., 1990; Carrington et al., 1991; Bittner et al., 1998; Gerber et al., 1999].

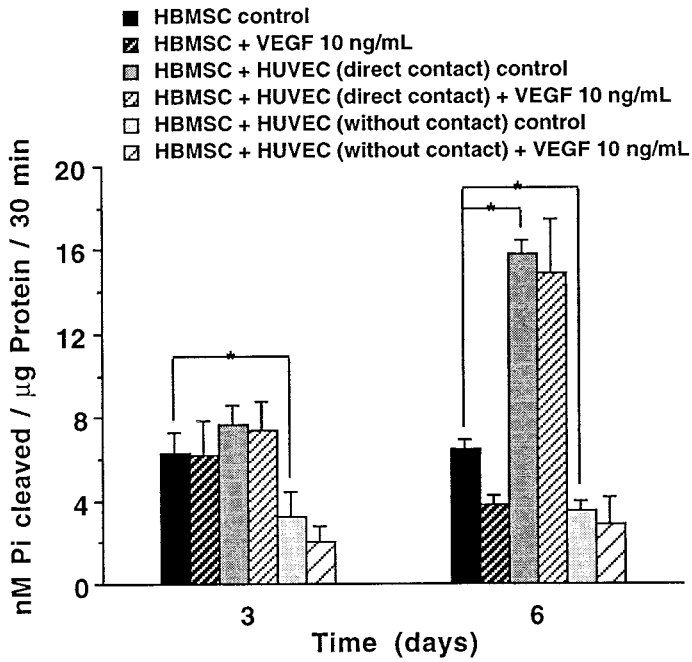


In contrast, a reduction in the number of sinusoids and arterial capillaries in the bone marrow is a typical feature of osteoporosis [Burkhardt et al., 1987].

Moreover, an increasing number of reports suggest that vascular endothelial growth factor (VEGF) is a physiological angiogenesis factor in highly vascularized tissues and that the expression of VEGF mRNA in endocrine tissues is regulated by trophic hormones [Berse et al., 1992; Cullinan-Bove et al., 1993; Ferrara et al., 1997]. Some of these papers have reported that potent stimulators of bone formation in vivo or in vitro like prostaglandin E1 (PGE1) and PGE2, 1,25-$(OH)_2D_3$, insulin-like growth factor I (IGF-I), PTH, induce VEGF mRNA expression in osteoblast-like cells [Harada et al., 1994; Goad et al., 1996]. All these reports suggest that VEGF is produced by osteoblast-like cells and that it is involved in the anabolic effects of osteotropic hormones in skeletal tissue [Wang et al., 1996, 1997; Schlaeppi et al., 1997; Gerber et al., 1999]. Although endothelial cells are present in the bone marrow, the mechanism by which they proliferate in skeletal tissue is not well elucidated; the fact that osteoblast-like cells synthesize and secrete VEGF may be the premise of an explanation.

However, the possibility that VEGF has a direct effect on differentiation, migration, or proliferation of osteoblast-like cells is debated, and studies on the interaction between endothelial cells and osteoblast-like cells are contradictory [Guenther et al., 1986; Leung et al., 1989; Villanueva et al., 1990; Midy et al., 1994; Jones et al., 1995; Decker et al., 1995; Wang et al., 1997]. The comparison between these studies is difficult because they reported the use of cells from different species (Rodent, Bovine, Human), from different origin [endothelial cells from microcirculation (skin or liver) or macrocirculation (aorta, umbilical vein)], and

Fig. 3. Proliferative effect of conditioned medium by one type of cell on the other cell-type. Cell proliferation was assessed by using [^{3}H]-thymidine incorporation in DNA during 15 days, as described in Materials and Methods. **a:** 50% (v/v) HBMSC-conditioned medium (—□—) induces a proliferative effect on HUVEC, in a comparable extent to that of VEGF (10 ng/mL;) (—○—) and in comparison with HUVEC control (—●—). **b**: 50% (v/v) HUVEC-conditioned medium (—○—) induces a proliferative effect on HBMSC in comparison with HBMSC control (—□—) or HBMSC exposed to VEGF at 10 ng/ml (—□—).



