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In vitro differentiation of mesenchymal stem cells into endothelial cells

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ABSTRACT

The aim of the investigation was to detect the in vitro differentiation of mesenchymal stem cells (MSCs) into cells of the endothelial lineage. MSCs were generated from mononuclear bone marrow cells from healthy donors separated by density gradient centrifugation. Cells were characterized by flow cytometry using monoclonal antibodies and were tested for their potential to differentiate various mesenchymal lineages. The isolated MSCs were positive for the markers CD105, CD73, CD166, CD90, and CD44 and negative for typical hematopoietic and endothelial markers. They were able to differentiate into adipocytes and osteocytes after cultivation in respective media. Differentiation into endothelial-like cells was induced by cultivation of confluent cells in the presence of 2% fetal calf serum and 50 ng/ml vascular endothelial growth factor (VEGF). Laser scanning cytometry analysis of the confluent cells in situ showed a strong increase of expression of endothelial-specific markers like KDR and FLT-1. The functional behavior of the differentiated cells was tested with an in vitro angiogenesis test kit where cells formed characteristic capillary-like structures. These predifferentiated cells provide new options for engineering of artificial tissues based on autologous MSCs and vascularized engineered tissues.

Keywords: Bone marrow cells, endothelial differentiation, Endothelial cell, Mesenchymal stem cells.

INTRODUCTION

Adult bone marrow contains two types of stem cells: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) [1]. Both cell types can be isolated from the mononuclear cell fraction of bone marrow aspirates, and HSCs can be further enriched by immunomagnetic isolation based on specific surface antigens like CD34 or CD133. MSCs lack a unique surface antigen that could be used for positive selection, and therefore the general strategy for the enrichment of MSCs is based on the adherence of cells to plastic dishes in medium with low serum [1-3]. *In vitro* MSCs grow as a homogenous population of adherent cells and express a set of marker proteins on their surface, including CD105 and CD73 [1-3], sometimes also referred to as SH2 and SH3, CD44, CD90, and CD29 [1-5]. Since these markers are not specific for MSCs, they are mainly characterized by their ability to differentiate into multiple mesenchymal lineages, including osteocytes, chondrocytes, adipocytes, and skeletal muscle cells under controlled *in vitro* conditions [2].Bone marrow Stromal Cells are adult stem cells typically found in bone marrow stroma(structural tissue)and other locations throughout the body[3,4].When we consider the various types of adult stem cells ,an advantage of mesenchymal stem cells over other types of adult stem cells, they are clinically accessible and thus relatively easy to obtain from the patient[5,6].Transplantation studies in several mouse models also showed an engraftment at ischemic lesions [7, 8]. Therefore, MSCs fulfill all criteria of stem cells, i.e., self-renewal, multi lineage differentiation, and *in vivo* reconstitution of tissue [9].

Progenitor cells for endothelial cells have been identified both in peripheral blood and in bone marrow. Peripheral blood endothelial progenitor cells (EPCs) can be isolated by magnetic bead selection on the basis of the CD34 antigen, and they were found to be positive for CD34, CD133, and vascular endothelial growth factor (VEGF)

receptor 2, sometimes also referred to as KDR or FLK1 [10]. EPCs originate in the bone marrow and can be mobilized either endogenously by tissue ischemia or exogenously by cytokine stimulation or HMG-CoA reductase inhibitors [11, 12]. CD133-selected cells from peripheral blood were also shown to have the capacity to differentiate into endothelial cells under defined conditions [13, 14]. Multipotent adult progenitor cells (MAPCs) were isolated from bone marrow by depleting hematopoietic cells from the bone marrow cell fraction and plating the resulting cells. MAPCs are positive for the VEGF receptors KDR and FLT1 and dimly positive for CD44 and CD133 [15, 16]. Besides their ability to differentiate in numerous mesenchymal tissues, they were also shown to differentiate in endothelial and neuronal cells *in vitro* and *in vivo* [16, 17], indicating a greater developmental potential of MAPCs compared with MSCs. The aim of our study was to test *in vitro* if bone marrow MSCs isolated by density centrifugation and positive for the markers CD105 (SH2) and CD73 (SH4) were capable of differentiation into endothelial cells. We have therefore established a protocol based on low-serum culture supplemented with VEGF. We can show that under these conditions, MSCs acquire several features of mature endothelium, including the expression of VEGF receptors, VE-cadherin, VCAM-1, and von Willebrand factor (vWF). They show also an enhanced ability to form capillary structures in semisolid medium. MSCs may therefore be an alternative source for endothelial progenitors for clinical therapies like tissue replacement or vascularization of artificial organs. In addition, the *in vitro* differentiation of MSCs might be a useful model for the elucidation of the role of VEGF for differentiation and maturation of endothelial cells.

