This is a postprint of an article published in
Klöpper, J., Lindenmaier, W., Fiedler, U., Mehlhorn, A., Stark, G.B., Finkenzeller, G.
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(2008) Microvascular Research, 75 (1), pp. 83-90.

High efficient adenoviral-mediated VEGF and Ang-1 gene delivery into osteogenically differentiated human mesenchymal stem cells

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Abstract

Survival of ex vivo constructed tissues after transplantation is limited by insufficient oxygen and nutrient supply. Therefore, strategies aiming at improvement of neovascularization of engineered tissues are a key issue in tissue engineering applications. This in vitro study aimed at exploring the usability of osteogenically differentiated human mesenchymal stem cells (MSCs) as carriers of the angiogenic growth factor genes vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) for therapeutic angiogenesis in bone tissue engineering. The ex vivo adenoviral vector mediated transduction into osteogenically differentiated MSCs revealed a highly efficient and long lasting expression of the transgenes. Biological activity of VEGF and Ang-1 secreted from transduced cells was confirmed by analyzing the sprouting, proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs) in response to conditioned medium obtained from transduced cells. The transduced osteogenically differentiated MSCs described in this report may be suitable for inducing neovascularization in bone tissue engineering applications.

Keywords: Tissue engineering; mesenchymal stem cell; VEGF; angiopoietin-1; HUVEC; adenovirus; gene transfer; osteogenic differentiation

Introduction

Angiogenesis, the sprouting of new blood vessels from preexisting ones, plays an important role in bone tissue engineering applications since survival of ex vivo constructed tissues after transplantation is limited by an insufficient oxygen and nutrient supply (Griffith et al., 2005; Young et al., 1996). Therefore, strategies aiming at improvement of vascularization of engineered tissues are of great interest. Most approaches in regenerative medicine attempting to induce neovascularization in hypoxic tissues are based on the administration of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) (Baumgartner et al., 1998; Schumacher et al., 1998; Henry et al., 2001). These molecules can either be applied in form of recombinant proteins or by means of a gene therapeutic approach based on the delivery of genes encoding these factors (Khan et al., 2003; Rosinberg et al., 2004).

In addition, cell-based therapies using genetically modified cells for transplantation purposes have been developed. Here, transfected cells can be used as a vehicle for proangiogenic secreted molecules, thus providing a transient source of growth factors following transplantation (Suzuki et al., 2001; Yang et al., 2007). This approach may help to maintain a therapeutic level of these growth factors in order to improve angiogenesis.

One of the most widely used growth factors to accomplish therapeutic angiogenesis is VEGF, an endothelial cell specific mitogen and survival factor (Takahashi et al., 1999; Gerber et al., 1998). It was shown that gene transfer of VEGF expression plasmids induced neovascularization and brought significant increased blood flow with restored vasomotion in collateral vessels (Bauters et al., 1994; Asahara et al., 1996) as well as at the microvascular level (Takeshita et al., 1998). However, it remains unclear whether the administration of VEGF alone is adequate to promote the formation of mature vessels that are lined with pericytes or vascular smooth muscle cells. Some reports suggest that excessive VEGF

expression induces pathological and immature vessel formation resulting in plasma leakage and tissue edema (Dvorak et al., 1999; Carmeliet et al., 1999). Therefore, stabilization of newly formed vessels is essential for establishing a long lasting vascular network. In this context, it has been demonstrated that angiopoietin-1 (Ang-1) plays an important role for stabilization and maturation of blood vessels by inducing the recruitment of perivascular mural cells such as pericytes and smooth muscle cells (Thurston et al., 1999; Thurston et al., 2000; Gamble et al., 2000). In the context of therapeutic angiogenesis it was recently shown that a combined gene transfer of VEGF and Ang-1 leads to the formation of functionally stable blood vessels with no signs of plasma leakage (Arsic et al., 2003).

We have previously described a three-dimensional spheroidal coculture model of human primary endothelial cells and human primary osteoblasts which was designed to improve angiogenesis in bone tissue engineering (Wenger et al., 2004). The present study aimed at exploring the usability of osteogenically differentiated mesenchymal stem cells (MSCs) as carriers of the angiogenic growth factor genes VEGF and Ang-1 for therapeutic angiogenesis in bone tissue engineering. The ex vivo adenoviral vector mediated transduction into osteogenically differentiated MSCs revealed a highly efficient expression of biologically active VEGF and Ang-1.

Materials and methods

Cell culture

Isolation of human mesenchymal stem cells (MSCs) was performed as described before (Mehlhorn et al., 2006). In brief, bone marrow aspirates were obtained from consenting healthy adult donors by an iliac crest biopsy. Mononuclear cells (MNCs) were purified by density gradient centrifugation with Biocoll Separating Solution (Biochrom AG, Berlin,

Germany). Subsequently, cells were filtered through 100-µm cell strainers (BD Labware, Franklin Lakes, NJ). MNCs were seeded in T175 culture flasks at a density of 5 x 10^5 cells / cm^2 in 25 ml of expansion medium (alpha-MEM, 10 % FCS, 50 µg/ml gentamicin, 5 ng/ml bFGF) at 37 °C, 5 % CO_2 in a humidified atmosphere. The medium was changed twice a week, washing out all nonadherent cells. Once adherent cells had grown to confluence, they were detached and reseeded at a density of approximately 2000 cells / cm^2 and cultivated for 2 further passages. MSCs were differentiated into osteogenic cell lineage by replacing the expansion medium with differentiation medium (DMEM, 10 % FCS, 50 µg/ml gentamicin, 0.1 µM dexamethasone, 10 mM β-glycerolphosphate, 50 µM l-ascorbic acid-2-phosphate) and further cultivation for 2 weeks. Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany) and were cultured in endothelial cell growth medium (ECGM, Promocell, Heidelberg, Germany) supplemented with 10% FCS and supplement mix at 37 °C, 5 % CO_2 in 75 cm^2 tissue culture flasks. HUVECs from passage 2 to 5 were used for experiments.

