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Vascular Endothelial Growth Factor (VEGF-A) Expression in Human Mesenchymal Stem Cells: Autocrine and Paracrine Role on Osteoblastic and Endothelial Differentiation

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Abstract Angiogenesis is essential in bone fracture healing for restoring blood flow to the fracture site. Vascular endothelial growth factor (VEGF) and its receptor have been implicated in this process. Despite the importance of angiogenesis for the healing processes of damaged bones, the role of VEGF signaling in modulation of osteogenic differentiation in human mesenchymal stem cells has not been investigated in great detail. We examined the expression of VEGF-A and VEGFR-1 in human adult mesenchymal stem cells derived from trabecular bone (hTBCs). VEGF-A was found to be secreted in a differentiation dependent manner during osteogenesis. Transcripts for VEGF-A were also seen to be elevated during osteogenesis. In addition, transcripts for VEGF-A and the corresponding receptor VEGFR-1 were upregulated under hypoxic conditions in undifferentiated hTBCs. To investigate the signaling of VEGF-A on osteogenesis recombinant hTBCs were generated. High expression of VEGF-A stimulated mineralization, whereas high expression of sFLT-1, an antagonist to VEGF-A, reduced mineralization suggesting that VEGF-A acts as autocrine factor for osteoblast differentiation. In addition, VEGF-A secreted by hTBCs promotes sprouting of endothelial cells (HUVE) demonstrating a paracrine role in blood vessel formation. In summary, an in vitro analysis of transgene effects on cellular behavior can be used to predict an effective ex vivo gene therapy. J. Cell. Biochem. 95: 827–839, 2005.

Key words: stem cells; human; VEGF-A; VEGF-R; hypoxia; osteogenesis; neoangiogenesis; adenovirus

Angiogenesis is a crucial part of bone formation and bone fracture healing [Collin-Osdoby, 1994]. In the developing skeleton the endochondral cartilage template is replaced by highly vascularized bone tissue [Carlevaro et al., 2000; Engsig et al., 2000]. Recent studies have suggested that vascular endothelial growth factor (VEGF), a potent angiogenic stimulator,

Abbreviations used: AA, amino acid; ALP, alkaline phosphatase; CFU, cell-forming units; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; EtOH, ethanol; FGF-2, fibroblast growth factor-2; FCS, fetal calf serum; hMSC, human mesenchymal stem cell; hTBC, human trabecular bone cell; m.o.i., multiplicity of infection; OD, optical density; OI, osteo-inductive medium; PBS, phosphate-buffered saline; PNPP, *p*-nitrophenyl phosphate; sALP, secreted form of alkaline phosphatase.

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may play an important role during endochondral bone formation. VEGF family members named VEGF-A, -B, -C, -D and PIGF-1, -2 have in common the ability to stimulate endothelial cell proliferation. The VEGF gene can be alternatively spliced, and in the case of VEGF-A resulting in gene products corresponding to amino acids (AA) 121, 165, 189, 206 main splice forms in humans. Each splice variant has specific characteristics. The VEGF family act through signaling via various type II tyrosine kinase receptors. VEGFR-1 (FLT-1) binds VEGF-A, -B, -PIGF; VEGFR-2 (KDR) binds VEGF-A, processed-C, -D, but not PIGF; VEGFR-3 (FLT-4) binds VEGF-C, -D. Neuropilin has no signaling function for VEGF and acts as co-receptor of VEGFR-2 and binds PIGF-2 and VEGF-A-165, but not A-121 [Neufeld et al., 1999]. VEGF is expressed in hypertrophic chondrocytes and osteoblasts in vivo [Deckers et al., 2000]. In the human marrow microenvironment a broad spectrum of cytokine gene expression including angiogenic factors has been reported [Sensebe et al., 1997]. In cell culture both VEGF-A and VEGFR have been shown to be expressed during human mesenchymal stem cell (hMSC) derived osteoblastogenesis [Furumatsu et al., 2003]. In addition, VEGF-A was shown to be a chemo-attractant for endothelial cells [Streeten and Brandi, 1990; Gerber et al., 1999] and for osteoclasts in vivo, invading into developing long bones [Engsig et al., 2000; Nakagawa et al., 2000].

The importance of angiogenesis is also reflected in processes of bone repair, which have, however, not been studied in great detail. After bone injury, an hypoxic zone is formed and it is thought that angiogenesis is stimulated to restore blood flow to the fracture site thus initiating the bone repair process [Glowacki, 1998; Ferguson et al., 1999]. It has been demonstrated in cell culture, that VEGF-A expression is specifically regulated by hypoxia and a number of inflammatory cytokines [Steinbrech et al., 2000; Akeno et al., 2001].

A more direct evidence for the physiological role of VEGF was found recently, since the administration of VEGF stimulates and the antagonist sFLT-1 suppresses bone repair, thus affecting angiogenesis [Maes et al., 2002; Street et al., 2002]. Using a cellular delivery system of muscle derived stem cell line, beneficial effects of VEGF of bone healing elicited by BMP-4 have been reported [Peng et al., 2002].

Genetic models with defects in normal bone cell function have been associated with impaired angiogenesis. Absence of osteoblast differentiation and disturbed chondrocyte differentiation were reported to impair bone vascularization [Ducy et al., 1997], suggesting that angiogenesis is controlled by osteoblasts. Further, the lack of VEGF isoforms in genetic models shows a completely disturbed vascular pattern associated with an impaired differentiation of hypertrophic chondrocytes and osteoblasts [Zelzer et al., 2001, 2002; Maes et al., 2002], suggesting that VEGFs not only mediate bone vascularization, but also affect differentiation of progenitors to hypertrophic chondrocytes and osteoblasts [Maes et al., 2004].

Different sources for mesenchymal stem cells in human have been described. In addition to bone marrow [Pittenger et al., 1999], a population of stem cells could be isolated from trabecular bone by outgrow [Sottile et al., 2002], by collagenase treatment of the cultured chip [Tuli et al., 2003], or by direct collagenase treatment [Mayer, 2004] and from adipose tissue [Zuk et al., 2002]. Experimentally, when carriers seeded with adult stem cells are implanted in vivo they form bone [Bruder et al., 1997, 1998; Kraus et al., 1999].

Neovascularization is critical for bone tissue engineering since implantation of voluminous grafts without sufficient vascularity results in cartilage and hypoxic cell death of engineered tissues. Accordingly, strategies that enhance angiogenesis should have positive effects on bone repair.

