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Role of Vascular Endothelial Growth Factor in the Communication Between Human Osteoprogenitors and Endothelial Cells

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ABSTRACT

Proper bone remodeling requires an active process of angiogenesis which in turn supplies the necessary growth factors and stem cells. This tissue cooperation suggests a cross-talk between osteoblasts and endothelial cells. This work aims to identify the role of paracrine communication through vascular endothelial growth factor (VEGF) in co-culture between osteoblastic and endothelial cells. Through a well defined direct contact co-culture model between human osteoprogenitors (HOPs) and human umbilical vein endothelial cells (HUVECs), we observed that HUVECs were able to migrate along HOPs, inducing the formation of specific tubular-like structures. $VEGF_{165}$ gene expression was detected in the HOPs, was up-regulated in the co-cultured HOPs and both Flt-1 and KDR gene expression increased in co-cultured HUVECs. However, the cell rearrangement observed in co-culture was promoted by a combination of soluble chemoattractive factors and not by $VEGF_{165}$ alone. Despite having no observable effect on endothelial cell tubular-like formation, VEGF appeared to have a crucial role in osteoblastic differentiation since the inhibition of its receptors reduced the co-culture-stimulated osteoblastic phenotype. This co-culture system appears to enhance both primary angiogenesis events and osteoblastic differentiation, thus allowing for the development of new strategies in vascularized bone tissue engineering. J. Cell. Biochem. 106: 390–398, 2009.

KEY WORDS: HUMAN STEM CELLS; ENDOTHELIAL CELLS; VEGF; CO-CULTURE; MIGRATION; OSTEOGENESIS

Bone remodeling consists of a balance of bone resorption and formation that occurs throughout life, not only in skeleton development but also in bone hemostasis and fracture healing. Bone formation is coordinated by osteoblasts and bone degradation is guaranteed by osteoclast activity. The formation and development of an active microvasculature is an essential stage for bone remodeling and fracture healing [Gerber and Ferrara, 2000; Carano and Filvaroff, 2003]. Angiogenesis, the formation of new blood vessels from pre-existing ones, allows for the restoration of blood flow to the fracture site and can modulate bone formation by the production of growth factors able to regulate osteoblastic activity, recruitment of stem cells and their orientation to the osteoblastic lineage [Fiedler et al., 2005]. Angiogenesis is modulated by a tight balance between pro- and anti-angiogenic factors and involves a cascade of events in which the migration of endothelial cells (ECs) is an initial event [Lamalice et al., 2007]. Chemotaxis refers to cell migration toward a gradient of soluble chemoattractants, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietins [Cross and Claesson-Welsh, 2001]. VEGF is a major promoter of both physiological and pathological angiogenesis that belongs to a family of homodimeric proteins consisting of six members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor [Cross et al., 2003; Ferrara et al., 2003]. VEGF-A exists in five different isoforms: $VEGF_{145}$, $VEGF_{189}$ and $VEGF_{206}$ which are able to bind to the extracellular matrix (ECM) through heparin, $VEGF_{121}$ which is soluble and $VEGF_{165}$, which is the most abundant form and can be both soluble and bound to the ECM [Neufeld et al., 1999]. These isoforms are able to activate two different tyrosine kinase receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) [Cross et al., 2003]. Their activation induces their phosphorylation and leads to the transduction of different signals promoting cell migration or osteoblastic phenotype

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induction by enhancing angiogenesis [Mayr-Wohlfart et al., 2002; Street et al., 2002; Clarkin et al., 2007]. Moreover, Bouletreau et al. [2002] demonstrated that VEGF is able to stimulate bone morphogenic protein (BMP-2) production, which then enhances fracture healing [Bouletreau et al., 2002].

To better understand the influence of angiogenesis on bone remodeling, we have developed a co-culture system in which human osteoprogenitors (HOPs) and ECs are in direct contact. These two types of cells can communicate through autocrine/paracrine soluble factors, cell-to-cell contact or through their interaction with the ECM. In previous studies, we showed that osteoblastic differentiation was regulated by cell-to-cell communication through gap junction and connexin43 activity [Villars et al., 2000, 2002; Guillotin et al., 2004]. Villars et al. [2000] demonstrated that exogenous VEGF had a positive effect on cell proliferation in this co-culture system.

This work aims to identify the paracrine role of VEGF in the communication between osteoblastic and endothelial cells. The cell-to-cell contact between HOPs and ECs is associated with the formation of specific cellular tubular-like networks. Our results showed that the cell rearrangement occurring in this co-culture was due to the migration of ECs through their attraction to the soluble factors secreted only in the co-culture conditions. Despite $VEGF_{165}$ and its receptors were up-regulated in the co-culture, this factor alone did not affect EC migration in isolated cultures. On the other hand, VEGF appeared to have a crucial role in co-culture-stimulated osteoblastic differentiation since inhibition of Flt-1 and KDR abolished ALP and Coll-I increased gene expression.

MATERIALS AND METHODS

CELL CULTURE

After informed consent was obtained, human bone marrow was obtained by aspiration from the femoral diaphysis or iliac bone from patients undergoing hip prosthesis surgery after trauma. Cells were separated into a single suspension by sequential passage through syringes fitted with 16-, 18-, and 21-gauge needles. The cells were then counted and plated at 10^5 cells/cm^2 in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), and incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C. HOPs were obtained after treatment with dexamethasone at 10^{-8} M for the first 2 weeks of culture to induce osteoblastic differentiation of adherent cells [Villars et al., 2000]. They express Cbfa1/Runx2, ALP and type I collagen but do not express osteocalcin which is a late marker.