Cellular staining

Osteogenically differentiated mesenchymal stem cells were plated in 12-well cluster plates, incubated for 3 days and then analyzed for alkaline phosphatase and osteocalcin expression and mineralization of the extracellular matrix (von Kossa staining). Cells were washed twice with PBS and fixed in ice-cold methanol. Cells were incubated for 30 min with blocking solution (10 % normal goat serum; Sigma, Deisenhofen, Germany) followed by incubation with 1:100 monoclonal mouse anti-osteocalcin antibody (Acris, Hiddenhausen, Germany) for 1 hour at RT, three washings and incubation with ready to use biotinylated goat anti-mouse immunoglobulin (Dako, Hamburg, Germany) for 30 min and DAB chromogen substrate for 5 minutes. Hematoxylin (1:1 in water) was used for a weak counterstain. For alkaline

phosphatase evaluation, cells were stained using the alkaline phosphatase kit from Sigma (Deisenhofen, Germany) following manufacturer guidelines. Mineralization of the extracellular matrix in the cultures was assessed using von Kossa stain. The matrix was washed with PBS, and cultures were treated with 5% $AgNO_3$ solution in the dark at 37°C for 30 minutes. The excess silver nitrate solution was then completely washed away using double-distilled H_2O and the culture plate was exposed to sodium carbonate/formaldehyde solution for a few minutes to develop color.

Adenoviral infection

The adenovirus for the expression of Ang-1 was generated using the Gateway technology and the Adenovrial Expression System from Invitrogen. Briefly, myc-tagged human Ang-1 cDNA (Fiedler et al., 2003) was amplified by PCR using primers containing *attB* sites and recombined into the pDONR vector according to the manufactors instructions. The constructs were subsequently recombined into the pAd/CMV/V5-Dest vector (Invitrogen) and adenoviruses were generated and purified using the ViraPower system (Invitrogen). Osteogenically differentiated human mesenchymal stem cells were infected with adenoviruses encoding $VEGF_{165}$ (VEGF Adv; Mayer et al., 2005) or Ang-1 at about 80 % confluency. 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 with a muliplicity of infection (MOI) of 1 – 300 pfu/cell in 1 ml of infection buffer at RT for 60 min. After infection, differentiation medium was added and the cells were incubated for the indicated time periods at 37 ^{o}C, 5 % CO_2 in a humidified atmosphere. Fluorescence microscopic analysis of GFP expression was carried out 3 days after infection. Negative control (non-infected cells) were treated equally, but did not receive any virus load.

Spheroid sprouting assay

The preparation of cell spheroids was performed as described previously (Korff & Augustin, 1998). Briefly, subconfluent monolayers of HUVECs were trypsinized and suspended in endothelial cell basal medium (ECBM, Promocell, Heidelberg, Germany) containing 20% methocel (Sigma, Deisenhofen, Germany). 500 cells per well were seeded into nonadherent round-bottom 96 well plates (Greiner, Frickenhausen, Germany) to assemble into a single spheroid per well within 24 h at 37 ^{o}C, 5 % CO_2 in humidified atmosphere. Spheroids were embedded into collagen type I (isolated from rat tail tendons) gels. For each gel, 40 spheroids were seeded into 700 µl collagen solution in non-adherent 24 well plates, with a final collagen concentration of 1,5 mg/ml. Freshly prepared gels were transferred into a humidified incubator (37 ^{o}C, 5 % CO_2) and after polymerization, 200 µl ECGM (Promocell, Heidelberg, Germany) supplemented with 10 % FCS and supplement mix (Promocell, Heidelberg, Germany) was added per well along with 200 µl of conditioned media from non-transduced or transduced MSCs. Vascular endothelial growth factor (VEGF) (R&D Systems, Wiesbaden, Germany) was added as indicated, in a final concentration of 25 ng/ml. After 48 h, gels were photographed and spheroid sprouting was assessed quantitatively. The assessment of the sprouting process was quantified by measuring cumulative length of all capillary-like structures originating from the central plain of a single spheriod using a digital system (analySIS, Soft imaging system GmbH, Muenster, Germany) connected to an inverted microscope. All in all, ten spheriods per group and per experiment were analyzed. Results for the different experimental groups were expressed as means $\pm$ SD.

Cell proliferation assay

Cell proliferation was assessed by using the CellTiter 96 Aqueous one solution cell proliferation assay (Promega, Heidelberg, Germany). In brief, 4000 cells per well were seeded in triplicate into 96-well plates in 80 µl ECBM supplemented with 5 % FCS in the presence or absence of 20 µl of conditioned medium from non-transduced or transduced MSCs. Recombinant VEGF was used as a positive control in ECBM, 5 % FCS in a final concentration of 30 ng/ml. After 3 days of cultivation at 37 ^{o}C, 5 % CO_2 in a humidified atmosphere, 20 µl of CellTiter 96 Aqueous One solution was added per well for another 4h. Microtiter-plates were then analyzed at 490 nm using an automated microtiter plate reader.

TUNEL staining

To assess apoptosis, 3000 HUVECs per well were seeded in triplicate in 96 well cluster plates in 80 µl ECBM, 1.5 % FCS in the presence or absence of 20 µl of conditioned medium from non-transduced or transduced MSCs. After three days, a TUNEL staining was performed with a commercially available kit (Roche, Mannheim, Germany), detecting fragmented DNA as a signal of apoptosis. Two drops of Antifade solution (BiomedDia, Zweibrücken, Germany) were added to each well before microscopic fluorescence photographs were taken with a ZEISS Axio Vision 100 microscope. For each experimental group, total cell number was counted and put into relation to the number of cells that displayed fluorescence in the nuclear core region.