In this study, we examined the synthesis of VEGF-A protein and its functionality during osteoblast differentiation of human mesenchymal stem cells derived from trabecular bone (hTBCs). We demonstrated that VEGF-A expression is increased during osteogenic differentiation and induces tube formation of endothelial cells. In addition we found that the synthesis of VEGF-A mRNA is hypoxy regulated, and the expression of the VEGFR-1 was found to coincide with the expression of the ligand. A postulated autocrine effect on osteogenesis was studied in recombinant stem cells expressing VEGF-A and the antagonist sFLT-1. VEGF-A high expressing cells enhance and sFLT-1 expressing cells suppress mineralization. This study illustrates that VEGF-A acts as an autocrine molecule on late phases of osteogenesis and as a paracrine molecule on endothelial

cells. With the coupling of gene delivery and mesenchymal stem cells the effect of a defined microenvironment can be examined in vitro on cellular behavior and used to predict an effective ex vivo gene therapy.

MATERIALS AND METHODS

Isolation of Progenitor Cells From Trabecular Bone

Bone specimens were obtained from females of the ages 50–80 years with osteoarthritis undergoing hip replacement (Herzogin Elisabeth Hospital, Braunschweig, Germany), with the approval of the local ethics committee. Subjects taking medications or having comorbid conditions or infections were excluded. The bone specimens of the size of 2 cm^3 were cut out from the inner part of the neck region and transferred into 30 ml Ringer solution in 50 ml vial (Falcon, BD Biosciences, Franklin Lakes, NJ), transported and mixed with sterilized 30 mg collagenase, Type 2 (Worthington), 30 mg chondroitin sulfate and 300 mg sorbite p.a.(Merck) in 15 ml Dulbecco's modified Eagle's medium (DMEM) (high glucose) (ICN) supplemented with 2 mM L-glutamine, 60.6 μg/ml ampicillin; 100 μg/ml streptomycin and rotated at 180 rpm at 37°C for approximately 12 h. The cell fraction was collected by centrifugation at 1,200 rpm for 5 min at 20°C in a Christ centrifuge and was subsequently washed twice with phosphate-buffered saline (PBS). The cell pellet was resuspended in 4 ml DMEM containing 15% fetal calf serum (FCS). A total number of nuclear cells of about 10^6 were plated in a 25 cm^2 flask (Falcon) and cultured at 37°C in a humidified chamber with 5% CO_2. After 5 days, the non-adherent cell fraction was removed by shaking and washed twice with PBS. The adherent cell layer was cultured an additional 6 days with media changes every 2 days. When cultures reached approximately 90% confluence, cells were harvested by incubation with a dilution of 1:10 of 5 g/L trypsin and 2 g/L EDTA (PAA-Laboratories) in PBS for 3–5 min at room temperature. The trypsin was inactivated by FCS and the cells were suspended in DMEM 10% FCS. For cryopreservation, 200,000 cells were suspended in 1 ml FCS containing 10% DMSO and slowly frozen in propanol at −70°C over night and stored in liquid nitrogen. From a frozen stock the cells were suspended in 5 ml DMEM 10% FCS and incubated over night. The non-adherent cells were then removed by changing the medium. Bone marrow hMSCs were obtained using standard protocol [Bruder et al., 1997].

To analyze the expression pattern under osteo-inductive medium (OI) conditions cells were cultured in OI medium (DMEM supplemented with 10% FCS, 10 mM β-glycero-2-phosphate, 50 μg/ml ascorbic acid and 10^{-7} or 10^{-8}M dexamethasone, Dex) for the indicated days.

To analyze the expression pattern in response to hypoxia cells were plated in 80 cm^2 flasks and grown under standard conditions to near confluence. The medium was replaced with DMEM 0.1% FCS and the cultures were exposed to hypoxia (2% oxygen) and normoxia (20% oxygen) for 24 h. SAOS-2, ACC-243, and COLO-800, ACC-183 were from DSMZ, Braunschweig, hFOB, 1.19, CRL-11372, A549, CCL-185 from ATCC. HEK 293 LP were purchased from Microbix.

In Vitro Angiogenesis Assay

Endothelial cell growth medium (ECGM), endothelial basal medium (ECBM), and the corresponding media supplements and were purchased from Promocell (Heidelberg, Germany). Human umbilical vein endothelial cells (HUVEC) were freshly isolated from human umbilical veins by collagenase digestion. Cells were cultured at 37°C, 5% CO_2, 100% humidity in the corresponding media containing 10% FCS (Biochrom, Berlin, Germany). Only HUVE cells cultured from passage 4 to 8 were used for experiments. Spheroids of a defined cell number were generated as described previously [Korff and Augustin, 1998; Korff et al., 2001]. Spheroids were cultured for at least 24 h and used for the corresponding experiments. In vitro angiogenesis was digitally quantitated by measuring the cumulative length of the sprouts that had grown out of each spheroid (ocular grid at 100× magnification) using the digital imaging software DP-Soft (Olympus, Germany) analyzing at least 10 spheroids per experimental group and experiment.

Western Blot Detection of VEGF-A From Recombinant hTBCs

For analysis of secreted VEGF-A and sFLT-1 the medium was replaced by DMEM without serum for 5 days and the supernatant lyophi-

lized. Protein samples were incubated for 10 min at 95°C in a sample buffer containing β-mercaptoethanol and loaded on 5% stacking gel and separated on a 15% separation gel. The broad range standard (7–200 kDa, BioRad) was used as a protein marker. Protein transfer on Immobilon™ PVDFMembran (Millipore) was performed using Trans-Blot®SemiDry Transfer Cell (BioRad) according to the manufacturer's protocol. The ECL-System (Amersham) was used for detection. Membranes were washed with H_2O and incubated with ECL-solution for 1 min. For visualization we exposed the membranes to X-ray films (X-Omat, Kodak) with an exponation time ranging from 5 s to 5 min in a dark chamber. For VEGF detection Anti-VEGF-Ab R6 was used. This antibody reacts with the N-terminus of all human VEGF isoforms.

RT-PCR Assays

Total RNA was isolated using a commercial RNA isolation kit (Tri reagent Molecular research centre). Approximately 1–5 μg of total RNA was transcribed to single stranded cDNA using reverse transcriptase according to the manufacturer(GibcoBRL).

Aliquots of the cDNA were amplified in 10 μl containing 25–50 pmol of a forward and reverse primer, 1× PCR Puffer 0.5 μl dNTP mix (10 mM) 1 μl primer (5 pmol), and 0.2 μl Taq polymerase (1 U) in 20 μl.