ECs were isolated from human umbilical cord vein as described by Bordenave et al. [1993] according to the procedure of Jaffe [1980]. Human umbilical vein endothelial cells (HUVECs) were cultured in IMDM supplemented with 20% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.4% (v/v) Endothelial Cell Growth Supplement/Heparin kit (PromoCell).

HOPs arising from the second subculture were co-cultured with HUVECs in IMDM containing 10% (v/v) FBS without growth factor. HOPs were co-cultured at a 1:2 ratio with HUVECs for 6–48 h, in the same phase seeding, while HOP and HUVEC isolated cultures were used as controls. Co-culture of HOPs and human gingival fibroblasts (HGFs) under the same conditions was used as a co-culture control.

CELL SEPARATION AFTER CO-CULTURE HOPS/HUVECS

To evaluate the specific effect on each cell type after co-culture, we used magnetic beads coupled with an antibody against CD31, a specific protein of ECs, which is able to separate HUVECs from HOPs [Guillotin et al., 2008]. Briefly, cells in co-culture were harvested by trypsin treatment and incubated with 10 CD31-labeled magnetic beads/HUVEC for 30 min at 4°C under gentle stirring. After five washings with PBS 1× containing 0.1% (w/v) bovine serum albumin (BSA) on a magnet, the supernatant fraction containing the co-cultured HOPs (co-HOPs) was then separated from the bead fraction containing the co-cultured HUVECs (co-HUVECs).

CELL IDENTIFICATION BY CYTOCHEMICAL AND IMMUNOSTAINING

Intracellular alkaline phosphatase (ALP) activity was detected in HOPs according to the technical support of the SIGMA diagnostic kit (85L-2). Immunostaining was performed in order to detect von Willebrand factor (vWf) in HUVECs raised to confluence. Isolated cultures of HOPs, HUVECs or co-cultures were fixed in 4% (w/v) paraformaldehyde for 20 min at 4°C and permeabilized in methanol 100% for 5 min at room temperature. Fixed cells were incubated for 30 min in HBSS containing 1% (w/v) BSA, then for 1 h at 37°C with primary antibody anti-vWf (rabbit anti-human, DAKO). Subsequently, cells were washed in HBSS and incubated with Alexa-488-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h at 37°C. At least five independent experiments were performed and cultures were examined with a fluorescence microscope (Nikon) equipped with the appropriate epifluorescence filter sets. Controls were performed without primary antibody.

TIME LAPSE VIDEOMICROSCOPY

Before the time lapse videomicroscopy experiment, HUVECs were incubated with 0.2 μg/ml of Dil-Ac-LDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein, Harbor Bioproducts, USA) in culture medium for a minimum of 4 h. Then, HUVECs stained with Dil-Ac-LDL and HOPs were seeded onto a glass slide in a specific chamber corresponding to the microscope (Leica, TCS SP5) and incubated at 37°C in a 5% CO_2 humid atmosphere with IMDM-10% FBS for 1 h in order to obtain adherent cells. The chamber was settled on a thermostable plate on the microscope which allows for a constant temperature of 37°C in the chamber. The microscope was programmed to take a picture every 10 min for 48 h in DIC and fluorescence to detect HUVECs. The reconstruction of the resulting movie was made with the LAS-AF (Leica Advanced Suite-Advanced Fluorescence) software. Three independent experiments were conducted.

SCANNING ELECTRON MICROSCOPY (SEM)

SEM was carried out in order to study cell morphology and organization on the HOPs/HUVECs co-cultures in direct contact. Samples were fixed by immersion in a 2.5% (v/v) glutaraldehyde in a cacodylate buffer (0.1 M, pH 7.4). Samples were washed twice with cacodylate buffer, then dehydrated by successive immersions in ethanol solutions (from 30% to 100%), then incubated in

hexamethyldisilazane (HMDS) for 30 s. Samples were coated by a gold layer and SEM analyses were carried out at 8 and 10 keV using a Hitachi (Tokyo, Japan) S-2500. SEM analysis was performed once and several fields of the cultures were observed.

CONDITIONED MEDIUM (CM)

HOPs were co-cultured with HUVECs for 6, 24, and 48 h as previously described. Supernatants obtained from these co-cultures were collected at the aforementioned time points, centrifuged for 5 min at 800*g* to eliminate cells and then frozen at −80°C until subsequent use. Isolated cultures of HOPs and HUVECs cultured for 24 h were incubated with these different CMs from co-cultures diluted in fresh IMDM-10% FBS at a 1:1 ratio. Control experiments were performed using either IMDM-10% FBS, or CMs arising from HOPs cultures at 6, 24, and 48 h. As for CMs from co-culture, these were mixed with IMDM-10% FBS at the same ratio (1:1) and incubated with HUVECs. At least four experiments were carried out for each condition.

SEMI-QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (Q-PCR)

Total RNA were extracted using the RNeasy™ Total RNA kit (QIAGEN) and 1 μg was used as template for single-strand cDNA synthesis with the Superscript pre-amplification system (Gibco) in a 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 at 42°C for 50 min, the reaction was stopped at 70°C for 15 min.