Quantitative real time RT-PCR

TaqMan RT-PCR was carried out as previously described (Medhurst et al., 2000). Total RNA was prepared using TRIzol reagent (Invitrogen). Total RNA (3 µg) was treated with 3 units of deoxyribonuclease I (DNase I) (Invitrogen) to digest genomic DNA contamination. Random-primed cDNA synthesis was performed using 3 µg of DNase I-treated total RNA and 50 units of StrataScript reverse transcriptase according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). TaqMan PCR assays were performed in 96-well optical plates on an ABI Prism 7700 Sequence Detection system (Applied Biosystems, Forster City, CA, USA) using Absolute QPCR ROX Mix (Abgene, Hamburg, Germany) according to the manufacturer's instructions. Oligonucleotide primers and TaqMan probes (Table 1) were designed using Primer Express (Applied Biosystems) according to company guidelines. The thermal cycling conditions were 95 ^{o}C for 15 min followed by 40 cycles at 95 ^{o}C for 15 sec and at 60 ^{o}C for 1 min. Data were analyzed using the relative standard curve method, with each sample being normalized to GAPDH to correct for differences in RNA quality and quantity. Results from three experiments are expressed as mean arbitrary units $\pm$ SD.

ELISA

Concentrations of $VEGF_{165}$ and angiopoietin-1 (Ang-1) were quantified in conditioned media of non-transduced or adenovirus transduced cells using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany).

Statistics

Statistically significant differences between groups were determined by using an unpaired Student´s *t*-test. Statistical significance was defined when $p < 0.05$.

Results

Human mesenchymal stem cells were isolated from bone marrow and expanded up to passage three. Thereafter, osteogenic differentiation was induced by cultivation of cells in differentiation medium for further two weeks. Osteogenic differentiation was confirmed by cellular stainings for the osteogenic markers alkaline phosphatase (AP) and osteocalcin (Fig. 1). Moreover, an osteoblastic cell type specific calcification of the extracellular matrix could be demonstrated by von Kossa staining (Fig. 1). These osteogenically differentiated human MSCs were used for subsequent transduction studies with adenoviruses encoding the angiogenic growth factor genes VEGF (VEGF Adv) and angiopoietin-1 (Ang-1 Adv). As shown in figure 2, a clear dose-relationship could be detected between the amount of VEGF adenovirus and the number of cells to be transduced. At a multiplicity of infection (MOI) of 300, a more than 4600-fold increase in VEGF expression could be detected by using a VEGF-ELISA. A MOI of 100 resulted in an about 240-fold increase in VEGF expression. Since we have noticed cytotoxic effects at higher viral titers, further experiments were conducted by using a MOI of 100. The transduction efficiency at this MOI was estimated to be about 80 % by counting GFP-positive cells (data not shown).

Expression efficiency was confirmed by quantitative real time RT-PCR (Fig. 3). Quantification of VEGF mRNA revealed high levels of VEGF gene expression in VEGF Adv transduced cells as well as in cells transduced simultaneously with VEGF and Ang-1

adenoviruses (Fig. 3a). The same was true for Ang-1 expression in cells transduced with Ang-1 Adv or cells transduced with VEGF and Ang-1 adenoviruses (Fig. 3b).

Time course experiments performed with osteogenically differentiated MSCs transduced simultaneously with VEGF and Ang-1 adenoviruses revealed long lasting transgene expression for up to 15 days of observation after transduction with a peak at day 8 (Fig. 4). Interestingly, expression kinetics of both genes were very similar as both genes showed a sharp decline in expression at day 9.

In order to test the biological activity of VEGF and Ang-1 secreted from transduced cells, we assessed sprouting, proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs) in response to conditioned medium obtained from transduced osteogenically differentiated MSCs.

The three-dimensional spheroid sprouting assay represents an in vitro angiogenesis assay, which is suitable for the detection of pro-angiogenic molecules, such as VEGF (Haspel et al., 2002). In the spheroid sprouting assay, spheroids generated from HUVECs were embedded into collagen type I gels and incubated for 48 h with control medium or conditioned medium (CM) from non-transduced or transduced cells. As a positive control, we have used recombinant VEGF (25 ng/ml) in control medium. As shown in figure 5, conditioned medium from non-transduced osteogenically differentiated MSCs was not able to increase HUVEC sprouting over baseline levels (unstimulated). Recombinant VEGF increased spheroid sprout length to 326.2 $\pm$ 91.3 µm. Similarily, CM from VEGF Adv transduced cells as well as from cells transduced simultaneously with VEGF and Ang-1 adenoviruses, led to a significant increase in sprout formation. In contrast, CM from Ang-1 transduced cells failed to significantly increase sprout formation of HUVEC.

Cell proliferation of HUVECs was assessed by using a colorimetric assay (Fig. 6). HUVEC proliferation was low in the unstimulated group but could be significantly elevated by addition of CM from non-transduced osteogenically differentiated MSCs which express

endogenous VEGF. However, the mitogenic response of HUVECs was significantly higher when cells were incubated with CM from VEGF transduced cells, Ang-1 transduced cells or cells transduced by both adenoviruses.

Finally, the apoptosis level of HUVECs under low serum conditions was quantified by TUNEL staining (Fig. 7). Cultivation of HUVECs under low serum conditions (1.5 % FCS) for 3 days provoked a strong apoptotic response with 40.4 $\pm$ 19.1 % of apoptotic cells. Addition of CM from cells transduced individually by VEGF or Ang-1 adenoviruses or by both viruses, strongly suppressed HUVEC apoptosis with a similar efficiency as recombinant VEGF, which was used as a positive control in this experiment.

In summary, our experiments have demonstrated high efficient transduction of the angiogenic growth factor genes VEGF and Ang-1 into osteogenically differentiated human mesenchymal stem cells. Moreover, by using various functional in vitro assays, we were also able to show that both angiogenic growth factors, secreted from transduced cells, were biologically active.

Discussion

In the present in vitro study, we intended to investigate whether osteogenically differentiated MSCs are suitable for adenoviral-mediated expression of the angiogenic growth factors VEGF and Ang-1 to achieve neovascularization in later in vivo experiments.