PCR reactions were performed using a Perkin Elmer thermocycler after an initial denaturation at 94°C for 2 min with the reaction profile 94°C for 30 s, annealing temperature as indicated for 2 min and DNA synthesis 72°C for 2 min. All reactions were terminated by incubation at 72°C for 10 min followed by 4°C. The amount of product accumulation was initially measured as a function of cell cycle number an equalized. All reactions were performed for 30 cycles. For each PCR amplification an aliquot of each product was electrophoresed in a 1% or 1.5% agarose gel. The gel was stained with ethidium bromide and photographed. The amount of product accumulation for each gene was then normalized to that of hypoxanthinphosphoribosyltransferase (HPRT). The following primers were used: for hHPRT: forward primer 5′GGTCAGGCAGTATAATCCAAAGA, backward primer 5′AGGCTCATAGTGCAAATAAACAGT, 404 bp, 52.2°C; hALP: forward primer 5′ACGTGGCTAAGAATGTCATC, backward primer, 5′CTGGTAGGCGATGTCCTTA, 475 bp, 55.5°C; hVEGF-A: forward primer 5′TGGATCCATGAACTTTCTGCT, backward primer 5′GAATTCACCGCCTCGGCTTGTC, 600 bp, 450 bp, 55°C; hVEGFR-1 (TMR), forward primer 5′GCACTACAGTATTAGCAAGCAA, backward primer 5′TTGTCCGAGGTTCCTTGAACA, 461 bp, 62°C.

Cytochemical and Quantitative Assays

For cytochemical assays cell monolayers cultivated in 24-well plates (Falcon) were washed with PBS, fixed with cold 100% ethanol (EtOH) for 15 min, washed with PBS and stained for alkaline phosphatase expression with 1:10 of the stock solution (8 mg/ml fast red and 4 mg/ml Natrium α-naphtylphosphate in 1M Tris-HCl, pH 8) for 1 h.

For determining mineralization cultures were incubated with 1 ml calcein (Sigma) 5 mg/L in H_2O over night at 4°C. After washing twice with PBS the fluorescence was measured in wells with 0.5 ml PBS in plate fluorimeter (Millipore Cytofluor 2350) at Ex 485 nm, Em 530 nm at sensitivity 4. Mineralization was expressed as fluorescence/μg protein. The values obtained with calcein were found to correlate to the values obtained by van Kossa staining.

To monitor the soluble ALP activity (sALP) during cultivation 50 μl of the supernatant were transferred in 96-well plate at the indicated time point and frozen. For measuring the sALP, 50 μl ALP solution (2× ALP buffer with 1 g/L para nitrophenyl-phosphate) was added and incubated at 37°C for 24 h and measured after 6, 12, and 24 h. Control wells contained the medium with ALP solution. The expression profile of the soluble ALP was found to correlate with the expression of the cellular ALP (unpublished results).

Monolayers lysed with 0.1% Triton X-100 over night and the protein content was determined using a protein assay kit (Pierce) with bovine serum albumin as a standard.

VEGF-A and sFlt-1 Sandwich ELISA

For detection of VEGF-A a highly sensitive specific sandwich ELISA was used according standard protocols and the methods described [Bando et al., 2004]. Briefly, the IgG fraction of VEGF-A immunisized rabbit was used for coating and the antigen-affinity purified and biotinylated anti-VEGF antibody was used as a

detector antibody. As a standard human rh VEGF165 was used in a concentration range between 0.1 and 5 ng/ml. For visualization streptavidin-HRP (horseradish peroxidase) conjugate was used (Endogen, Woburn, MA) followed by the addition of TMB (tetra-methylbenzidine; Roche Mannheim, Germany). After stopping the reaction with 1M H_2SO_4, the absorbance was measured at 450 nm and subtracted by 620 nm background value with an ELISA reader. Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicates. Serum samples and cell culture samples and supernatants from recombinant cells were serially diluted from 1:2 and 1:500 respectively with a sample buffer. The measurements of sFlt-1 secretion from infected cells has been described before [Hornig et al., 1999]. ELISA data were performed in 1% FCS with 10^5 cells.

Generation of Recombinant Cells by Adenoviruses

Recombinant adenoviruses were constructed by insertion of mono- or bicistronic expression cassettes into adeno cosmid pAdcos45, containing an E1, E3 deleted viral genome. The expression cassette containing a CMV-promotor and the 3′regulatory sequence of pGreenLantern (GibcoBRL) was amplified by PCR to add XbaI and SwaI/Psp1406I restriction sites to the 3′and 5′ ends, respectively, and cloned to yield pGEMGreenLantern. For the construction of bicistronic expression cassettes the poliovirus IRES element was inserted in front of the GFP gene. Insertion of the gene of interest (VEGF-A-165 and sFLT-1) in front of the IRES element yielded the bicistronic expression cassette. The expression cassettes isolated as Psp1406I-XbaI fragment were ligated into ClaI-XbaI digested pAdcos45, packaged in vitro and transduced in *E. coli* DH5. This procedure results in pAdcosGFP, pAdcosVEGF-A-165IRESGFP, and pAdcossFLT-1IRESGFP. For the production of the corresponding adenoviruses two 80 cm^2 of 293 cells were transfected using the Ca–phosphate coprecipitation method with 10 μg of cosmid DNA each. After 7–14 days viral plaques were formed. The recombinant adenovirus was propagated as described [Graham and Prevec, 1995].

For infection with the adenoviruses cells (hTBCs, A549, hFOB) were cultured until 80% confluence was reached. Prior to infection, cells were washed with infection buffer (PBS 2% FCS) three times and incubated at RT for 10 min. Infection was carried out normally in 80 cm^2 culture flasks (NUNC) with a multiplicity of infection (m.o.i.) of 50–200 pfu/cell in 5 ml at RT for 90 min. After infection 20ml DMEM containing 5%–10% FCS was added and the cells were incubated for 3 days. After this incubation period, cells were washed three times with DMEM 10% FCS. Fluorescence microscopic analysis of GFP expression was carried out 5 days after infection.

Statistical Analysis

Statistics were performed with the Microsoft excel program. Values are given as mean ± SD. Image analysis was performed with the Wincam program, Cybertech. Statistical analysis was performed with ANOVA comparing differences between groups with $P \leq 0.05$ considered significant.

RESULTS

Increased Synthesis of VEGF-A During Osteogenesis

To explore the relationship between angiogenesis and osteogenesis in vitro we analyzed the osteogenic potential on cellular level of hTBCs. Culture from a given donor in early passages was comprised predominantly of fibroblastoid cells with a high proliferative potential. Following exposure to OI medium nearly all clonal colonies were ALP positive and appeared more intensively stained when compared to the cells cultured without Dex. The morphology of cells changed to a broader cuboidal shaped form in cultures treated with Dex (Fig. 1). Therefore, the majority of the cell population appeared to differentiate into osteoblasts.