MATERIALS AND METHODS

Isolation and Culture of Bone Marrow-Derived Human MSCs

Bone marrow samples were collected from healthy donors (age 23-47 years) at The Bone Marrow Transplantation Center of the University Hospital Carl Gustav Carus, Dresden, after obtaining informed consent. The study was approved by the local institutional review board. MSCs were isolated and cultured according to modifications of previously reported methods [1-3]. Briefly, 5-7 ml bone marrow aspirate was diluted 1:5 in phosphate-buffered saline (PBS) containing 0.5% human serum albumin.

A 20-ml aliquot was layered over a Percoll solution and centrifuged at 900 *g* for 30 minutes at room temperature. Mononuclear cells at the interface were recovered, pressed through a 100-µm nylon cell strainer and washed twice in PBS 0.5% HSA. All cells were seeded into 75- cm2 flasks containing Dulbecco's-modified Eagle's Medium- Low Glucose, penicillin 10 U/ml, streptomycin 100 µg/ml (both from Biochrom), and 10% fetal calf serum (FCS).

MSC cultures grew at 37°C in 5% CO2. Nonadherent cells were removed after 24 hours by washing with PBS 0.5% HSA. The medium was changed subsequently every 4 days. Two weeks later the culture reached 90% confluency. MSCs were recovered using 0.25% Trypsin- EDTA and replated at a density of 5,000-6,000 cells per cm^2 of surface area as passage 1 (P1) cells [3].

Differentiation of M SCs into Osteocytes and Adipocytes

For osteogenic differentiation, a 70% subconfluent culture of MSCs from passages P0-P2 was used. Cells were incubated in osteogenic medium with 10–7 M dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate. The medium was replaced twice a week. After day 15 in differentiation medium, cell colonies displayed bone-like nodular aggregates of matrix mineralization. The mineral deposition could be visualized by Kossa staining for calcium [1]. Briefly, cell layers were fixed with 10% formalin (Sigma) for 30 minutes, incubated with 2% silver nitrate solution (weight/volume [w/v]) for 15 minutes in the dark, and developed with 1% pyrogallol. The layer was washed thoroughly with deionized water. The alkaline 379 Differentiation of MSCs into Endothelial Cells phosphatase in osteogenic differentiated cells was determined by using the histochemical, semiquantitative kit for alkaline phosphatase. For adipogenic differentiation, MesenCult medium with adipogenic stimulatory supplements was used. The medium was replaced every 3-4 days for 21 days.

Endothelial Cell Differentiation

Confluent cells were cultivated in the presence of 2% FCS and 50 ng/ml VEGF for 7 days. Medium was changed every 2 days.

Immunohistochemistry

For von Willebrand staining, cells were fixed with methanol at -20°C for 10 minutes and rinsed with PBS. Samples were incubated with an antibody against vWF for 30 minutes, rinsed with PBS, and incubated with a labeled secondary goat anti-mouse antibody

Flow Cytometry

Cells were trypsinized, washed with PBS, and incubated with antibodies against CD34, CD45, CD44, CD73, CD90, VE-cadherin, VCAM-1 (all Becton-Dickinson), CD105, CD117 (Dianova), KDR and FLT-1 (both Sigma), and CD133-1. Analysis was performed with a FACScalibur flow cytometer (Becton Dickinson).