Adult mesenchymal stem cells have a high therapeutic potential as an autologous cell source for various applications in regenerative medicine and tissue engineering. In particular, these cells are of high interest for bone tissue engineering, since autologous MSCs can be easily isolated from a patient´s bone marrow, show a high proliferation potential and can be ex vivo differentiated toward the osteoblastic lineage without difficulty (Marion & Mao, 2006). As already demonstrated elsewhere (Jager et al., 2005; Rodriguez et al., 2004), osteogenic

differentiation of expanded MSCs was inducible by simply changing the growth medium to differentiation medium (containing dexamethasone, β-glycerolphosphate and ascorbic acid). Osteogenic differentiation was verified by monitoring the expression of the osteogenic differentiation markers alkaline phosphatase (AP) and osteocalcin and by assessing mineralization of the extracellular matrix.

The osteogenically differentiated MSCs were used for adenoviral transduction studies with adenoviruses encoding VEGF and Ang-1. Since we intend to use these genetically manipulated cells in subsequent in vivo experiments for improving vascularization in bone tissue engineering applications, we have also performed cotransfection studies with both viruses in order to deliver both angiogenic growth factors simultaneously.

The transduction efficiencies and the release of the expression products were very high with an approximately two order of magnitude increased transgene expression in relation to non-transduced cells. Furthermore, the transduced cells showed a long lasting expression of the transgenes for up to 15 days with peak levels at about 8 days after transduction. The time course of transgene expression reported in our study is very similar to previously published results describing adenoviral transduction of VEGF into skeletal myoblasts (Ye et al., 2003). The transient nature of transgene expression is characteristic for adenovirus-mediated transduction. We assume that a transient expression of the angiogenic growth factors would be sufficient to induce the angiogenic process. Of note, permanent transgene expression may in fact result in deleterious effects in vivo. Therefore, we believe that the cell-based delivery of angiogenic growth factors using adenoviral transduced osteogenically differentiated MSCs is a safe method for gene delivery to bone tissues.

As shown by the results from the HUVEC sprouting, proliferation and apoptosis assays, VEGF and Ang-1 proteins secreted from transduced cells were biologically active. Secreted VEGF was able to induce an angiogenic response in the HUVEC spheroid sprouting assay, induced HUVEC proliferation and acted as a survival factor in the apoptosis assay. Similar to

VEGF, Ang-1 secreted from transduced cells inhibited endothelial cell apoptosis. This is in line with other reports describing Ang-1 as an anti-apoptotic growth factor for endothelial cells (Kim et al., 2000a; Papapetropoulos et al., 2000).

In the proliferation assay decribed in this study, Ang-1 produced by transduced cells showed a robust mitogenic effect on HUVECs. The role of Ang-1 as an endothelial cell mitogen is still a matter of debate. Some authors describe Ang-1 as a potent mitogen of endothelial cells (Kanda et al., 2005), while others showed that Ang-1 failed to induce proliferation (Hayes et al., 1999) or induced proliferation of endothelial cells only very weakly (Koblizek et al., 1998). The relatively strong mitogenic activity of Ang-1 reported in our study may be explained by the high concentration of this growth factor present in the conditioned medium of transduced cells or by synergy in the mitogenic activity of Ang-1 and other undefined growth factors secreted by osteogenically differentiated MSCs.

In contrast to VEGF, Ang-1 expressed in transduced MSCs failed to elicit HUVEC sprouting. This result is unexpected, since this ligand has previously been reported to induce sprouting of a great variety of primary endothelial cell types such as pulmonary artery endothelial cells (Kim et al., 2000b), bovine aortic endothelial cells (Hayes et al., 1999), adrenal-cortex-derived microvascular endothelial cells (Koblizek et al., 1998) and HUVECs (DeBusk et al., 2004) in various in vitro angiogenesis assays based on fibrin or collagen matrices. The fact that we have not been able to detect an angiogenic activity in conditioned medium of Ang-1 transduced MSCs in our spheroid sprouting assay may be explained by the massive overexpression of this ligand. Therefore, titration experiments would be necessary to identify the optimal concentration of Ang-1 to elicit this particular cellular response. However, the finding of robust cellular responses in terms of HUVEC survival and proliferation clearly supports the notion that Ang-1 produced by transduced osteogenically differentiated MSCs is biologically active.

In summary, we have demonstrated that adenoviral transduced osteogenically differentiated MSCs are able to express and secrete high amounts of biologically active VEGF and Ang-1. The transduced osteogenically differentiated MSCs described in this paper may be suitable for inducing neovascularization in bone tissue engineering applications.

Acknowledgments

We thank B. vom Hoevel for excellent technical assistance and A. Momeni for critical reading of the manuscript. This work was supported by funding through the Deutsche Forschungsgemeinschaft (STA-472/1-1).

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Figure Legends

Fig. 1. Morphology and immunohistochemical characterization of non-differentiated (control) and osteogenically differentiated human MSCs. Phase contrast images of non-differentiated (a) and osteogenically differentiated (b) MSCs. Immunohistochemical analysis of non-differentiated (c, e) and osteogenically differentiated (d, f) MSCs for alkaline phosphatase (c, d) and osteocalcin (e, f). Verification of mineralization of the extracellular matrix by von Kossa staining (g, h). Magnification 200 x.

Fig. 2. Determination of VEGF expression as a function of multiplicity of infection (MOI) by ELISA. 2 x 10^5 cells per well were seeded in 6 well cluster plates and transduced with VEGF Adv at various MOIs. VEGF was detected in supernatants of the cells after 3 days of infection. Bars represent means $\pm$ SD of three experiments. Statistically significant differences between groups are indicated for $p<0.0003$(*).

Fig. 3. Determination of VEGF (a) and Ang-1 (b) mRNA expression by quantitative real time RT-PCR in osteogenically differentiated MSCs after transduction with VEGF Adv, Ang-1 Adv or after transduction by both viruses at MOIs of 100 for each virus. As controls, non-transduced cells were used. Bars represent means $\pm$ SD of three experiments. Statistically significant differences between groups are indicated for $p<0.01$(*).