To assess the synthesis of VEGF-A during the progression of osteogenesis cells were continuously cultured at high density in OI medium for 7 weeks. For measuring the secretion of VEGF the medium was replaced at the indicated time points by DMEM 1% FCS and cultured for 6 days. The expression of ALP was used as an osteogenic marker. Our observation of correlation of expression profile of the soluble ALP (sALP) to the expression of the cellular ALP (cALP) secreted VEGF-A could be determined in the same sample as sALP. During cultivation, the expression of sALP is transiently increasing

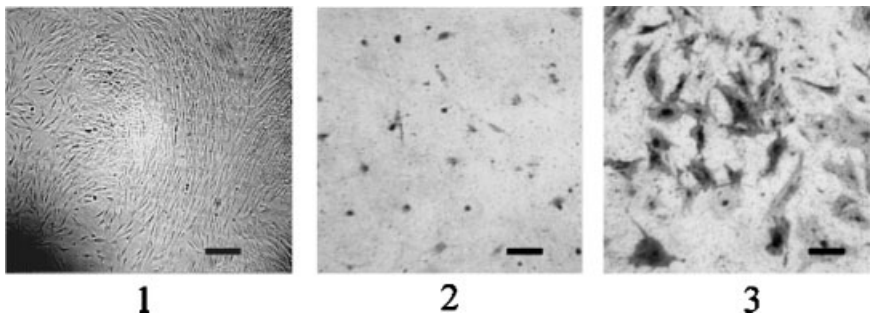

Fig. 1. Increased ALP expression under osteo-inductive conditions. hTBCs express ALP under osteo-inductive medium (OI) conditions. Photomicrographs of cultures stained for alkaline phosphatase activity. **1**: Secondary culture of hTBCs from trabecular bone from a 60-year-old woman shows fibroblast-like cell layer; (**2**) clonal culture under OI conditions grown for 3 weeks without 10 nM Dex reveals absence of staining for ALP; (**3**) clonal culture under OI conditions with 10 nM Dex reveals positive staining of cuboidal cells for ALP with different intensities. Bar represents 100 μm (1), bar represents 300 μm (2, 3). The experiment shown is representative for three different donors.

about fivefold and peaks after 3 weeks and decreasing at the end of the culture. The level of VEGF-A under undifferentiated conditions was low. Following exposure to OI media it increased more than threefold to approximately 900 pg/ml/ 10^5 cells/6 days after 3 weeks and remained elevated for 6 weeks. These results indicate that VEGF-A is maximally secreted at the late phase of osteogenic differentiation when extracellular matrix is mineralized (Fig. 2).

Increased Expression of mRNA for VEGF-A and VEGFR-1 During Osteogenesis and Under Hypoxia

Having demonstrated an increase in the production of VEGF-A during osteogenesis we proceeded to examine the VEGF-A mRNA synthesis in undifferentiated and differentiated cultures. As previously shown the culture of hTBCs under OI conditions at 3 weeks expressed genes of the BMP and PTH signaling system known to be specifically expressed in early and middle phases of osteogenesis. The expression of BMP-2 and the corresponding receptors BMPR-I (ALK-3, -6), BMPRII and PTHrP, PTH-R was significantly increased during osteogenesis. The expression of osteonectin, osteopontin and collagen I was similar in both conditions. A representative chondrocytic marker, collagen II, was not observed [Mayer, 2004]. Transcripts of VEGF-A 121 and 165 isoforms were analyzed during the period of 7 weeks by RT-PCR analysis and found to arise significantly, preferentially transcripts of VEGF-A-121 during osteogenesis. Transcripts of ALP were found to arise transiently during osteogenesis. An expression of VEGFR-1 could not be demonstrated reproducibly under these conditions (Fig. 3).

VEGF-A and VEGFR-1 mRNA expression has been shown in a variety of cells to be regulated by hypoxia. To assess the effect of

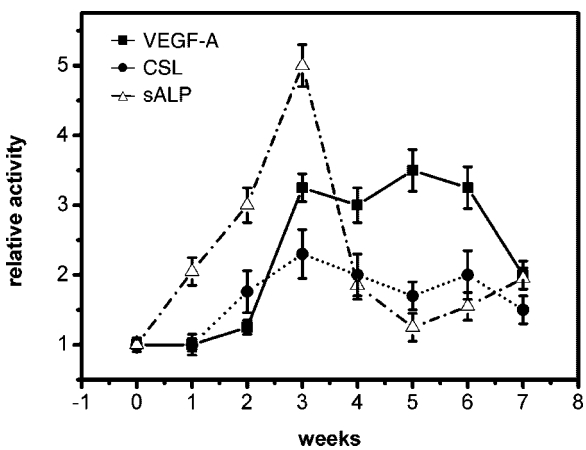

Fig. 2. Increased expression of VEGF-A during osteogenesis. VEGF-A protein and angiogenic activity is increased in parallel during osteogenesis. Secondary culture from a representative donor was plated in 24-well plates and at confluence with 10^5 cells grown in 1 ml under OI conditions with 10% FCS, and at the indicated time points the medium was replaced by OI medium with 1% FCS and cultured for 6 days. In the conditioned medium sALP (-△-) was measured as an indicator for osteogenesis with *p*-nitrophenyl phosphate (PNPP); and the secreted VEGF-A was determined by ELISA (-■-) and the cumulative sprouting length (CSL) was measured on HUVE cells (-●-). The value for sALP increased from $0{,}122 \pm 0{,}01$ at 0 weeks to $0{,}610 \pm 0{,}045$ at 3 weeks and decreased to 0.150 ± 0.020 at 5 weeks at OD = 405 nm. The value for VEGF-A increased from 300 ± 0.035 pg/ml to approximately 900 ± 45 pg/ml. The cumulative sprouting length (CSL) in 10 spheroids increased from 500 ± 50 μm to $1{,}200 \pm 100$ μm. n = 4, The data shown are representative for cultures of five different donors.