Laser Scanning Cytometry Analysis of MSCs

Cells were grown in chamber slides; stained with antibodies against KDR, FLT-1, VCAM-1, and VE-cadherin; rinsed with PBS; and stained with a secondary goat anti-mouse antibody labeled with fluorescein isothiocyanate (FITC; Dianova). After that, cells were again rinsed with PBS and fixed with 4% paraformaldehyde (Sigma) in PBS for 15 minutes at room temperature. Cells were again rinsed with PBS and permeabilized with 0.1% Triton (Sigma) in PBS for 5 minutes at room temperature. Subsequently, cells were rinsed with PBS and stained with 0.1 µg/ml TO-PRO-3 Iodide (Molecular Probes; Eugene, OR; http://www.probes.com) in PBS for 1 hour. Finally, cells were rinsed and mounted on a microscopic slide and subsequently analyzed on a LSC 2 laser scanning cytometer with an air-cooled 15-mW 488-nm argon-ion laser for FITC excitation and with a 25-mW 633-nm helium-neon laser for TO-PRO-3 Iodide excitation using a 20× objective. FITC signals were detected through a 505-530-nm green filter and TO-PRO-3 signal through a 650-nm long-pass filter. Contouring of cells was achieved by nuclear staining with TO-PRO-3 Iodide. Photomultiplier tube (PMT) settings for PMT voltage, offset, and gain were 30%, 2070, and 255, respectively, for green and 15%, 2070, and 255, respectively for far-red. As minimal area 10 µm2 were defined and 12 pixels were added to cover the whole cell. Data were acquired and analyzed with WinCyte acquisition software.

***In Vitro* Angiogenesis**

Analysis of capillary formation was performed using the in vitro angiogenesis kit according to the manufacturer's instructions. Fifty microliters of gel matrix solution were applied into one well of a 96-well plate and incubated for 1 hour at 37°C. Cells were then trypsinized and 5×10^3 cells were suspended in 50 µl of the DMEM containing various concentrations of VEGF and plated onto the gel matrix and incubated for 2 hours. Cells were counted by eye for the formation of capillary structures. The percentages of formed capillaries were calculated from two independent experiments.

RESULTS

Cell Culture of MSCs

MSCs were isolated according to standard techniques for the isolation of mononuclear cells from bone marrow using density gradient centrifugation. Phase contrast microscopy from cells in passage P0 demonstrated a fibroblast-like, spindle- shaped morphology (Fig. 1). In later passages (>P5), the spindle-shaped cells began to display a broadened, flat morphology. Therefore, endothelial experiments were performed only on cells from P1 to P5. Cells were tested with flow cytometry for the presence or absence of characteristic hematopoietic and endothelial markers. MSCs typically expressed the antigens CD105 and CD73, which are also recognized by the SH2 and SH3 antibodies described by Pittenger *et al*., [2].