Five microliters of cDNA diluted at a 1:80 ratio were loaded in a 96-well plate. SYBR-Green Supermix® (2X iQ™, BioRad) was added to the final concentration of 50 mM KCl, 20 mM Tris–HCl pH 8.4, 0.2 mM of each dNTP, 25 units/ml iTaq™ DNA polymerase, 3 mM $MgCl_2$, SYBR™ Green I, 10 nM fluorescein, and stabilized in sterile distilled water. Primers of P0, $VEGF_{165}$, Flt-1, KDR, ALP and type-I collagen (Coll-I) were used at the final concentration of 200 nM; their sequences are summarized in Table I. Data were analyzed with the iCycler IQ™ software and compared by the ΔΔCt method. Each Q-PCR was performed in triplicate for PCR yield validation. Results were expressed relatively to gene expression level of HOPs after 6 h of culture, which was standardized to 1. Three independent experiments were performed and significance was calculated by the Student test ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

$VEGF_{165}$ DETECTION USING ELISA ASSAY

$VEGF_{165}$ released by each type of cell was quantified using a specific ELISA assay (Quantikine, R&D Systems). Supernatants from three independent cultures of HOPs, HUVECs and co-cultures were collected and submitted to the immunoenzymatic detection, according to the protocol described by the supplier. Results are expressed in pg of $VEGF_{165}$ per μg of total protein. IMDM-10% FBS was used as control.

VEGFR-1 AND VEGFR-2 NEUTRALIZING ANTIBODIES

Co-culture of HOPs and HUVECs was performed as described above with culture medium containing neutralizing antibodies or not. Specific neutralizing antibodies against human VEGFR-1 or VEGFR-2 from R&D systems were used at 10 and 0.5 μg/ml respectively. Cell organization was observed and cells were harvested after 6 and 48 h of culture and treated for Q-PCR analyses. These investigations were conducted on three independent experiments.

RESULTS

ENDOTHELIAL CELL MIGRATION IS INDUCED BY SOLUBLE FACTORS SECRETED ONLY BY CO-CULTURE HOPS/HUVECS

A cell rearrangement of HOPs and HUVECs was observed when they were co-cultured in direct contact, whereas no rearrangement was found when cultured separately (HOPs or HUVECs, Fig. 1A). Between 6 and 48 h in isolated culture, HUVECs formed a typical cobblestone confluent monolayer and HOPs formed a homogeneous multilayer. However, direct contact co-cultures of these two cell types were clearly organized in specific tubular-like network after 48 h (Fig. 1A). Moreover, this specific network was not observed when HOPs were co-cultured with HGFs. Staining of each cell type with vWF and ALP confirmed that co-cultured HOPs and HUVECs were organized in cellular multilayers (Fig. 1B).

HOPs/HUVECs in co-culture were monitored for 48 h using time lapse videomicroscopy, permitting us to follow cell organization within the same culture area. To differentiate HOPs from HUVECs in co-culture, HUVECs were preliminarily labeled with Dil-Ac-LDL before cell seeding with HOPs. We observed that ECs were able to

TABLE I. Primer Sequences Used in Q-PCR

Transcript	GenBank	Primers sequences	TM (°C)
P0	BC015690	Forward 5′-ATGCCCAGGGAAGACAGGGC-3′ Reverse 5′-CCATCAGCACCACAGCCTTC-3′	65
$VEGF_{165}$	AB021221	Forward 5′-TATGCGGATCAAACCTCACCA-3′ Reverse 5′-CACAGGGATTTTTCTTGTCTTGCT-3′	58
Flt-1	AF063657	Forward 5′-CAGGCCCAGTTTCTGCCATT-3′ Reverse 5′-AAGGTCGAGTCGCACCAGCAT-3′	65
KDR	AF063658	Forward 5′-CCAGCAAAAGCAGGGAGTCTGT-3′ Reverse 5′-ACAGACACAGTAGCCTCACTATAGG-3′	62
ALP	BC021289	Forward 5′-AGC CCT TCA CTG CCA TCC TGT-3′ Reverse 5′-ATT CTC TCG TTC ACC GCC CAC-3′	64
Coll-I	NM000089	Forward 5′-GGA ATG AGG AGA CTG GCA ACC-3′ Reverse 5′-TCA GCA CCA CCG ATG TCC AAA-3′	65

migrate and seemed to move along HOPs and their corresponding filopodia (Fig. 1C). SEM images confirmed that ECs used osteoblastic cells as a ''matrix'' upon which they could spread (Fig. 1D).

We then focused on the possible autocrine/paracrine role of soluble factors produced by the co-culture that could promote this specific cell organization. HUVECs or HOPs were cultured in the presence of conditioned medium (CM) from co-cultured HOPs/HUVECs after 6, 24, or 48 h. The incubation of HOPs with the CMs from co-culture did not interfere with cell organization, whatever the time point of the CM used (6, 24, 48 h, Fig. 2B–D). However, HUVECs incubated with CM from 24 h of co-culture (Fig. 2G) exhibited a cell rearrangement similar to the tubular-like network observed in co-culture (Fig. 1A). The same cell organization was also observed when HUVECs were cultured in presence of CM from 48 h of co-culture (Fig. 2H). Finally, we demonstrated that the incubation of HUVECs with CM from HOP culture alone had no effect on cell organization (Fig. 2J–L).