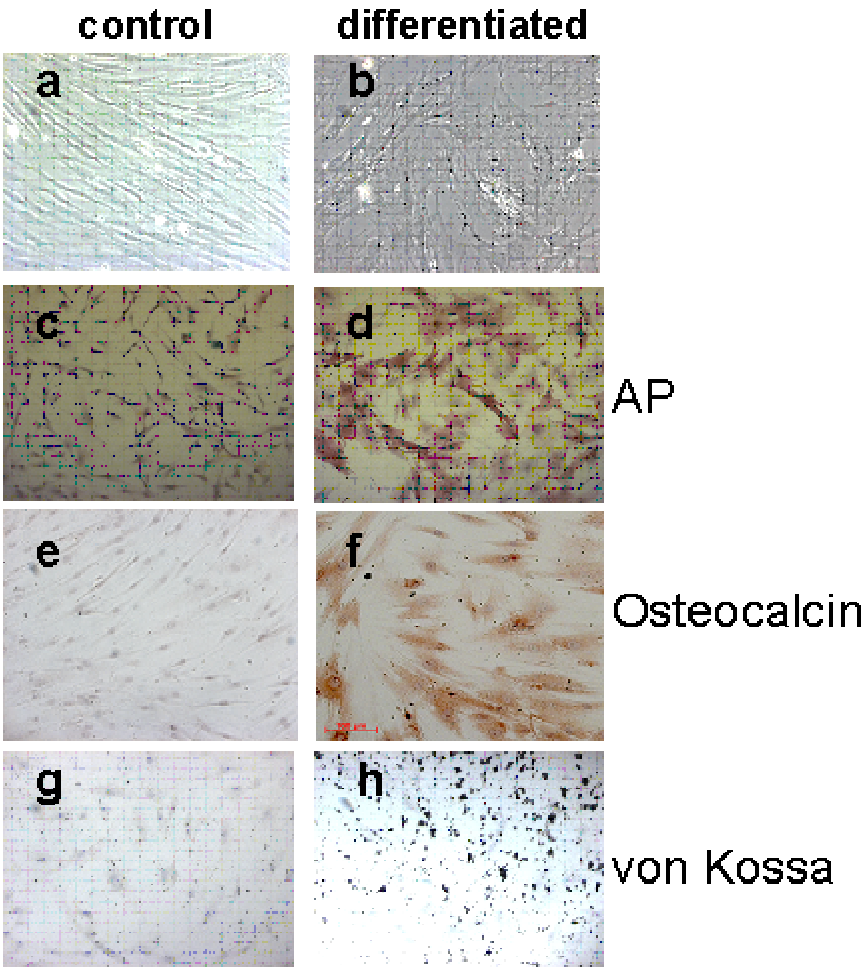
Fig. 4. Time course of VEGF (a) and Ang-1 (b) protein expression determined by ELISA. 2 x 10^5 cells per well were seeded in 6 well plates and transduced with VEGF Adv and Ang-1 Adv at a MOI of 100, respectively. As controls, non-transduced cells were used. Data points represent protein levels produced during 24 h intervals. Means + SD from three experiments are shown.

Fig. 5. Quantification of sprout formation of HUVEC spheroids in collagen gels grown in the absence of conditioned medium (CM) (unstimulated), grown in the presence of CM from non-transduced osteogenically differentiated MSCs (CM control) or grown in the presence of CM from cells transduced individually by VEGF Adv and Ang-1 Adv or transduced by both viruses simultaneously (CM VEGF/Ang-1 Adv). As a positive control, recombinant VEGF (25 ng/ml) was used (rec. VEGF). Cumulative length of all sprouts originating from the central plain of an individual spheroid was measured by semiautomatic analysis after 48 h of growth. Mean values $\pm$SD of 10 individual spheroids per experimental group are shown. Statistically significant differences between groups are indicated for $p<0.0001$(*).

Fig. 6. Proliferation of HUVECs grown in the absence of conditioned medium (CM) (unstimulated), grown in the presence of CM from non-transduced osteogenically differentiated MSCs (CM control) or grown in the presence of CM from cells transduced individually by VEGF Adv and Ang-1 Adv or transduced by both viruses simultaneously (CM VEGF/Ang-1 Adv). As a positive control, recombinant VEGF (30 ng/ml) was used (rec. VEGF). Proliferation was measured by a colorimetric assay after 3 days of growth. Mean values $\pm$ SD from three experiments are shown. Statistically significant differences between groups are indicated for $p<0.013$(*).

Fig. 7. Quantification of HUVEC apoptosis by TUNEL staining. HUVECs were grown under low serum conditions (1.5 % FCS) either in the absence (unstimulated) or presence of CM from non-transduced (CM control) or CM from cells transduced individually or in combination with VEGF Adv and Ang-1 Adv. As a positive control, recombinant VEGF (30 ng/ml) was used (rec. VEGF). After three days of growth, TUNEL staining was performed and the percentage of apoptotic cells was calculated for each group. Shown are means $\pm$ SD

from three experiments. Statistically significant differences between groups are indicated for $p<0.03$(*).