	% of HBMSC control		+ VEGF 10 ng/ml (% of same culture model control)		
	Direct co-culture with HUVEC	Indirect co-culture with HUVEC	HBMSC alone	Direct co-culture with HUVEC	Indirect co-culture with HUVEC
Day 3	+15.2% ±24.4%	-48.5% ±35.3% [*]	-12.8% ± 31.5%	+9.8% ±18.1%	-36.9% ±59.9%
Day 6	+144.5% ±18.0% [*]	-44.9% ±13.6% [*]	-41.2% ±0.6% [*]	-5.8% ±21.0%	-20.3% ± 48.2%

Fig. 4. Alkaline phosphatase activity of HBMSC cultured. Enzymatic activity was quantified according to Materials and Methods. Results are expressed in nM of Pi cleaved in 30 min per µg of proteins. Data are expressed as mean ± standard deviation (n = 4; $*P < 0.05$).

osteoblast cells from calvaria or trabecular bone. Moreover, the culture medium or treatment conditions are different (medium supplemented with ascorbic acid, β-glycerophosphate, VEGF, $1,25(OH)_2D_3$, hydrocortisone, EGF, mitomicyne C, . . .). Moreover, cells were used either at confluence, or in exponential proliferation phase, with or without cell replication inhibitors, and FCS. Finally, cell cultures were performed in several culture models (with or without direct contact, with conditioned medium, in vitro or in vivo with a porous cell chamber). Then, the conclusions of these papers lead to different interactions between endothelial and osteoblast cells. Wang et al. [1997] reported that osteoblastic cells increase proliferation of endothelial cells, and endothelial cells increase proliferation and Al-P activity of osteoblastic cells in all of their culture models (with or without direct contact). Jones et al. [1995] have demonstrated that osteoblastic cells increase proliferation of endothelial cells, and endothelial cells increase proliferation, but decrease Al-P activity, osteocalcin, and mineralised nodule formation of osteoblastic cells in all of their culture models (with or without direct contact). Gunther et al. [1986] have shown that conditioned medium by endothelial cells increase proliferation of osteoblastic cells without increase of collagen synthesis. The in vivo studies of Villanueva et al. [1990] and that of Decker et al. [1995] lead to the same conclusion: endothelial cells increase bone formation and extracellular matrix mineral-