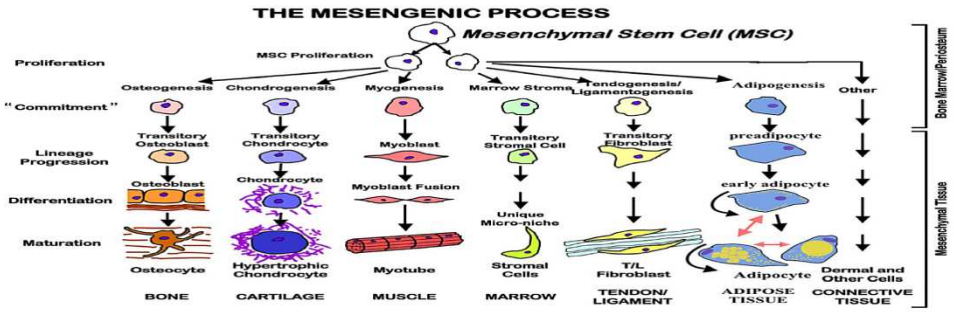


Fig 1: Over view of stem cell production

Furthermore, cells expressed CD166, CD90, and CD44 (Fig. 2A). They were negative for typical lymphocytic markers like CD45 and CD14 and for the early hematopoietic markers CD34 and CD133. Undifferentiated MSCs neither expressed VEGF receptors KDR and FLT-1 nor VE cadherin and VCAM-1(Fig 3A). The ability of the MSCs to differentiate into osteocytes and adipocytes was tested in all cultures from various donors. When cultured in osteogenic medium for 15 days, Oswald, Boxberger, Jørgensen et al. 380 the morphology changed: A) on day 1, a nearly confluent spindleshaped layer; B) day 5-7, cells are positive for APase (data not shown) and form nodular aggregates, and C) day 12-15, cells began to mineralize their matrix and were positive for Kossa staining (Fig. 2a).

They were also able to differentiate into adipocytes, and cells accumulated different amounts of lipid vacuoles (Fig. 2b) after cultivation in adipogenic medium.

Differentiation of MSCs into Endothelial-Like Cells

We introduced differentiation into endothelial-like cells by cultivating confluent MSCs in the presence of 2% FCS and 50 ng/ml VEGF for 7 days. Cell morphology showed no difference compared with undifferentiated MSCs. Immunohistochemical staining for vWF was chosen for the basal characterization of endothelial-like cells. Undifferentiated MSCs showed almost no specific staining for vWF, but after 7 days of cultivationthe overall fluorescence intensity of the differentiated MSCs was markedly enhanced. Also, Weibel-Palade bodies were visible in differentiated MSCs (Fig. 3Ba). Fluorescence-activated cell sorter (FACS) analysis confirmed the expression of vWF (Fig. 3Bb). The ability to form capillaries in semisolid medium was tested with an *in vitro* angiogenesis kit. Undifferentiated and differentiated MSCs were trypsinized and seeded on top of the ECmatrix gel solution. Cells were cultivated in the presence of two different concentrations.

Fig 2A: FACS analysis of typical CD markers present on MSCs.

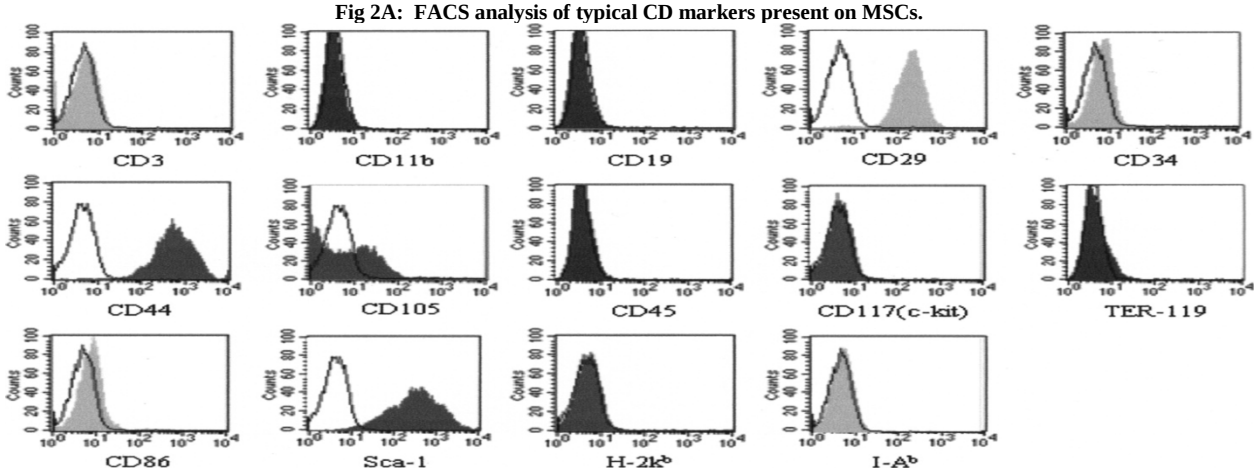
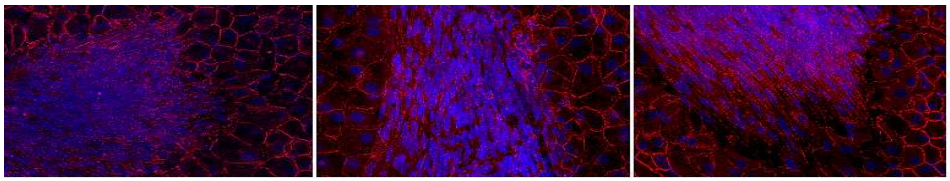