$VEGF_{165}$ IS SECRETED BY HOPS AND COULD ACTIVATE VEGF RECEPTORS IN CO-CULTURED HUVECS

We wondered whether VEGF could be one of the secreted factors present in the co-culture supernatant contributing to cell migration. First, we measured the level of $VEGF_{165}$ gene expression in each cell type by Q-PCR. Total RNA were extracted from HOPs, HUVECs, co-cultured HOPs and co-cultured HUVECs, obtained after co-culture and subsequent cell separation using immunomagnetic beads. As shown in Figure 3A, HUVECs did not express $VEGF_{165}$ either in isolated cultures or when co-cultured with HOPs. Moreover, $VEGF_{165}$ mRNA expression was detected in HOPs isolated culture and was found to increase with time. Interestingly, cell contact between HOPs and HUVECs up-regulated $VEGF_{165}$ gene expression in co-cultured HOPs (Fig. 3A).

The amount of $VEGF_{165}$ protein secreted in the corresponding supernatants of HOPs, HUVECs and their co-culture was also quantified. Surprisingly, ELISA assay revealed that $VEGF_{165}$ was detected only in the supernatant of osteoblastic cells, but not in HUVECs or in co-culture HOPs/HUVECs (Fig. 3B). The quantification of $VEGF_{165}$ secreted by isolated cultures of HOPs, HGFs and co-cultures of HOPs/HGFs was also performed as control. $VEGF_{165}$ was found to be present in the supernatant of these three different types of cultures (Fig. 3B) including that of the HOPs/HGFs. To further investigate the role of $VEGF_{165}$ signaling in co-cultured HOPs/HUVECs, we focused our efforts on the analysis of mRNA expression of Flt-1 and KDR in the different culture models. Gene expression of both receptors remained constant with time in HOPs and HUVECs alone and in co-cultured HOPs (Fig. 4). However, the expressions of Flt-1 and KDR were significantly up-regulated in co-cultured HUVECs compared to HUVECs alone (Fig. 4).

ROLE OF VEGF IN OSTEOBLASTIC DIFFERENTIATION

To determine the specific role of VEGF in our co-culture system, HOPs, HUVECs and HOPs/HUVECs in co-culture were incubated with neutralizing antibodies against VEGF receptor 1 and 2. The inhibition of Flt-1 or KDR did not block the cell rearrangement in co-culture (data not shown). Furthermore, the addition of exogenous $VEGF_{165}$ (20 ng/ml) in HUVECs culture medium did not stimulate the formation of tubular-like structures as observed in co-culture (data not shown). On the other hand, the osteoblastic markers ALP and Coll-I were up-regulated in co-culture conditions (Fig. 5), an effect that was abolished by incubation with a specific Flt-1 neutralizing antibody. Moreover, neutralization of KDR led to a down-regulation of both osteoblastic markers in co-cultured HOPs/HUVECs when compared to untreated cells (Fig. 5).

DISCUSSION

Under our previously described conditions, the association of osteoprogenitors arising from bone marrow stromal cells in direct contact with HUVECs led to stimulation of osteoblastic differentiation [Villars et al., 2000, 2002; Guillotin et al., 2004]. In this work, we demonstrated that HOPs and HUVECs in direct co-culture also led to a cell rearrangement giving rise to tubular-like networks. This cellular network was also observed when HOPs were in co-culture with other types of ECs, such as endothelial precursor cells isolated from blood cord (EPCs) and endothelial cells from human saphenous vein (HSV) [Xin et al., 2001; Guillotin et al., 2004; Unger et al., 2007]. However, direct co-culture of HOPs and HGFs did not interfere with cell organization, thus in direct opposition to the data presented by Sorrell et al. [2008]. Under our conditions, this cellular network formation appears to be specific of ECs.

In this article, we focused on cell communication through paracrine/autocrine factors, which could exert a chemoattractive effect on ECs. We analyzed the effect of factors secreted in the culture medium of HOPs or co-cultured HOPs/HUVECs on cell migration. We showed that only the soluble factors secreted in the co-culture conditions induced EC migration and tubular-like network formation. Direct contact between HOPs and HUVECs thus appears necessary for the secretion of chemoattractive factors. Possible candidates for the promotion of EC migration include epidermal growth factor (EGF), bFGF, transforming growth factor β-1 (TGFβ-1), hepatocyte growth factor (HGF) or VEGF [Rousseau et al., 2000; Xin et al., 2001; Lebrin et al., 2005].

We hypothesized that cell-to-cell contact through gap or adherens junctions could be involved in the secretion of soluble factors, including VEGF, which could influence angiogenesis [Carmeliet and Collen, 2000; Suarez and Ballmer-Hofer, 2001; Walker et al., 2005; Lampugnani et al., 2006]. We already observed cell-to-cell communication establishment between HOPs and HUVECs in co-culture through homotypic and heterotypic gap junctions [Villars et al., 2002; Guillotin et al., 2004] and a regulation of the cadherin signaling pathway (data not shown). Taken together, it seems plausible that these two modes of cell-to-cell communication could participate in the cell migration events in this co-culture system, in combination with the release of soluble factors.