control
differentiated
a
b
c
d
AP
e
f
Osteocalcin
g
h
von Kossa
Fig.1

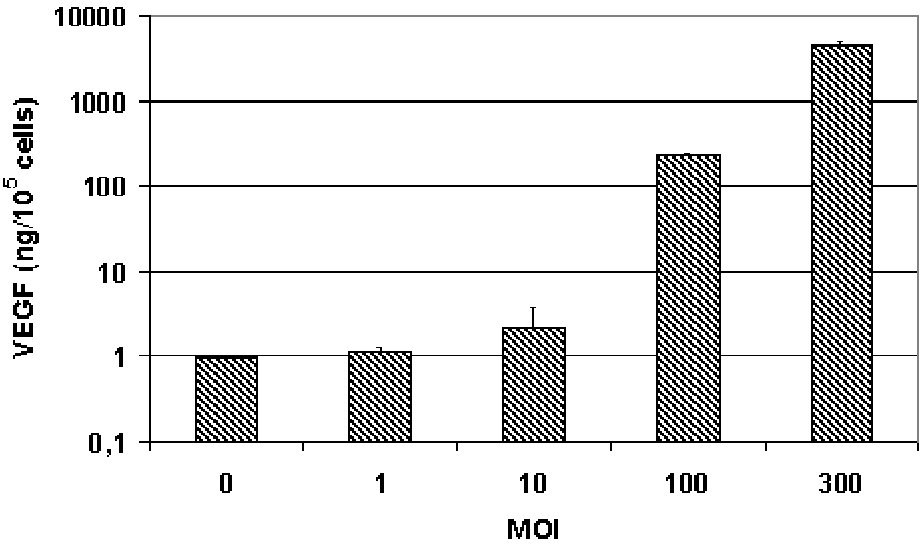


Fig. 2

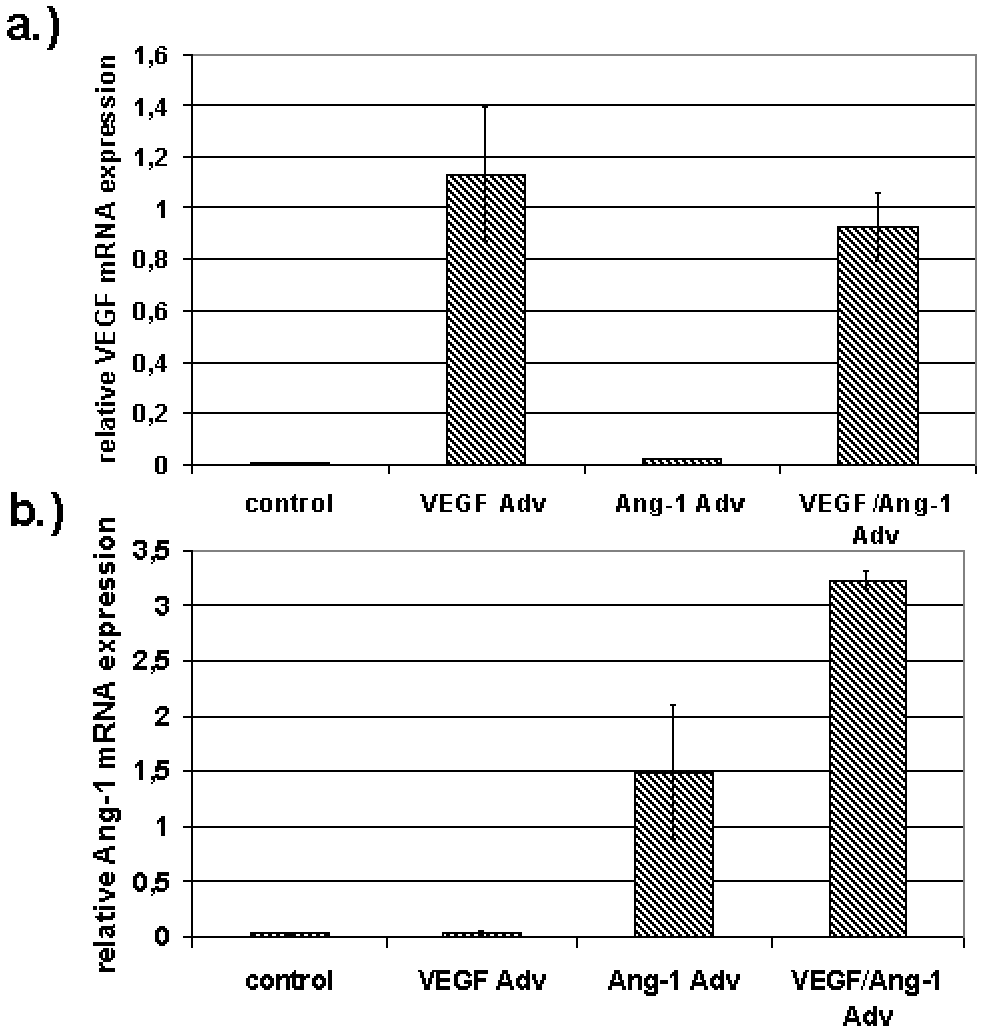

a.)
relative VEGF mRNA expression
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
control
VEGF Adv
Ang-1 Adv
VEGF /Ang-1 Adv
b.)
relative Ang-1 mRNA expression
3,5
3
2,5
2
1,5
1
0,5
0
control
VEGF Adv
Ang-1 Adv
VEGF/Ang-1 Adv