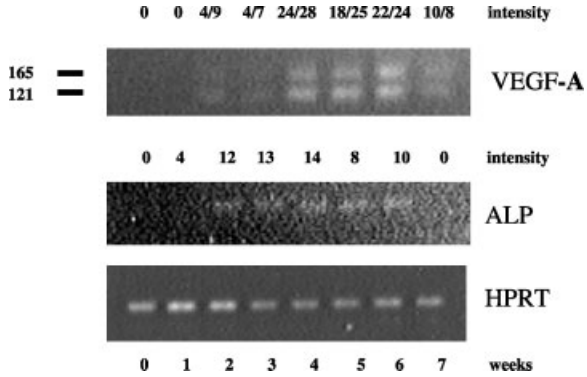

Fig. 3. Increased expression of mRNA for VEGF-A during osteogenesis. VEGF-A-isoforms -121 preferentially and -165 are osteogenesis dependent upregulated. Cells were plated in 6-well plates for the indicated time under OI conditions. RNA was isolated from the cultures and after standardization for HPRT expression, equal amounts of cDNA were subjected to PCR. The relative percentage of intensities of the bands is shown. The data shown are representative for cultures of three different donors. RT-PCR-product of 600 bp for VEGF-A-165 and slightly more the product of 459 bp for VEGF-A-121 increased from 4 to 6 weeks under OI conditions. The product of 475 bp for ALP increased under OI conditions from 1 to 6 weeks and peaks at 4 weeks.

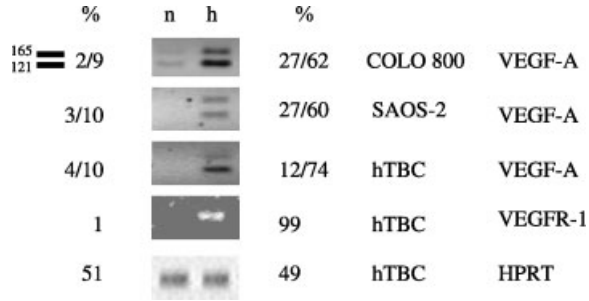

Fig. 4. Increased expression of mRNA for VEGF-A and VEGFR-1 under hypoxic conditions. VEGF-A-isoforms -121 and -165, preferentially VEGF-A-121 and VEGFR-1 are upregulated by hypoxia. Cells were plated in 80 cm^2 flasks and grown under standard conditions to near confluence. The medium was replaced with Dulbecco's modified Eagle's medium (DMEM) 0.1% FCS and the cultures were exposed to normoxia (n) (20% O_2) and hypoxia (h) (2% O_2) and for 24 h. RNA was isolated from hTBCs, COLO-800, SAOS-2 and after standardization for HPRT expression, equal amounts of cDNA were subjected to PCR. RT-PCR-product of 600 bp for VEGF-A-165 and slightly more the product of 459 bp for VEGF-A-121 and the product of 461 bp for VEGFR-1 increased under hypoxic conditions. The relative intensities of the bands is shown. The data are representative for cultures of three different donors.

hypoxia on hTBCs nearly confluent cultures were exposed in DMEM with 1% FCS to hypoxia of 2% O_2 for 24 h. RT-PCR-products for VEGF-A isoforms and VEGF-R1 of cells under hypoxic conditions were compared with cells maintained at normoxia of 20% O_2. As shown, hypoxia induced a significant increase in VEGF-A mRNA isoforms 121 and 165 in hTBCs to a similar level as in the osteosarcoma cell line SAOS-2 and in control cell COLO-800. VEGF-A transcripts were hardly detectable under normoxia in all of the tested cells. The expression of the VEGFR-1 were also seen to be induced by hypoxia in hTBCs. Altogether, the expression of both isoforms 121 and 165 of VEGF-A and VEGFR-1 were upregulated by hypoxic conditions in undifferentiated hTBCs (Fig. 4). In conclusion, the study demonstrates that transcripts of VEGF-A-121 and -165, preferentially -121 arise, when the osteoblastic phenotype is present and under hypoxic conditions.

High Expression of VEGF-A and sFLT-1 by Adenoviral Transduction

Having demonstrated the expression of VEGF-A in the proliferative phase under hypoxic conditions and in an osteogenic differentiation dependent manner, we were interested to prove a direct effect of continuously produced VEGF-A on osteogenesis. We therefore generated recombinant hTBCs overexpressing VEGF-A-165 and as control, the soluble receptor sFLT-1 to inhibit the endogenous VEGF-A activity. By insertion upstream of the marker gene for green fluorescence protein (GFP) the gene is expressed under the control of the CMV-promoter and GFP can be used to estimate both the transduction efficiency as well as the expression of the inserted gene.

The transduction efficiency of different cells by the recombinant Ad 45 VEGF-A165 IRES-EGFP and the Ad 45 sFLT-1 IRES-EGFP and the control construct Ad 45 GFP was very high. It was estimated to be above 85% by FACS analysis (data not shown) and 90% by counting the GFP positive cells (Fig. 5).

To assess the expression level and the maintenance during osteogenesis the products derived from the Ad 45 VEGF-A-165 IRES-EGFP and Ad 45 sFLT-1 IRES-EGFP transfected cells were analyzed at two different time points, once at day 3 when the proliferative phenotype was present and at day 15 under OI conditions, when the osteoblastic phenotype was expressed, by specific ELISA. To determine the influence of the cell type on the expression level in addition to hTBCs A549 as positive control and human osteoblast cell line hFOB

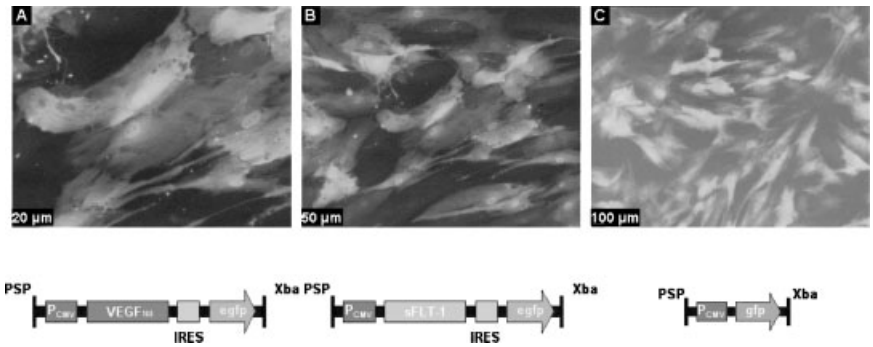

Fig. 5. High gene transfer by recombinant adenoviruses expressing EGFP. EGFP-fluorescence indicates an high transfection efficiency as well as a high expression of transgenes from the mono- or bicistronic expression cassettes. Fluorescence microscopic analysis of confluent cell layers of hTBCs transfected with (**A**) Ad 45-VEGF-A 165-IRES-EGFP and (**B**) Ad 45-sFLT-1-IRES-EGFP and (**C**) as a control Ad COS-45-GFP constructs.