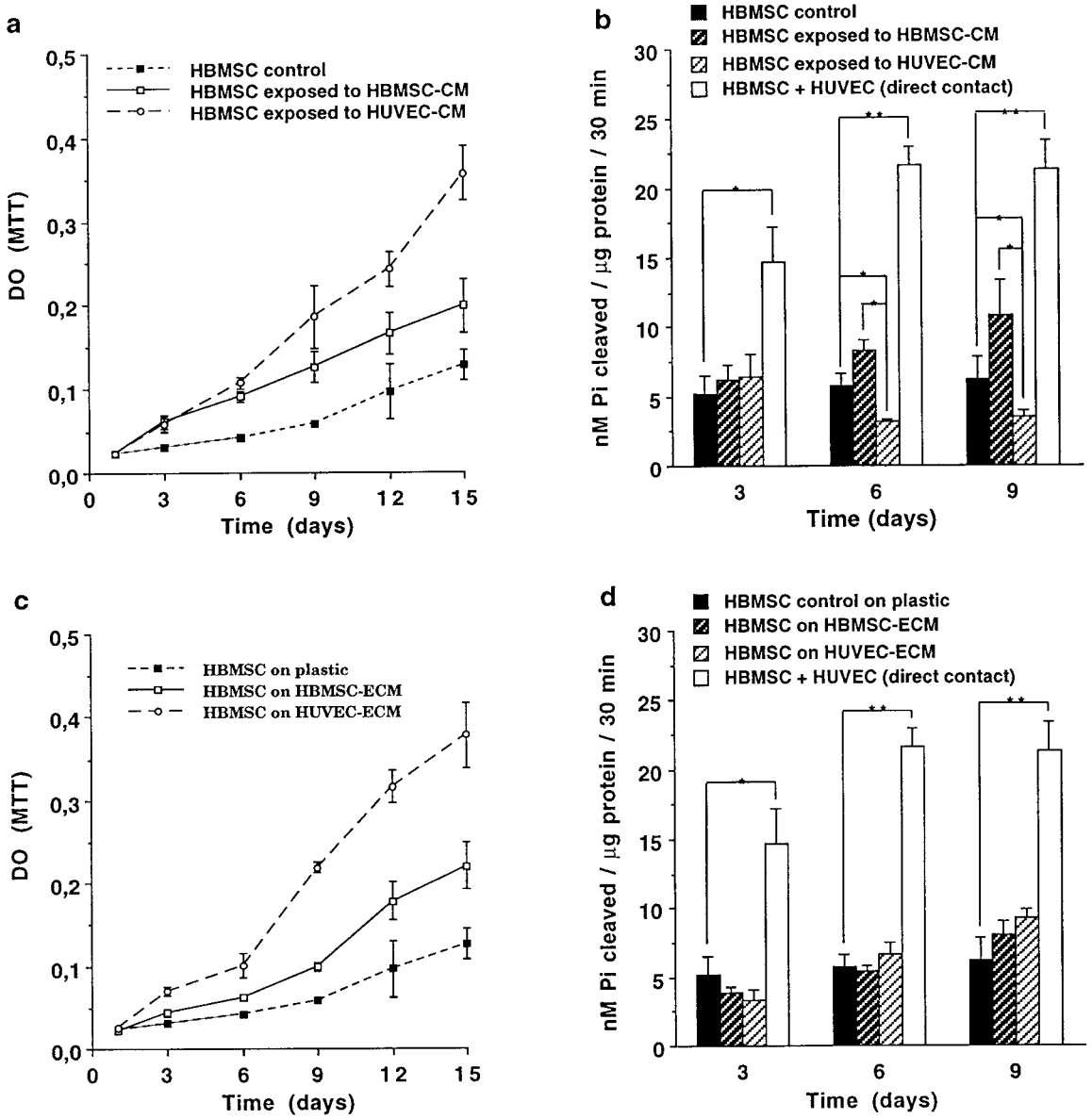
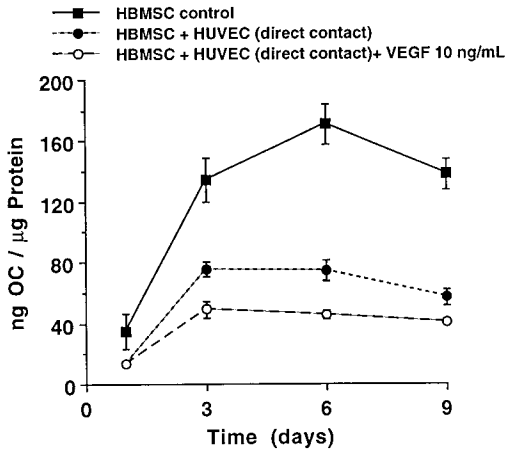


Fig. 5. **a:** Proliferative effect of conditioned medium by HUVEC on HBMSC. Cell proliferation was assessed by using MTT test during 15 days, as described in Materials and Methods. 50% (v/v) HUVEC-conditioned medium (—○—) induces a proliferative effect on HBMSC in comparaison with HBMSC control (—■—) or HBMSC exposed to their own conditioned medium (—□—). **b**: Alkaline phosphatase activity of HBMSC cultured alone (■), or in presence of HUVEC-CM (▨) or their own conditioned medium (▨), and co-cultured with HUVEC with direct contact (□) for 3, 6, and 9 days. Data are expressed as mean ± standard deviation (n = 4) of nM of Pi cleaved in 30 min by µg of proteins. (*$P < 0.05$, **$P < 0.04$). **c**: Proliferative effect of conditioned medium by one type of cell on HBMSC. Cell proliferation was assessed by using MTT test during 15 days, as described in Materials and Methods. HUVEC-extracellular matrix (—○—) induces a proliferative effect on HBMSC in comparaison with HBMSC cultured on plastic control (—■—) or HBMSC cultured onto their own extracellular matrix (—565—). **d**: Alkaline phosphatase activity of HBMSC cultured alone on plastic dishes (■), or cultured onto HUVEC-ECM (▨) or their own extracellular matrix (▨), and co-cultured with HUVEC with direct contact (□) for 3, 6, and 9 days. Data are expressed as mean ± standard deviation (n = 4) of nM of Pi cleaved in 30 min by µg of proteins. *$P < 0.05$, **$P < 0.04$).

isation associated with an increase of neovascularization.