Fig. 3

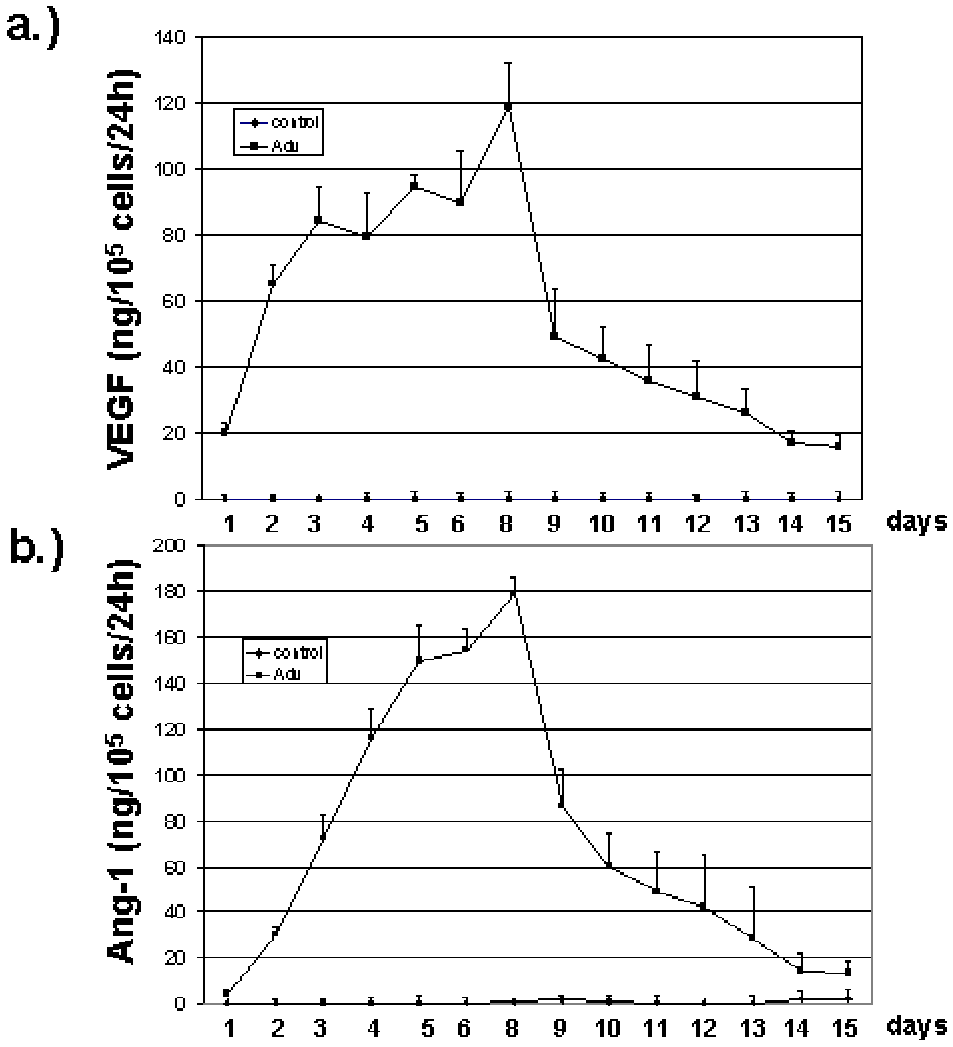

a.)
VEGF (ng/10^5 cells/24h)
control
Adu
0
20
40
60
80
100
120
140
1 2 3 4 5 6 8 9 10 11 12 13 14 15
days
b.)
Ang-1 (ng/10^5 cells/24h)
control
Adu
0
20
40
60
80
100
120
140
160
180
200
1 2 3 4 5 6 8 9 10 11 12 13 14 15
days

Fig. 4

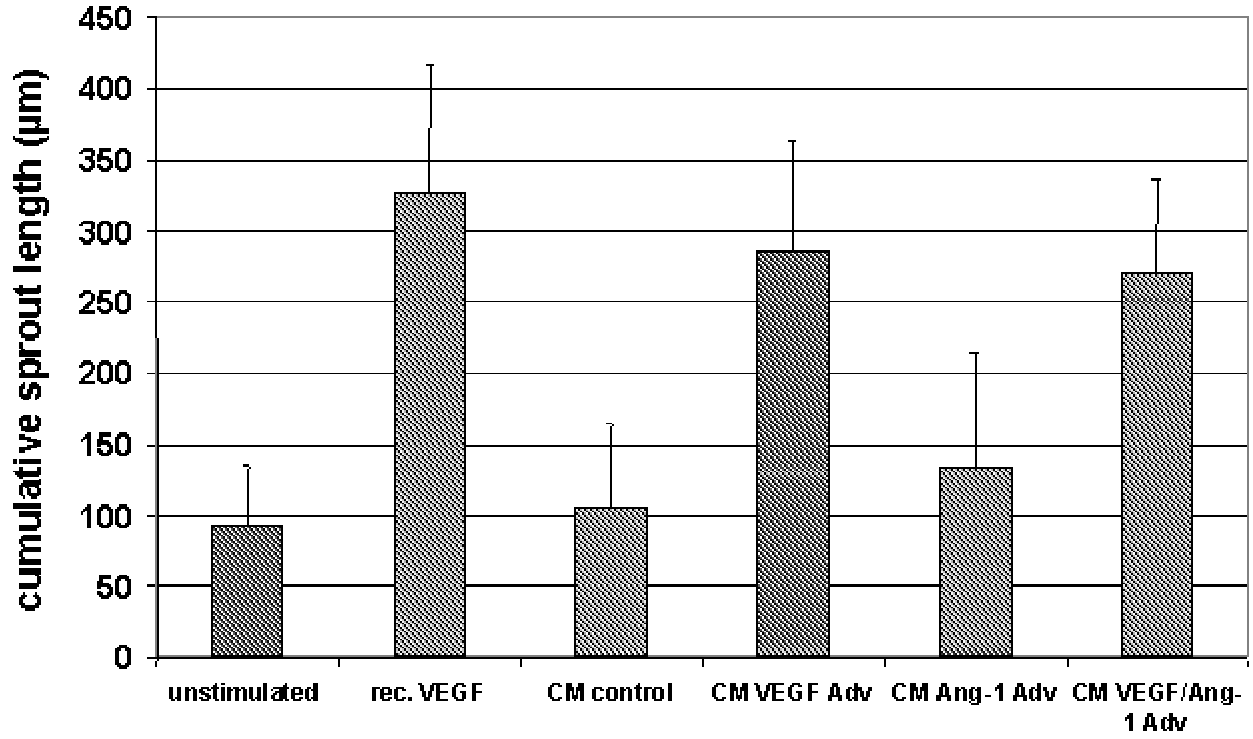

cumulative sprout length (µm)
450
400
350
300
250
200
150
100
50
0
unstimulated
rec. VEGF
CM control
CM VEGF Adv
CM Ang-1 Adv
CM VEGF/Ang-1 Adv