have been transfected. To determine further the effect of the viral input hTBCs were transfected with 100 and 250 m.o.i. As shown in Figure 6 VEGF-A was highly expressed in all transfected cells within the range of 78 ng/ml to 600 ng/ml/10^5 cells, that is more than 100-fold higher than in the controls with approximately 300–1,000 pg/ml/10^5 cells. No sFLT-1 could be detected in untransfected cells. The relative expression of recombinant sFLT-1 was within a similar order as VEGF-A. The expression level of VEGF-A and sFLT-1 was found to be constant over time in hTBCs. Furthermore no significant differences in the level of expression of VEGF-A and sFLT-1 at higher m.o.i was observed, indicating a maximal transfection efficiency with a m.o.i. of 100 pfu/cell. In conclusion VEGF-A-165 and sFLT-1 could be continuously and efficiently delivered from different cells using an adenoviral vector for gene transfer.

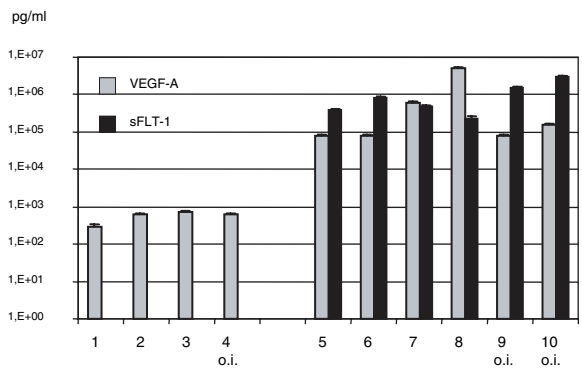

Fig. 6. High expression of VEGF-A- 165 and sFLT-1 by recombinant cells. VEFG-A and FLT-1 is highly expressed in different transfectants. Expression in transfectants is approximately more than 2 orders of magnitude higher than in the controls. VEGF-A ▒ and sFLT-1 ■ concentrations (pg/ml/ 10^5 cells) in conditioned medium. Supernatant of untransfected cells (**1**–**4**): (1) hTBCs, (2) hFOB, (3) A 549; (4) hTBCs. Supernatants of transfected cells (**5**–**10**): (5) hTBCs, 100 m.o.i., (6) hTBCs, 250 m.o.i., (7) hFOB, 100 m.o.i., (8) A549, 100 m.o.i., (9) hTBCs, 100 m.o.i., (10) hTBCs, 250 m.o.i. Cells were cultured for 3 or 15 days (OI), respectively. The values of VEGF-A between the different cells are in the range of 300–1,000 pg/ml/10^5 cells and increase in hTBCs three times under OI conditions. sFLT-1 could not be detected in untransfected cells. The values for VEGF-A and sFLT-1 are in the range of 100–5,000 ng/ml/10^5 cells and approximately more than two orders of magnitude higher than in the controls.

To verify the recombinant VEGF-A-protein the medium and the cell lysate of the recombinant A549 cells was analyzed. The immunoblot shows an immunopositive band with an estimated molecular weight of approximately 22.5 kDa corresponding to the glycosylated monomer and a band of lower MW, corresponding to the unglycosylated monomer molecule and a band with an estimated molecular weight of 45 kDa, corresponding to the homodimeric form. The untransfected cells show no bands on corresponding positions (Fig. 7). These data show that VEGF-A-165 is synthesized as an intact molecule by transfected cells.

Autocrine Effect of VEGF-A on Osteogenesis

To evaluate a direct effect of VEGF-A on osteogenesis recombinant hTBCs expressing either VEGF-A-165 or sFLT-1, an antagonist to VEGF-A, and control cells were cultured under OI conditions. The specific cALP was transiently expressed to a similar level in both, the recombinant and control cultures and peaked at day 20. The mineralization of the extracellular matrix in recombinant overex-

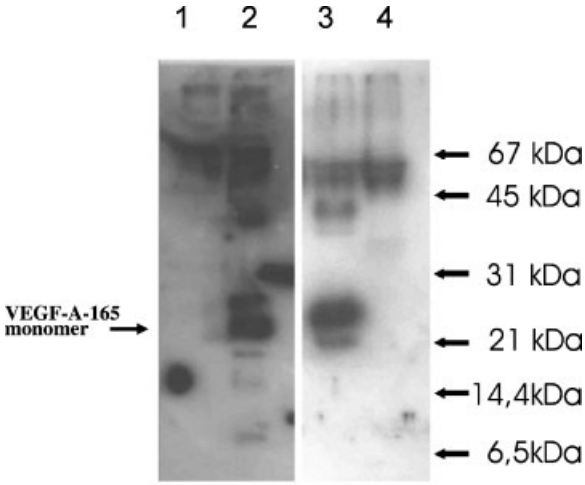

Fig. 7. VEGF-A protein analysis of recombinant cells. The 22.5 kDa band representing the monomeric, the 45 kDa band representing the dimeric form of VEGF-A-165 protein was detectable both in conditioned medium and the cell lysate of the VEGF-A transgene and not in protein lysates of the control. Western blot analysis of secreted VEGF-A-165 of recombinant cells. Aliquots from A 549 cell lysate transfected with control Ad-cos45-GFP infected A 549 cells (**1**) and Ad 45-VEGF-A 165-IRES-EGFP (**2**) and from conditioned medium from Ad 45-VEGF-A 165-IRES-EGFP cells (**3**), and control Ad-cos45-GFP (**4**) were subjected to 15% SDS–PAGE, blotted and stained with anti VEGF-A antibody.