A promising approach to the future study of the interaction between bone cells and endothelial cells would be to develop methods for the culture of endothelial cells from sites of active bone formation or bone marrow, so that, the physiology of these endothelial cells may be studied and their effect on bone cells compared with the effects seen in this study with HUVEC



Osteocalcin (% of HBMSC) control)

	Co-culture control	Co-culture + VEGF 10 ng/ml
Day 3	-59.0% ± 9.7%	-85.0% ± 9.5%
Day 6	-96.3% ± 6.2%	-125.2% ± 10.1%
Day 9	-81.1% ± 5.2%	-97.4% ± 8.1%

Fig. 6. Osteocalcin synthesis in HBMSC cultured. Osteocalcin synthesis was quantified by radioimmunoassay using the OSTK.PR® kit and results are expressed as mean ± standard deviation (n = 6) of ng of osteocalcin by µg of proteins.

[Garlanda et al., 1997; Jackson et al., 1997; Craig et al., 1998].

Several methods have been established for the study of cellular interactions in vitro, and they differ in complexity and in potential for experimental manipulation. Culture of different cell types together in direct contact is likely to support maximum interaction of cells but fails to isolate the response of the cell type of interest. We developed here different cell culture models between these two differentiated human primary cells in order to study the cell interaction between HUVEC and HBMSC and the function of VEGF in these intercommunications. The present study used four in vitro methods: 1) bone cells were cultured in direct contact with endothelial cells; 2) bone cells were grown on the same plate in medium shared with endothelial cells, but contact between the two cell types was prevented by the use of porous dish inserts; 3) media were conditioned by one type of cell and added to the other cell type; and 4) bone cells were grown onto endothelial extracellular matrix.

We demonstrate that only the direct contact between HBMSC and HUVEC enhances the osteoblastic phenotypic markers, i.e., alkaline phosphatase activity. Neither HUVEC-CM, nor indirect contact with HUVEC, nor extracted matrix from HUVEC exert the same effect on bone cell differentiation. In the opposite, while Al-P activity was increased by the direct contact of HUVEC with HBMSC, these human bone primary cells, when they were co-cultured with HUVEC without a direct contact, exhibit a decrease in the enzymatic activity which occurred after 3 days of co-culture when compared to bone cells cultured alone. These results are in accordance with those obtained by Jones et al. [1995].

Addition of VEGF to all of these culture systems did not act onto the bone cell differentiation. However, both HBMSC and HUVEC express the corresponding receptors to VEGF, the flt-1 and KDR mRNA, which would have supported, respectively, their response to VEGF. VEGF even at 40 ng/ml did not increase the HUVEC-induced alkaline phosphatase activity, or did not restablish its decrease observed in co-culture of HUVEC with HBMSC without direct contact.

Since the endothelium, which is of mesenchymal origin, is in close contact with the bone marrow, we had supposed that the communication between endothelial cells and stromal cells is highly complex. VEGF secreted spontaneously by HBMSC and stimulated by calcitropic hormones ($1,25\text{-}(OH)_2D_3$ at 10-8 M, 24 h) may activate HUVEC via the binding of VEGF to their receptors, and then cause an activation of HBMSC themselves as described by Wang et al. [1997]. On the contrary, in that paper, VEGF did not take part in an activation loop between endothelial cells and stromal cells differentiation.

However, while VEGF stimulated the proliferation of HUVEC, it did not exert any effect on HBMSC proliferation. In the same manner, VEGF secreted by HBMSC and present in the corresponding conditioned medium (HBMSC-CM), alone or probably associated with other cytokines or growth factors produced by these cells, are able to increase the cell proliferation of HUVEC. Reciprocally, HUVEC-CM increased the cell proliferation of HBMSC, while VEGF did not show the effect.