Fig. 5

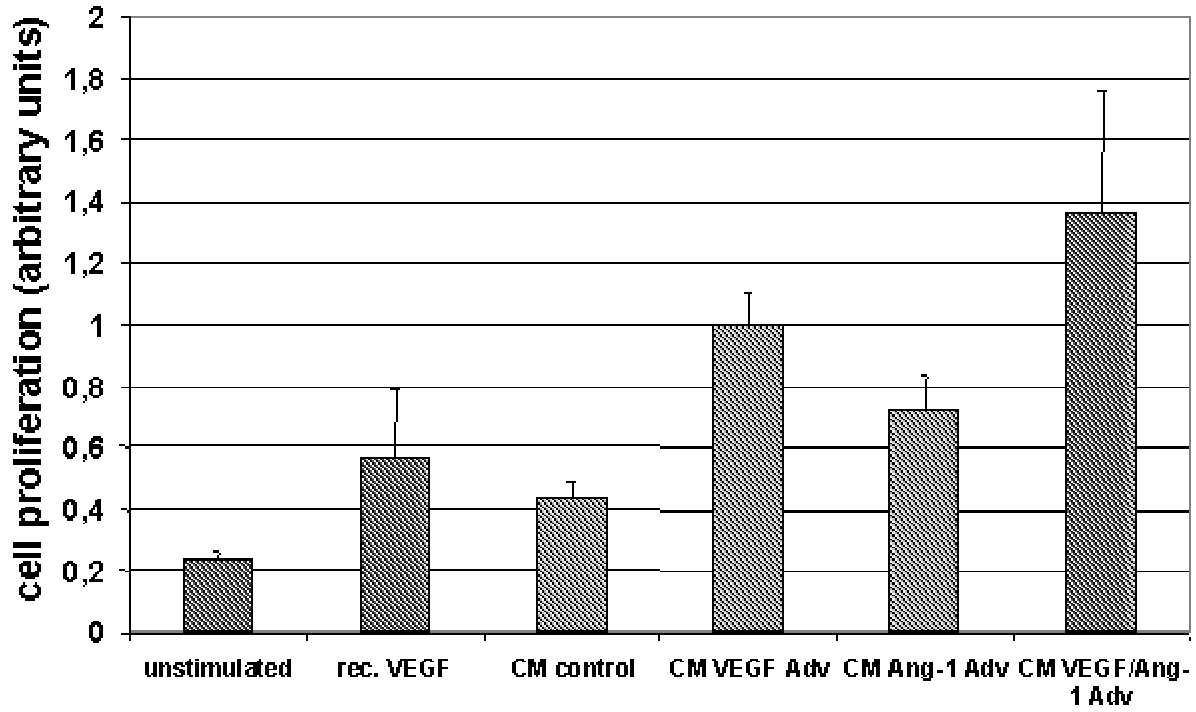

cell proliferation (arbitrary units)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
unstimulated
rec. VEGF
CM control
CM VEGF Adv
CM Ang-1 Adv
CM VEGF/Ang-1 Adv

Fig. 6

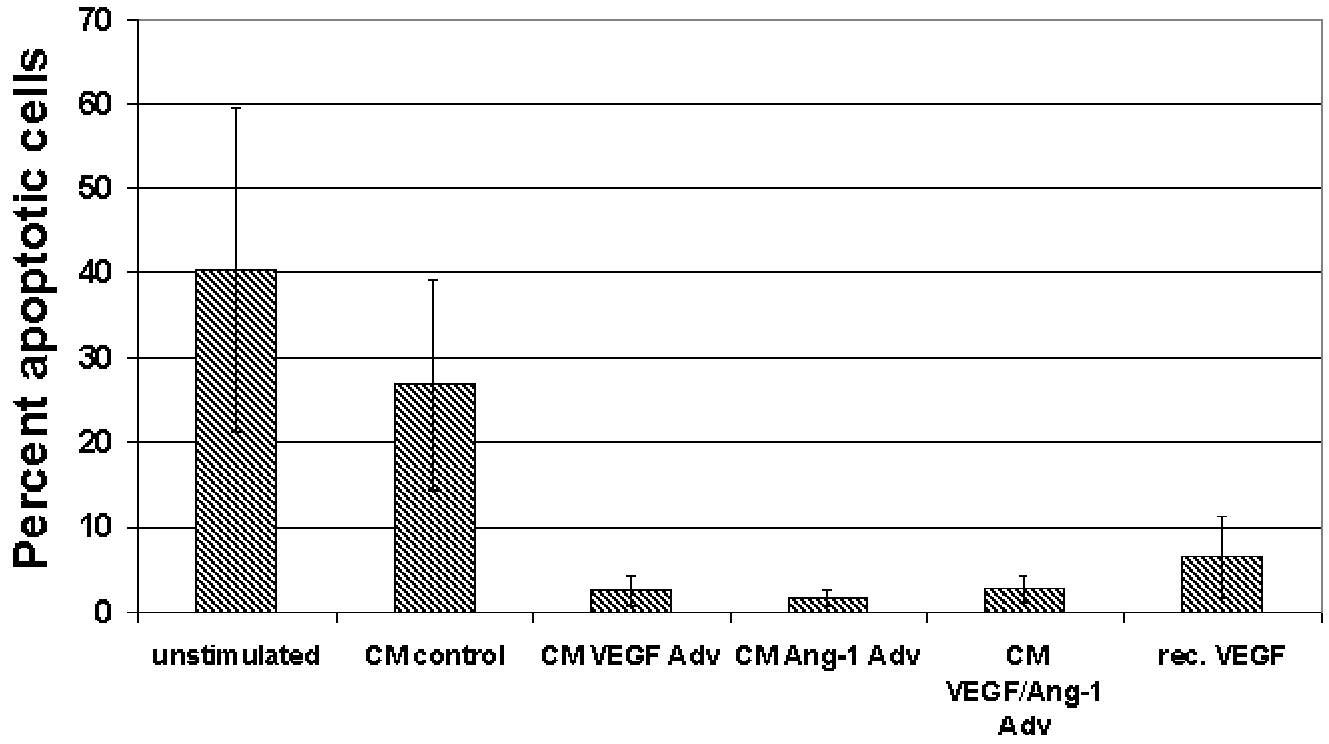

Percent apoptotic cells
70
60
50
40
30
20
10
0
unstimulated
CM control
CM VEGF Adv
CM Ang-1 Adv
CM VEGF/Ang-1 Adv
rec. VEGF

Fig. 7