Fig2: B) 30×. a) After differentiation into osteocytes after von Kossa staining. b) After adipogenic differentiation



Differentiation of MSCs into Endothelial Cells of VEGF and once without VEGF. The undifferentiated MSCs showed very few capillaries after 2 hours and most of the cells stayed round in the medium. When cultivated in the presence of VEGF, more tube-like structures were visible. Undifferentiated MSCs showed a substantial formation of capillary structures when cultivated for 2 hours in the presence of 50 ng/ml VEGF. After differentiation more than 80% of MSCs form capillary structures both in the presence and in the absence of VEGF.

For the quantification of endothelial- specific marker expression *in situ* after immunophenotyping, a laser-scanning cytometry (LSC)- based protocol was introduced. LSC is an alternative method to flow cytometry, especially for the analysis of small cell numbers. For contouring of cells, nuclear staining with the DNA stain TO-PRO-3 Iodide was chosen, and specific cellular antigens were stained with an FITC-labeled antibody. LSC analysis of differentiated MSCs showed expression of the VEGF receptors KDR and FLT-1 and also VE-cadherin and VCAM-1. These data show a substantial increase of expression of endothelial-specific marker molecules on MSCs after differentiation with VEGF. Taken together, our data indicate that cultivating in the presence of VEGF leads to a substantial expression of endothelial-specific markers on MSCs.