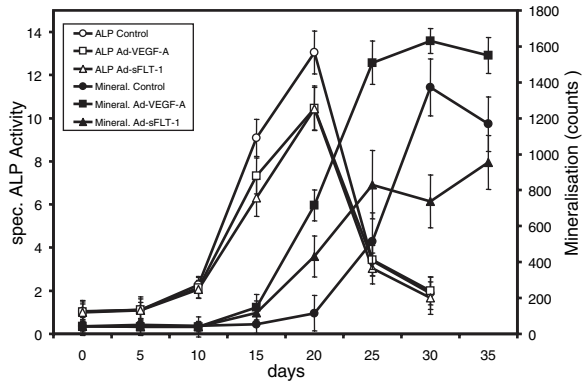

Fig. 8. Autocrine effect of VEGF-A and sFLT-1 on regulation of osteogenesis. The mineralization is enhanced in VEGF-A-165 transfectants and retarded in sFLT-1 transfectants versus control. hTBCs transfected with Ad 45-VEGF-A 165-IRES-EGFP and Ad 45-sFLT-1-IRES-EGFP and as a control untransfected cells were cultured in 24 well plates at confluence and subsequently grown under OI conditions with 10% FCS. (-○-), ALP control; (-□-), ALP Ad-VEGF-A; (-△-), ALP Ad-sFLT-1;(-●-), mineral control; (-■-), mineral Ad-VEGF-A; (-▲-), mineral Ad-sFLT-1. The expression of specific ALP activity and mineralization was determined at the time points s indicated. The cellular ALP was assayed using PNPP. The protein was determined using a BCA protein assay kit. The mineralization was determined using the calcein assay. The data shown are representative for three independent experiments ±SEM. n = 4.

pressing VEGF-A-165 cultures however was increased already at day 20 about sixfold at a level, reached in the untransfected control culture at day 27, and remained constant during the culture at an elevated level. In the recombinant sFLT-1 over expressing culture, on the other hand, the extent of mineralization was significantly retarded during the late phase compared to the control culture, probably by blocking of endogenous VEGF-A through binding by sFLT-1 (Fig. 8). These data of enhanced mineralization by continuous overexpressing of VEGF-A and of reduced mineralization by blocking the endogenous VEGF-A by overexpressing of sFLT-1 present evidence that VEGF-A is an autocrine acting osteogenic factor.

Paracrine Effect of VEGF-A on Angiogenesis

To examine the role of VEGF-A in the paracrine interaction of endothelial cells and adult stem cells an endothelial cell spheroid system was used. This cellular assay system is known to be responsive to endothelial growth factors including VEGF-A. Low endothelial sprouting activity was detectable in the conditioned medium of undifferentiated cells, and a slightly enhanced activity in cells under OI conditions (Fig. 9), which correlates to the ELISA data as shown in Figure 6. Further, when we analyzed the angiogenic activity during osteogenesis the angiogenic activity shows a trend to increase during osteogenic differentiation. The values are in parallel to the secreted VEGF-A protein measured by ELISA in Figure 2 the expression of transcripts of VEGF-A isoforms as shown in Figure 3. This suggests that human mesenchymal stem cells secrete in a differentiation dependent manner VEGF-A as an angiogenic factor. Furthermore, we were interested to determine the angiogenic activity of the transgenes expressing VEGF-A. An highly stimulated vessel formation was found in all of cells expressing the transgene VEGF-A-165 and the amount of the angiogenic activity was found to be approximately 100-fold higher than in the untransfected control cells. No significant differences has been found in transgenes of 3 and 15 days, suggesting a continuous synthesis of angiogenic activity during the culture period (Fig. 9). These data are consistent with the ELISA data as shown in Figure 6. Evidence is presented that VEGF-A is a paracrine acting angiogenic factor.

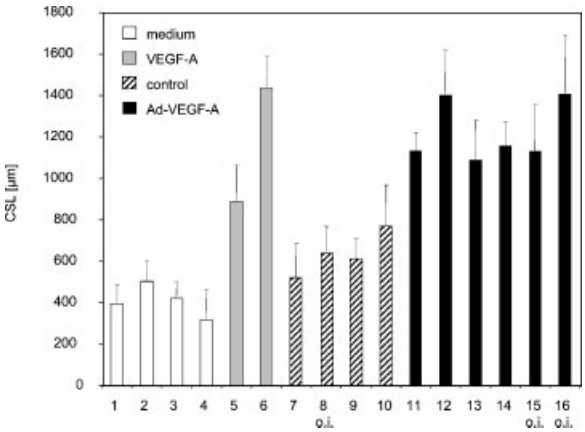

Fig. 9. Paracrine effect of VEGF-A on vessel formation of HUVE cells. The efficiency in inducing angiogenesis is more than two magnitudes higher in VEGF-A-165 transfectants than in the controls. CSL (µM) of medium □ (**1**–**4**), of VEGF-A ▒ (**5**, **6**), of untransfected cells ▨ (**7**–**10**), and Ad VEGFA transfected cells ■ (**11**–**16**). Medium, (1) DMEM; (2)+5% FCS; (3)+10% FCS; (4) DMEM + Pen/Strep; (5)+10 ng VEGF-A; (6)+25 ng VEGF-A; supernatants of untransfected cells (7) hTBCs, 30 pg; (8) hTBCs, 62 pg; and (9) hFOB, 60 pg; (10) A 549, 70 pg; supernatants of Ad 45-VEGF-A 165-IRES-EGFP transfected cells (11) hTBCs, 100 m.o.i. 8 ng; (12) hTBCs, 250 m.o.i. 8 ng; (13) hFOB, 100 m.o.i. 25 ng; (14) A549, 100 m.o.i. 25 ng; (15) hTBCs, 100 m.o.i. 8 ng; (16) hTBCs, 250 m.o.i.,15 ng. Cells were cultured for 3 days or 15 days (OI), respectively. For measuring the effect on neoangiogenesis HUVE cells were grown in spheroids 1,000 cells/spheroid in 1 ml and treated with 100 µl aliquots and the cumulative sprouting length was measured in 10 spheroids in micrometers. The indicated amount determined by ELISA have been assayed. The data are from 10 spheroids. $P < 0.001$ compared to the corresponding control. The assay experiments were done twice.

DISCUSSION

Blood supply is a crucial part of bone formation and fracture healing. Recent studies suggested that VEGF, a potent angiogenic stimulator, may play an important role during these processes in stimulating the restoration of blood flow to the fracture site, thus initiating the bone repair process [Glowacki, 1998; Ferguson et al., 1999].

Recently, attempts have been made to use adult stem cells for bone fracture healing. Implantation of ex vivo expanded stem cells has been demonstrated to affect bone regeneration in non-union fracture models [Kraus et al., 1999]. However, significant variations in the extent of healing have been described [Bruder et al., 1997]. It has been suggested that an incomplete engraftment may be due to heterogeneity of the transplanted cell population or, alternatively, to the inability of the host to support the engraftment. Despite the importance of angiogenesis in the process of bone fracture healing this issue has not been addressed in great detail, in particular on human mesenchymal stem cells.

To address the full potential of stem cell based therapy protocols for bone fracture healing the role of adult stem cells from human in the process of angiogenesis was analyzed. In the present study we have shown the following:

(1) Production of VEGF-A during the osteoblast differentiation.
(2) Regulation of transcripts of VEGF-A isoforms 121 and 165 and VEGFR-1 during osteogenesis and hypoxia.
(3) Modulatory effects on mineralization of autocrine produced VEGF-A or sFLT-1.
(4) New vessel formation in vitro by paracrine produced VEGF-A.