If VEGF was partly considered as one of the most angiogenic factors produced by osteoblasts, additional angiogenic factors were also thought to play a role such as FGF or IGF-I which are also produced by osteoblasts and which could activate the proliferation of endothelial cells. In response to these stimuli, vascular endothelial cells may synthesize and secrete soluble mediators. In 1978, it was first reported that endothelial cells produce cytokines or growth factors with cell growth-promoting activity, of which the latter have mitogenic effects upon several connective cells, such as smooth muscle cells, and bone marrow fibroblasts. Moreover, cell-growth promoting activity of conditioned medium from bovine aortic endothelial cells have been also reported and contains a potent mitogen to which bone cells respond [Bianchi et al., 1985; Guenther et al., 1986]. Among these soluble mediators, growth factors and cytokines such as FGF, IGF-I, IL-1, IL-6, CSFs (colony stimulating factors) of the G, GM, and M subtypes, arachidonic acid metabolites like prostacyclin, small peptides like endothelin-1, gaseous and free radical messengers like nitric oxide and superoxide anions [Collin-Osdoby, 1994], could be candidates to induce cell proliferation and/or cell differentiation. These regulatory molecules have been found in other studies to control the recruitment, proliferation, differentiation, function, and/or survival of various cells including osteoblasts and bone-resorbing osteoclasts [Matusuno et al., 1998]. However, the cytokines or growth factors produced by HUVEC present in the conditioned medium enhance cell proliferation of HBMSC, but do not increase their cell differentiation even in the presence of VEGF.

In our direct co-culture model the effect of HUVEC on HBMSC differentiation was ambivalent: co-culture induced an increase in Al-P activity in HBMSC, but a decrease in osteocalcin synthesis. A variable effect of a stimulus on osteoblastic marker expression could be explained by a sequence of gene expression according to their differentiation state [Stein et al., 1990; Katagiri et al., 1990; Collin et al., 1992; Vilamitjana-Amédée et al., 1993; Harris et al., 1994; Gori et al., 1999]. This has been particularly studied on cultures of rat calvaria-enriched bone. Osteocalcin expression is predominant in well-differentiated cells, while alkaline phosphatase is early expressed in the bone cell culture. The differentiation stage of these cells is an important condition for obtaining an appropriate response to stimulation by growth factors or others stimuli [Stein et al., 1990]. Our results suggest that the direct contact of HUVEC with HBMSC acts early in the cell differentiation process, at a stage of culture in which the cells are still able to proliferate. Endothelial cells themselves could be considered in this cell culture model as "osteoinductive" mediators instead of acting in bone cell maturation.

The communication pathways between bone cells and endothelial cells are also supported by their common response to local or systemic factors such as parathyroid hormone (PTH), progesterone, estrogen (E2), IGF-I and IGF-II, basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) [Cullinan-Bove et al., 1993]. Dexamethasone has been found to disrupt the normal control of microcapillary invasion into the growth plate cartilage zone of rabbits [Brown et al., 1990]. IL-1, a pleiotropic mediator which acts as a potent bone-resorbing agent, activates endothelial cells via autocrine/paracrine pathways to elicit the production of cell surface adhesion proteins, prostacyclin (PGI2), plasminogen activator inhibitor (PAI), and CSFs [Collin-Osdoby, 1994]. TNF (Tumor Necrosis Factor) and IL-1 independently induce the synthesis of another bone-resorbing factor and an angiogenic cytokine (IL-6) in endothelial cells [Mantovani et al., 1990, 1998]. Moreover, proliferation of HUVEC is stimulated by the bone-resorptive inhibitor and vasoactive neuropeptide calcitonin gene-related peptide (CGRP) with an accompanying increase in cAMP [Haegerstrand et al., 1990]. Finally, many of the same systemic and local mediators that are known to affect osteoblasts, osteoclasts, and bone remodeling cells arise from and/or activate endothelial cells.

This leads to the hypothesis that bone vascular endothelial cells may be essential contributing members of the intricate communication pathways operative in bone that link various cell types via diffusible signalling molecules, and perhaps by cell contact-mediated mechanisms as well.

Our results support this last hypothesis. In conclusion, in contrast to previous results obtained on other osteoblast cell lines which could exhibit phenotypic and physiologic differences [Bellows et al., 1998], the relationship

between human primary endothelial cells and human primary osteoprogenitor cells which leads to differentiation, is not modulated by VEGF treatment, but absolutely required a direct contact. This cell-cell interaction could involved either membrane proteins like adhesion molecules or intercellular "gap" junction that mediate intercellular exchange of regulatory ions, small molecules including second messengers (cAMP, Ca^{2+}) [Lecanda et al., 1998].

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