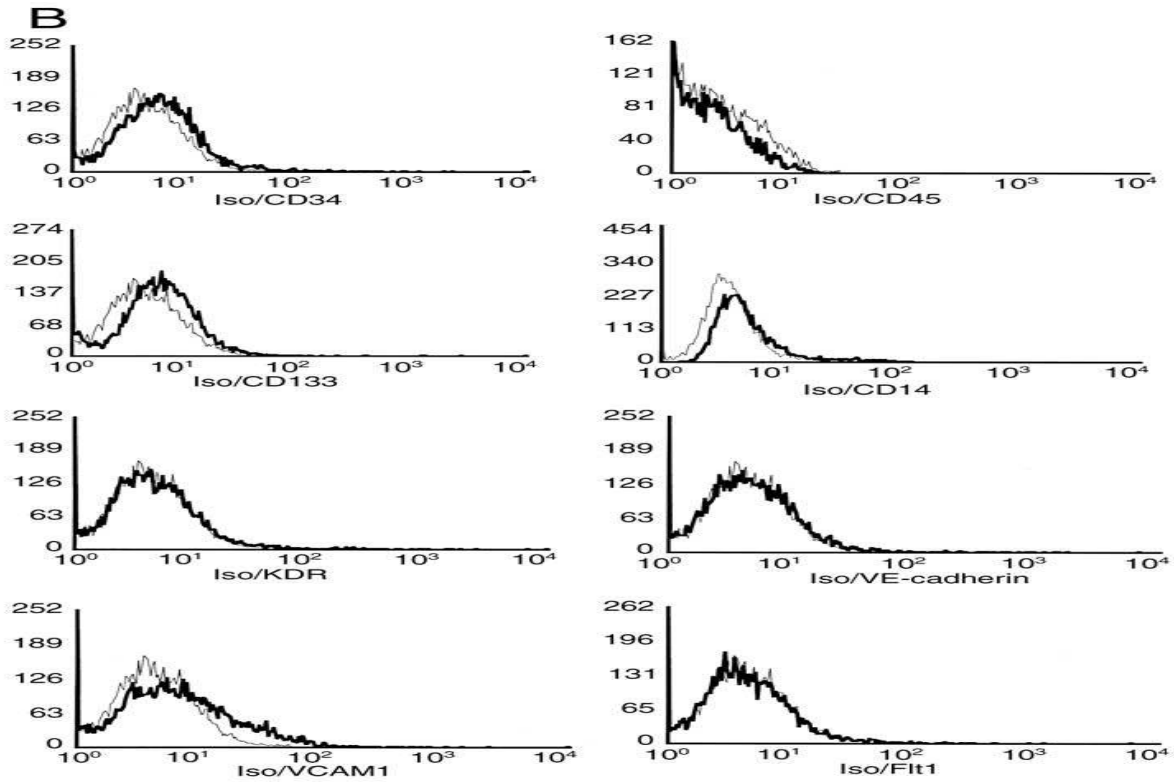
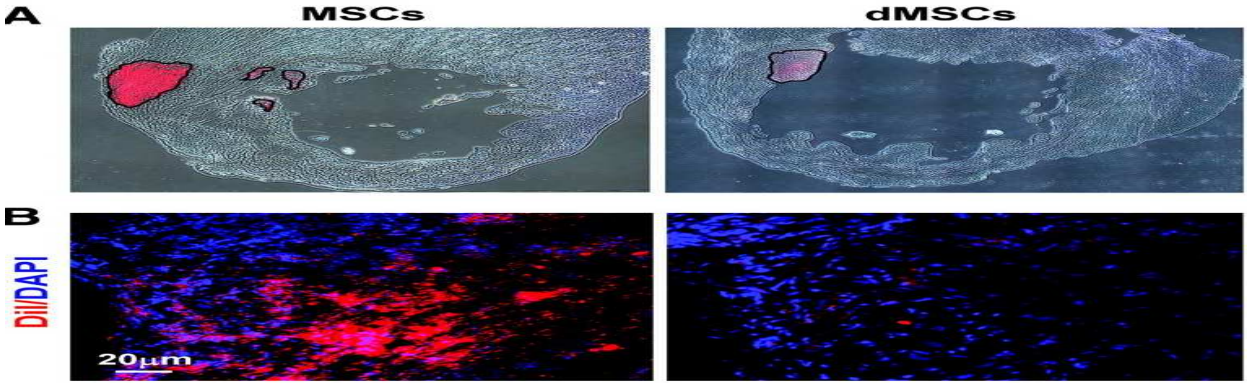


Fig 3A: FACS analysis of typical hematopoietic and endothelial markers which are not expressed by MSCs.

Fig 3B: a) von Willebrand staining of differentiated MSCs.
b) FACS analysis of differentiated MSCs stained with Wf.



DISCUSSION

The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for an autologous or allogeneic cell therapy. Clinical trials for the treatment of osteogenesis imperfecta [18], metachromic leukodystrophy, and Hurler syndrome [19] have proven the therapeutic relevance of transplanted MSCs. The use of autologous vascular endothelial progenitor cells seems attractive for the development of engineered vessels as well as for the vascularization of engineered tissues, and may also be useful to augment vessel growth in ischemic tissue [5, 9]. Our study shows for the first time that human bone marrow- derived CD105+ CD73+ MSCs are capable of differentiating into endothelial cells in vitro, which make them attractive candidates for the development of autologous tissue grafts. Serial analysis of gene expression (SAGE) revealed that single cell-derived colonies of MSCs expressed mRNAs of multiple cell lineages, including characteristic epithelial