Several studies also suggested that the ECM produced by HOPs plays a fundamental role in the sequestration and therefore secretion of chemotactic factors [Dallas et al., 2002; Ortega et al., 2003]. Direct contact co-culture could then stimulate the release of these chemotactic factors, which could in turn activate specific EC

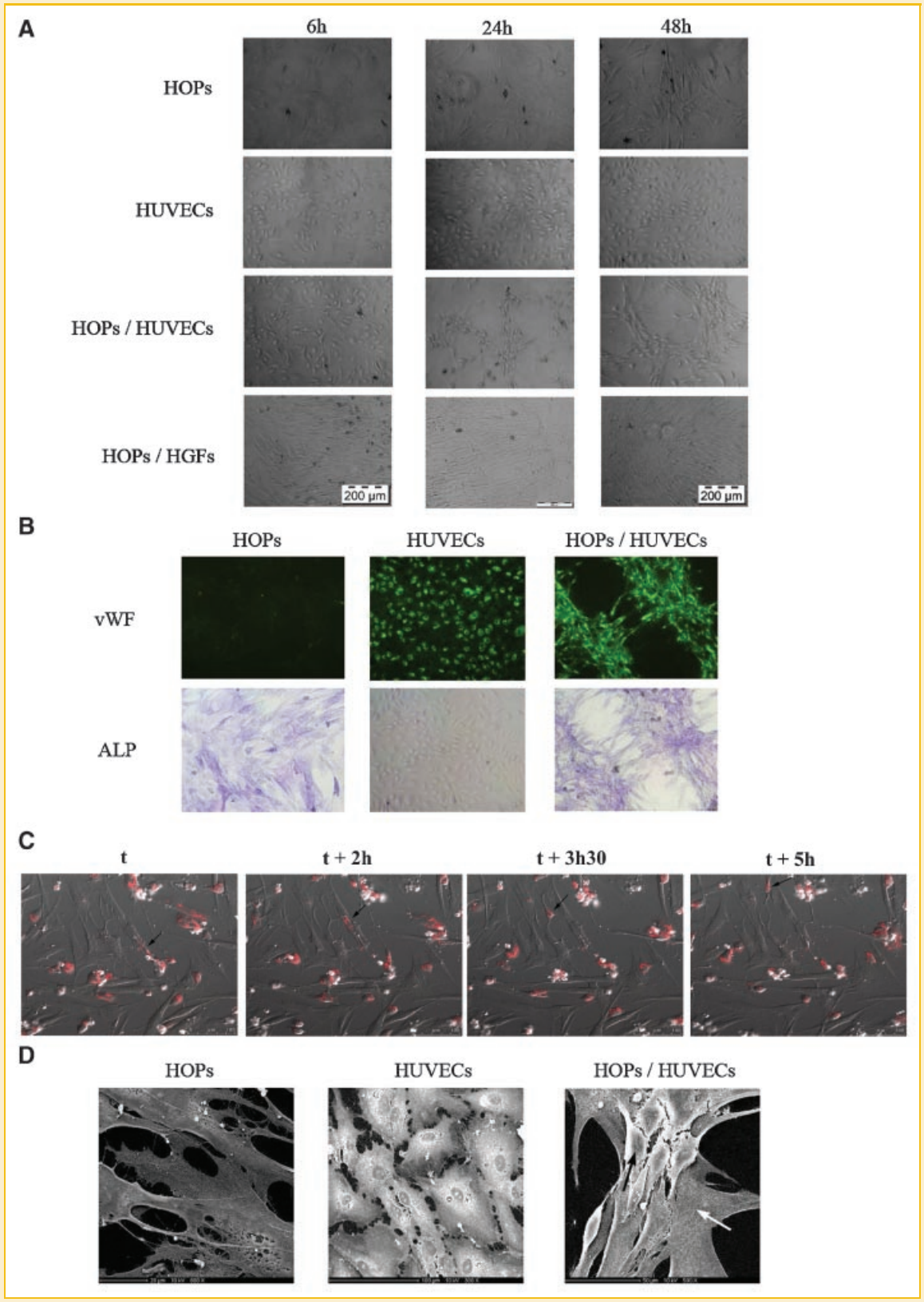
A
6h
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Fig. 1.

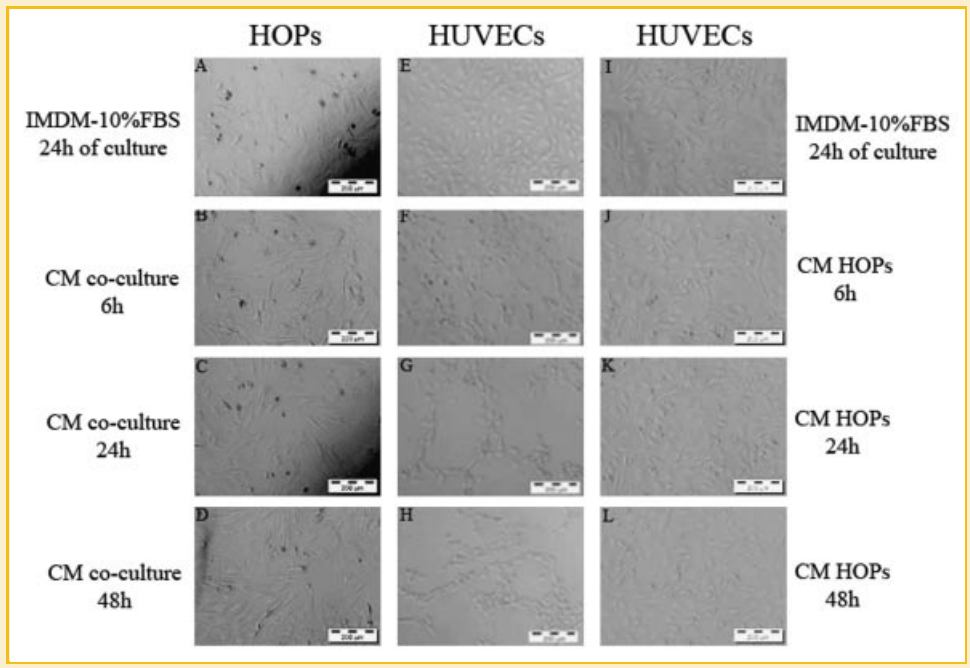

Fig. 2. Effect of released factors on endothelial cell migration A,E,I: HOPs (A) or HUVECs (E,I) were incubated for 24 h with control medium IMDM-10% FBS. B–D: HOPs isolated cultures were incubated for 24 h with conditioned medium (CM) from co-cultures after 6, 24, and 48 h. F–H,J–L: HUVEC isolated cultures were incubated for 24 h with CM from co-cultures 6, 24, and 48 h (F–H) or with CM from HOPs cultures 6, 24, and 48 h (J–L). We noticed that factors secreted only by the co-culture can induce ECs migration and the cell rearrangement similar to the tubular-like networks as observed in co-culture.