Different protocols have been described for the isolation of osteoprogenitor cells by outgrow [Sottile et al., 2002] and subsequent collagenase treatment [Tuli et al., 2003] or by direct collagenase treatment [Mayer, 2004]. As demonstrated, our cultures have fibroblasic morphology and display stem cell characteristics indicated by the presence of a CD13+, CD44+, CD90+, CD147+, and CD14−, CD45−, CD144− cell populations. They stably retain an undifferentiated phenotype during extensive proliferation. The sequential analysis of cell-forming units (CFUs) released by collagenase digestion suggests no significant contamination by the cells derived from bone marrow. In this report, we demonstrate that monolayers of human trabecular bone cells (hTBCs) are comprised predominantly of a fibroblastic spindle shaped cells which undergo at low density under OI conditions into osteoblasts expressing ALP. When we cultured hTBCs at high density following exposure to OI medium we show here that VEGF-A protein is expressed at low levels at the beginning of osteoblast differentiation and increased and remains constant during terminal differentiation. An osteogenesis dependent increase of VEGF-A synthesis was also found in hFOB and Saos-2 (not shown). The osteogenesis dependent increase of VEGF secreted by primary hTBCs from 300 to 900 pg/ml/10^5 cells in 5 days in our primary hTBCs, is in line of data reported in different osteogenic cell lines from 300 to 1,000 pg/ml/24 h for 2.5×10^6 MC3T3-E1 cells [Saadeh et al., 2000]

and from 250 to 1,000 pg/ml for KS483 cells [Deckers et al., 2000]. For hMSCs derived from bone marrow an increase from 4.42 to 7.48 ng/10^6 cells/48 h at 3 weeks Dex treatment had been reported [Furumatsu et al., 2003].

Further recording the increase of VEGF-A protein an increased mRNA expression of VEGF-A-isoforms can be expected. Of the known VEGF-A isoforms PCR products of the two most common isoforms A-121 and A-165, representing the soluble and the heparin binding form of VEGF-A protein respectively, could be demonstrated. The expression of VEGF-A isoforms -121 and -165 in our human primary cells is consistent with the expression profile of the mouse homologs VEGF-120 and -164 in the osteogenic KS483 cell line, which were shown to increase during osteogenesis and to be maximally expressed during mineralization [Deckers et al., 2000]. In hMSCs derived from bone marrow the expression of different isoforms have been reported by using different methods with a preferential increase of the A-121 isoform by RT-PCR analysis along osteoblastic differentiation [Furumatsu et al., 2003]. In our study we could demonstrate a preferential expression of the A-121 isoform during osteogenesis. No expression of VEGFR-1 and -2 has been reported in osteoblast like cell line MC3T3.E1 [Harper et al., 2001] and recently in human MSCs [Furumatsu et al., 2003]. Deckers et al. [2000] reported that the expression of VEGFR-1 and -2 increased along with the mineralization of KS483 cells. In our study we could also not demonstrate the expression of VEGFR-1 and -2 reproducible during osteoblastogenesis by RT-PCR analysis.

Furthermore a preferential increase of expression preferentially of VEGF-A-121 with -165 in different cells including hTBCs under hypoxic conditions in hTBCs has been found. The differential expression of VEGF-A-121 during osteogenesis in human mesenchymal cells derived from trabecular bone and bone marrow suggests that human mesenchymal stem cells use that the same splicing mechanism for VEGF-A, which seems to be different to that in mouse cell lines. In addition the expression of the VEGF-A under hypoxic and osteogenic conditions suggests that two different mechanisms are involved in gene regulation of VEGF-A transcription. The expression pattern for VEGF-A and VEGFRs suggest that in human mesenchymal stem cells signaling of VEGF-A via VEGFR-1, -2 might depend on the differentiation state. Further studies involving the differential expression of other members of the VEGF family and the receptors and coreceptors are required.

Genetic mouse models provide additional data for the concept of an osteogenic dependent mechanism of VEGF signaling. In the absence of osteoblast differentiation when mainly undifferentiated osteoprogenitor are present in the CBF-A KO model, the vascularization of the bone is impaired [Ducy et al., 1997]. Impaired differentiation of chondrocytes and osteoblasts in the absence of distinct VEGF isoforms [Zelzer et al., 2001, 2002; Maes et al., 2004] support our findings of a distinct role of VEGF for normal differentiation of osteoblasts. Recently by using the genetic stem cell approach the importance of VEGF-A on bone formation and bone fracture healing was demonstrated acting in synergy with BMP-4 and affecting different steps of cartilage and bone formation, suggesting multiple action mechanisms of VEGF [Peng et al., 2002].

With using adenoviral gene transfer for the production of recombinant cells expressing VEGF-A and the antagonist sFLT-1 a direct role of VEGF-A in the process of late phases of osteoblast differentiation could be demonstrated suggesting an autocrine loop mechanism. VEGF-A has been shown to have no direct effect on cell proliferation [Furumatsu et al., 2003] and on expression of ALP as BMP-2 in mesenchymal stem cells without Dex treatment (unpublished results). Our finding of enhanced mineralization via an autocrine loop and the described positive effect on bone growth in vivo suggests an direct action of VEGF-A on the mineralization process. The effect of enhanced ossification by VEGF-A and retarded ossification by the administration of sFLT-1/Fc chimera receptor was recently reported in an organ culture model of metatarsals [Maes et al., 2002]. The physiological role of sFLT-1 is not exactly known, but it is likely to be a negative regulator of VEGF availability or it may prolong the different activities associated with this protein [Hornig et al., 1999a; Hornig and Weich, 1999b; Neufeld et al., 1999]. Our results may suggest that sFLT-1 functions act as a negative regulator of VEGF availability.

Although benefits of stem cell therapy approach have been reported for bone healing, the effect of the product delivered from the

cellular vehicle has not been studied so far in detail. Recently it has been shown that the cellular vehicle used for delivery of the therapeutic gene product strongly affects the efficacy of bone gene therapy [Turgeman et al., 2001; Peng et al., 2002].

In this regard our study investigated in cell culture parameters which influence the effectiveness of mesenchymal stem cells derived from aged humans for the delivery of a therapeutic gene product and revealed autocrine and paracrine functions for VEGF-A. This study supports the hypothesis, that in vitro analysis of the cellular behavior in response to a therapeutic gene product can be used to predict effectivity of an effective ex vivo gene therapy.

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