Oswald, Boxberger, Jørgensen et al. 382 and endothelial molecules like Epican and Keratins 8 and 10 [20]. These data suggest that the in vitro differentiation potential of MSCs is not restricted to mesodermal lineages but also transdifferentiation of MSCs into other lineages like endothelial could be realized in vitro and in vivo. The formation of endothelial tissue (vasculogenesis) is a process in which the embryo angioblasts are differentiated from mesodermal cells and organized to form a primitive vascular network [21]. Angiogenesis, the formation of new

blood vessels by sprouting from pre-existing vessels, occurs in many situations such as embryonic development and pathological conditions like tissue ischemia. Although the molecular mechanisms responsible for vasculogenesis and angiogenesis are currently not fully understood, the pivotal role of VEGF for both processes is evident [22, 23]. Hence, VEGF is part of all cocktails for the in vitro differentiation of either endothelial progenitor cells or hematopoietic stem cells into endothelial cells in vitro [10, 13-15]. Several populations of bone marrow-derived cells have the potential to differentiate into endothelial-like cells. CD133+ HSCs cultivated at high cellular density and in the presence of endothelial growth factors like VEGF were shown to acquire endothelial features [13, 14], and CD34+ HSCs isolated from peripheral blood can differentiate into endothelial cells in vitro [10] and contribute to vascularization in animal models [7]. A distinct population of adult stem cells called MAPCs were described by the *Verfaillie* group [15]. They are capable of differentiating into endothelial cells in vivo and in vitro [16, 17], but this subset of multipotent cells is probably a different population than the MSCs isolated by plasticadherence. All cited model systems were derived of KDRpositive cells or at least KDR-dim cells, whereas MSCs in our system were KDR negative and CD133 negative, which clearly separates them from the MAPCs used by the *Verfaillie* group and also from EPCs [10, 12]. The major advantage of MSCs is the vast number of cells that can be achieved from one bone marrow aspirate. MSCs were shown to be genetically stable over many passages [2]. One major criticism of studies describing plasticity of bone marrow stem cells is the heterogeneity of the cell population.

Differentiation of MSCs into Endothelial Cells although we cannot rule out the possible existence of subpopulations of committed cells, it appears unlikely since cells do not proliferate during the differentiation in presence of 2% FCS and VEGF. Differentiation experiments with single-cell-derived MSCs will ultimately prove the plasticity of MSCs. In our differentiation system MSCs acquire major characteristics of mature endothelial-like expression of vWF, VEGF receptors 1 and 2 (FLT-1 and KDR), VEcadherin, and VCAM-1. Cells do not express CD31 and CD34 after a 7-day differentiation, which indicates that these markers are obviously later expressed in endothelial maturation. Elongation of differentiation time will probably also lead to an upregulation of these markers. After differentiation, the formation of capillary-like structures in semisolid medium was markedly enhanced when cells were cultivated without VEGF. Recent studies have shown that murine stroma cells can also be differentiated into vasculature-forming cells under hypoxic conditions or when genetically transduced to express VEGF [24, 25]. We also found that MSCs form tubelike structures when cultivated in semisolid medium; the presence of VEGF markedly enhanced this behavior. Interestingly, the numbers of capillary-like cells in this assay were strongly enhanced in predifferentiated MSCs. Hypoxia upregulates several genes involved in angiogenesis like basic fibroblast growth factor, VEGF, the VEGF receptors KDR and FLT-1, and components ofthe plasminogen system [26]. Differentiation of MSCs with VEGF also up regulates the expression of the VEGF receptors KDR and FLT-1, which play a major role in angiogenesis *in vivo* and contribute together with matrix-metalloproteases to the formation of capillary-like structures *in vitro*.

CONCLUSION

Our findings may support the development of tissue engineered vascular grafts based on autologous MSCs. Differentiated MSCs could also be beneficial in the engineering of complex tissues, where vascularization of the tissue is an essential feature for the successful engraftment. Clinical studies will have to prove whether the systemic application of predifferentiated endothelial MSCs may have positive effects in patients with small vessel diseases. In addition, tissues derived from autologous MSCs might engraft easier when the blood supply can be improved by vascularization of artificial bone, cartilage, or other tissues.

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