function such as cell migration [Wang and Keiser, 1998; Miralem et al., 2001; Sun et al., 2001; Ortega et al., 2003; Lamalice et al., 2007; Sorrell et al., 2008]. Preliminary investigations concerning the study of ECM protein regulation, such as matrix metalloproteinases or their inhibitors, revealed a regulation of these factors and a need for clarification [Guillotin et al., 2008]. Here, SEM and time lapse videomicroscopy analyses confirmed that ECs are able to migrate along HOPs using either their filopodia or this newly synthesized ECM. In addition, ECs migrated and organized themselves into a tubular-like network when in co-culture, as when they were cultured on matrigel (BD Biosciences, data not shown).

Having observed EC migration and tubular-like cellular network formation induced by soluble factors, we asked whether VEGF could be one of these secreted factors. Since $VEGF_{165}$ can be soluble and constitutes the predominant form of VEGF produced by the cells, we decided to quantify its expression. HUVECs proved not to express $VEGF_{165}$ mRNA either in isolated cultures or in co-cultures, a phenomenon already observed in a previous study [Villars et al., 2000]. The $VEGF_{165}$ gene was highly expressed by HOPs when in co-culture but the protein was not detected in the co-culture supernatant. We hypothesized that the protein was either not generated or was bound to the ECM and not available in soluble form. $VEGF_{165}$ protein was present in the supernatant of the control cultures, HOPs, HGFs and co-culture of HOPs/HGFs. We therefore concluded that $VEGF_{165}$ can be synthesized and secreted into the culture medium of the HOPs/HUVECs co-culture. Moreover, after 6, 24, or 48 h, very little or no matrix is synthesized by the cells. $VEGF_{165}$ immunostaining appears to be mainly intracellular (data not shown) but we cannot exclude that one part of the secreted $VEGF_{165}$ is bound to the pericellular ECM in the co-culture system and would be sequestered in the ECM in a longer time period. However, because $VEGF_{165}$ seems to be intracellular, the hypothesis supporting that this protein could be internalized and used for cell signaling in HUVECs is plausible.

Fig. 1. Observation of cell migration by microscopy. A: Cultures of HUVECs, HOPs, HGFs, and co-cultures of HOPs/HUVECs or HOPs/HGFs in direct contact. Each observation was performed after 6, 24, and 48 h of culture. Specific tubular-like networks were formed after 24 h of co-culture HOPs/HUVECs but not when HOPs were co-cultured with HGFs (magnification 100×). B: Detection of the localization of each type of cell by ALP activity and immunostaining of vWf in HOPs, HUVECs and co-cultures after 48 h of culture. The stainings confirm that the cells are organized in a multilayer (magnification 100×). C: Time lapse videomicroscopy proceeded after 20 h of seeding of HOPs co-cultured with HUVECs labeled with Dil-Ac-LDL. Arrows show that ECs are able to migrate along HOPs or their extracellular matrix (magnification 200×). D: SEM analyses of HOPs, HUVECs and co-culture after 48 h of culture. White arrow shows osteoblastic cell and black arrow shows EC spread over HOPs.

