Human adipose tissue stromal vascular fraction cells differentiate depending on distinct types of media

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Abstract. *Objectives:* Angiogenesis, the process of formation of blood vessels, is essential for many physiological as well as pathological processes. It has been shown that human adipose tissue contains a population of non-characterized cells, called stromal-vascular fraction (SVF) cells, which are able to differentiate into several lineages. The aim of this study was to determine conditions for promoting differentiation of human adipose tissue progenitors towards endothelial cells, as well as to show that SVF cells cooperate with differentiated endothelium in capillary network formation. *Materials and methods:* Stromal vascular fraction cells were isolated according to modified Hauner's method and after adaptation they were cultured in pro-angiogenic or pro-adipogenic medium. Cells were characterized by presence of surface antigens by flow cytometry, and by expression of genes characteristic for endothelial cells or for adipocytes, quantitative real-time polymerase chain reaction. A number of tests were performed to verify their differentiation. *Results:* Differentiation of human SVF cells towards endothelium was stimulated by the presence of serum and absence of adipogenic factors, documented by the pattern of gene expression as well as different functional *in vitro* assays. SVF cells were found to work together with human umbilical vein endothelial cells to form capillary networks. *Conclusions:* Here, we show that differentiation of SVF cells to endothelial cells or adipocyte-like cells depended on the medium used. Our work provides a clear model for analysing the differentiation capacity of SVF cells.

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INTRODUCTION

Several recent publications have indicated that in the body, adipose tissue mass is dependent and regulated by neo-angiogenesis (Rupnick *et al.* 2002; Hausman & Richardson 2004). It has been also demonstrated, that a certain number of undifferentiated cells residing in mature organs play an important role in tissue remodelling and repair during adolescence. However, such cells may also participate in pathological processes associated with inflammation and/or carcinogenesis (Halvorsen *et al.* 2000).

Adipose tissue is an active endocrine organ secreting, among other adipokines, various pro-angiogenic substances, cytokines and growth factors (reviewed by Trayhurn & Beattie 2001). It has been reported that human adipose tissue also contains a population of non-characterized cells that are able to undergo adipogenic, osteogenic, chondrogenic or myogenic differentiation *in vitro*. Such cells, called stromal-vascular fraction (SVF) cells, can be used for repair of tendons and bone as well as skeletal muscle (Planat-Benard *et al.* 2003; Bacou *et al.* 2004; Fraser *et al.* 2006; Gomillion & Burg 2006; Klein *et al.* 2006). It has been suggested that cardiomyocyte-like and endothelial-like cells may be obtained from adipose tissue-derived stromal cells (Bacou *et al.* 2004; Planat-Benard *et al.* 2003, 2004); also there are findings that progenitor cells isolated from adipose tissue may contribute to its remodelling (Bacou *et al.* 2004). The demonstration in animal models that SVF cells have the capacity to promote neovascularization of ischaemic tissue highlights the concept that adipose tissue might be an additional source of endothelial progenitor cells for this purpose (Planat-Benard *et al.* 2003; Bacou *et al.* 2004; Rodriguez *et al.* 2005).

There is evidence that endothelial cells and pre-adipocytes have common progenitors (Planat-Benard *et al.* 2003); pre-adipocytes enhance endothelial cell proliferation (Rehman *et al.* 2004) and secrete vascular endothelial growth factor (VEGF) in hypoxic conditions (Rehman *et al.* 2004), and endothelial cells increase adipose tissue progenitors' proliferation rate (Hutley *et al.* 2001). Wosnitza and co-workers have shown, that adipose tissue contains a population of CD31⁻ as well as CD31⁺ cells, and both cell populations could be differentiated into adipocytes as well as endothelial cells; this could indicate a potential of transdifferentiating endothelial cells to adipocytes (Wosnitza *et al.* 2007). Here, we confirm differentiation of human stromal adipose tissue cells towards adipocyte-like cells or pro-angiogenic-like endothelial cells that may co-operate with differentiated endothelial cells to build a capillary network. The cell culture model that we present here could also be used to study changes in differentiation status of SVF cells isolated from human adipose tissue.