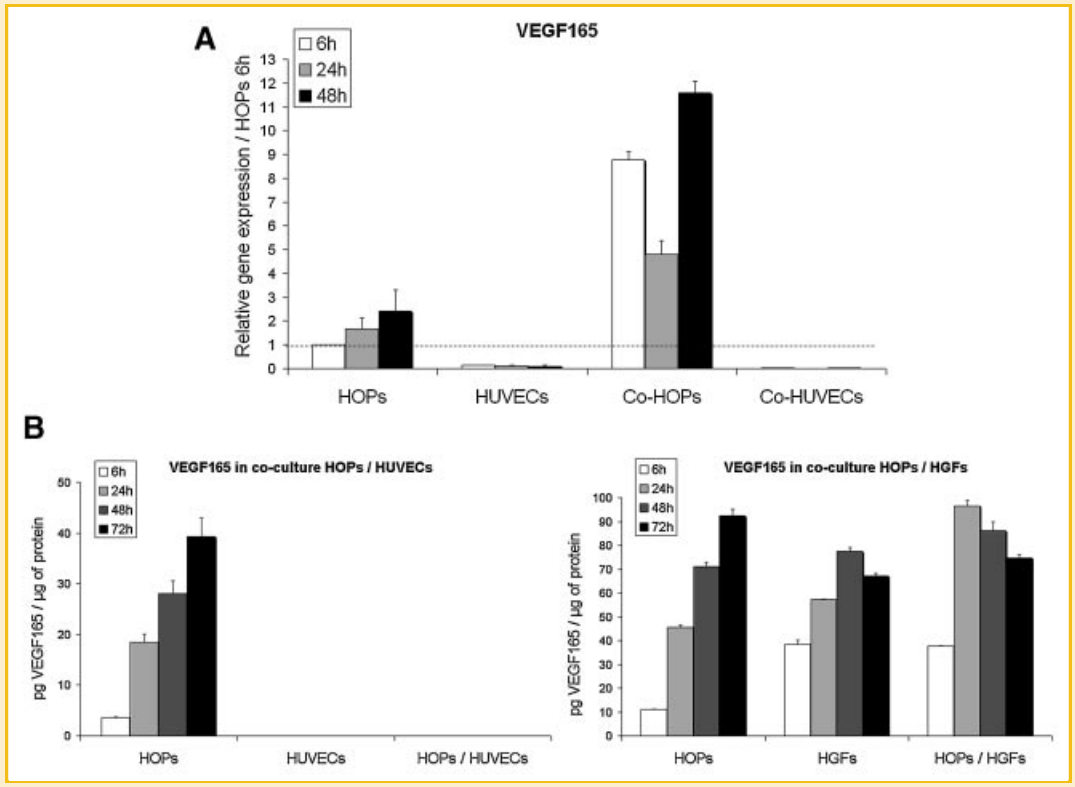

Fig. 3. Regulation of mRNA expression of $VEGF_{165}$ in co-culture. A: mRNA expression of $VEGF_{165}$ quantified by Q-PCR in HOPs and HUVECs isolated cultures and in HOPs and HUVECs arising from cell separation after co-culture (respectively co-HOPs and co-HUVECs). Data were normalized to P0 mRNA expression of each condition and were quantified relative to $VEGF_{165}$ mRNA expression in HOPs after 6 h of culture, which were standardized to 1. $VEGF_{165}$ gene expression increased with time in HOPs and was up-regulated in co-cultured HOPs. B: Quantification of $VEGF_{165}$ protein by ELISA assay in supernatant of HOPs, HUVECs, and co-culture of HOPs/HUVECs after 6, 24, 48, and 72 h. $VEGF_{165}$ was also quantified in control cultures of HOPs, HGFs and co-culture HOPs/HGFs at the same time points. The data are expressed in ng of $VEGF_{165}$ per µg of protein in cell lysate. $VEGF_{165}$ was not detected in the supernatant of co-culture HOPs/HUVECs, while its mRNA was expressed. Co-culture control of HOPs/HGFs confirms that $VEGF_{165}$ can be secreted and is probably immediately used by the cells in the co-culture HOPs/HUVECs.

Moreover, the gene expression of Flt-1 and KDR was significantly up-regulated in co-cultured HUVECs compared to HUVECs alone. Taken together, these data suggested that $VEGF_{165}$ protein would be secreted by co-HOPs and might be immediately used by the cells in co-culture HOPs/HUVECs to activate intracellular cell signaling and ECs migration [Gerhardt et al., 2003; Clarkin et al., 2007].

The data resulting from the addition of exogenous $VEGF_{165}$ showed that this factor alone was not sufficient to induce EC migration occurring in co-cultured HOPs/HUVECs. A combination of soluble factors produced by the two cell types in direct contact is necessary. However, VEGF appeared to be crucial for the enhancement of osteoblastic differentiation in co-culture conditions.

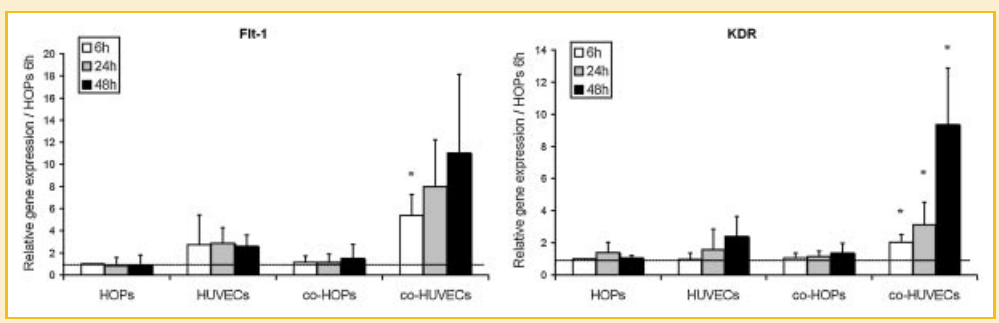

Fig. 4. mRNA expression of Flt-1 and KDR. Gene expression of VEGF receptors, Flt-1 and KDR, in HOPs, HUVECs, co-HOPs and co-HUVECs after 6, 24, and 48 h of culture were quantified by QPCR. Data were normalized to P0 mRNA expression of each condition and were quantified relative to Flt-1 or KDR gene expression of HOPs after 6 h of culture, which were standardized to 1. Flt-1 and KDR gene expression was significantly up-regulated in HUVECs when they were co-cultured with HOPs. Value means ± SD of three separate experiments ($^{*}P \leq 0.05$, against HUVECs in corresponding time points).

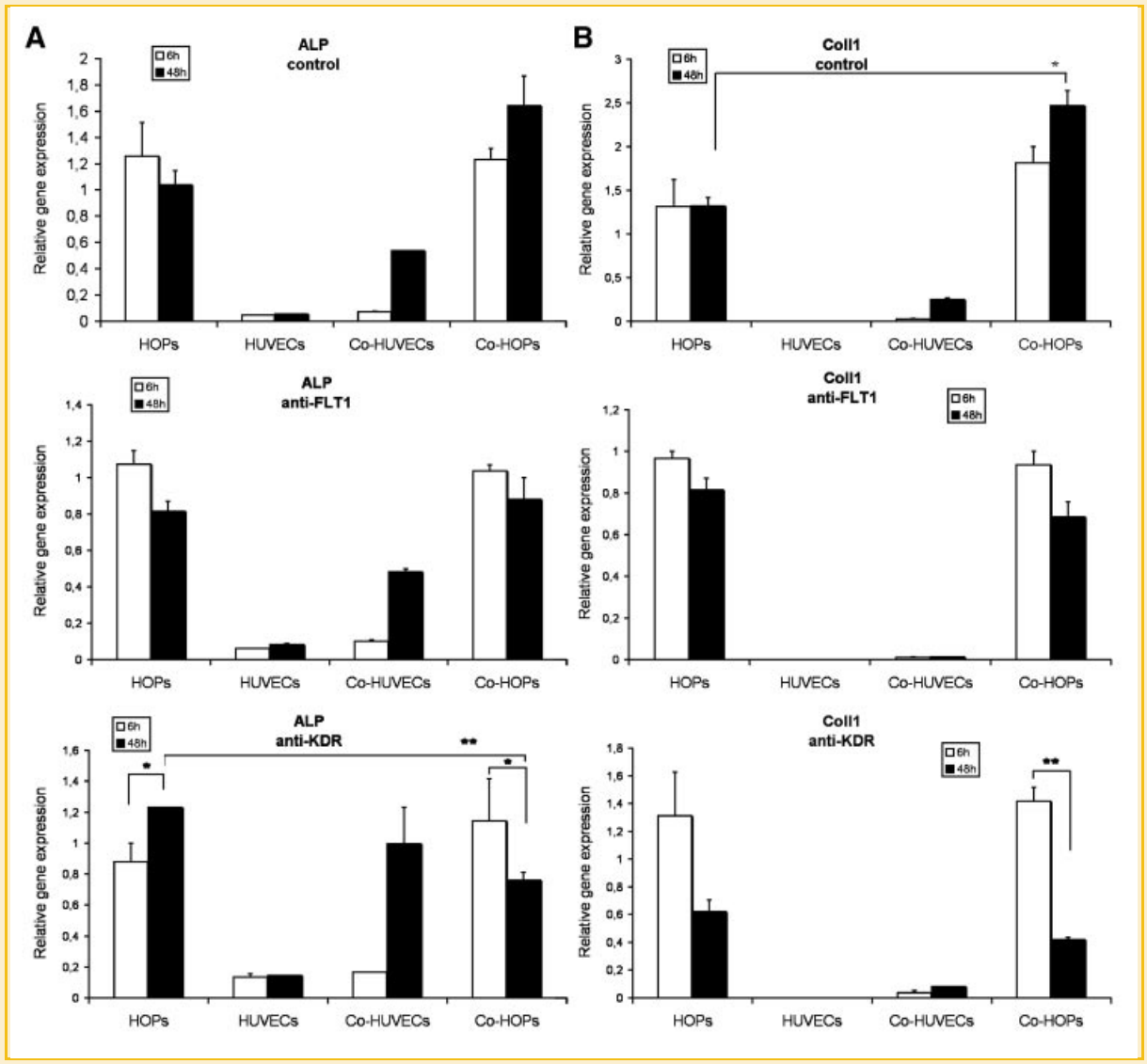

Fig. 5. Effect of VEGFR neutralizing antibodies on ALP and Coll-I gene expression. Gene expression of ALP and Coll-I, in HOPs, HUVECs, co-HOPs and co-HUVECs cultured for 6 and 48 h with or without neutralizing antibodies against Flt-1 or KDR. Data were normalized to P0 mRNA expression of each condition and were quantified relative to ALP or Coll-I gene expression of HOPs after 6 h of culture, which were standardized to 1. Flt-1 neutralizing antibody inhibited the increase of ALP and Coll-I in co-HOPs and neutralization of KDR led to a down-regulation of both factors. Value means ± SD of three separate experiments ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

Previous studies have already shown that osteoblastic differentiation is increased in co-cultured HOPs/HUVECs compared with HOPs alone [Villars et al., 2000, 2002; Guillotin et al., 2004]. In the present study, ALP and Coll-I up-regulations in Co-HOPs after 48 h of co-culture, compared with HOPs cultured alone were dependent on the Flt-1 and KDR pathways, as already proposed by Wang et al. [1997].

In conclusion, our results show that the tubular-like network observed in co-cultured HOPs/HUVECs is associated with EC migration, and is promoted by soluble factors secreted only in co-culture conditions. Even though $VEGF_{165}$ and its receptors were up-regulated in the co-culture, $VEGF_{165}$ alone did not affect EC migration under our conditions. A combination of soluble factors is necessary for the co-culture cell rearrangement, and might include bFGF, TGF-β, or HGF [Xin et al., 2001; Lebrin et al., 2005]. These soluble factors, which were secreted only when HOPs and HUVECs were co-cultured in direct contact, could be dependent upon the gap junctional activity established between the two cell types. In addition, the ECM could sequester and therefore release these components upon EC activity. Finally, VEGF appeared to play a crucial role in co-culture-stimulated osteoblastic differentiation, since inhibition of Flt-1 and KDR abolished ALP and Coll-I up-regulations.

This co-culture system appears to enhance both primary angiogenesis events and osteoblastic differentiation, and will thus allow us to set up new strategies for vascularized bone tissue engineering.

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