MATERIALS AND METHODS

Materials

All reagents for tissue culture were purchased from Sigma-Aldrich (Steinheim, Germany), except for foetal bovine serum, which was purchased from Gibco (Grand Island, NY, USA), MatrigelTM from Calbiochem (San Diego, CA, USA) and EBM medium from Clonetics (San Diego, CA, USA). Reagents for gene expression were from Invitrogen Life Technologies (Carlsbad, CA, USA) and Qiagen (Valencia, CA, USA). Primary monoclonal antibodies against CD45 (FITC)/CD14(R-PE) were from Caltag Laboratories (Burlingame, CA, USA); VE-cadherin/FITC from Bender MedSystems (Vienna, Austria); CD133/PE from MACS (Bergisch Gladbach, Germany), Miltenyl Biotec GmbH,

Table 1. Composition of media used in the experiments

	Inoculation medium	Adaptation medium	Angiogenic medium	MDI	Adipogenic medium
Basal medium	DMEM/F12 (Sigma)	DMEM/F12 (Sigma)	Endothelial growth medium (EGM; Clonetics)	DMEM/F12 (Sigma)	DMEM/F12 (Sigma)
Foetal bovine serum	10%	–	2%	–	–
Pro-angiogenic supplements and growth factors	–	–	EGM SingleQuots (Clonetics)	–	–
Pro-adipogenic supplements and growth factors	–	human transferrin–10 µg/mL, human insulin–66 nM, hydrocortisone–100 nM	–	IBMX–0.5 nM, dexamethasone–0.25 nM, insulin–66 nM	human transferrin–10 µg/mL, human insulin–66 nM, hydrocortisone–100 nM, triiodothyronine–1 nM

CD34/PE and CD31/PE from Becton Dickinson (San Jose, CA, USA). The ELISA kit for proliferation assays was purchased from Roche (Warsaw, Poland) and HTS FluoroBlock™ Insert cell culture inserts, for migration assays, was purchased from Falcon (Lincoln Park, NJ, USA).

Cell isolation and culture

All work was performed with the permission of the Polish Ethics Commission (no. KBET/56/B/2006) and all patients signed a consent acceptance form before being included in the study.

Isolation and culture of human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins according to the method of Jaffe *et al.* (1973). The cells were then cultured in angiogenic medium (Table 1) for up to six passages.

Isolation of stromal-vascular fraction progenitor cells from human adipose tissue

The primary progenitor stromal-vascular fraction (SVF) cells were isolated from human adipose tissue according to a modified Hauner's method (Hauner *et al.* 2001). Extraneous subcutaneous specimens (5–10 g) were obtained from female patients (age 35–55 years) undergoing gynecological surgery. These were cleared of remaining connective tissue and blood vessels, then collagenase digestion was performed (solution: 200 U/mL, 3 mL/g tissue; approx. 60–90 min, shaking, at 37 °C). Samples were centrifuged at 700 *g* for 10 min at room temperature. The supernatant was discarded and the cell pellet was re-suspended in inoculation medium (IM, Table 1) before being filtered through a 100-µm nylon mesh and centrifuged again (700 *g*, 10 min, room temperature). The resulting pellet was again re-suspended in fresh medium and was filtered through a 40-µm nylon mesh; cells were collected and counted before being seeded in inoculation medium (IM) and incubated for 24 h in a humidified CO₂ incubator (Juan) at 37 °C.

Culture and differentiation of SVF cells

Twenty-four hours after isolation of the SVF cells from adipose tissue, inoculation medium (IM) was changed to fresh adaptation medium (AM, Table 1) (Hauner *et al.* 2001). Cells were cultured for up to two passages (5–10 days of culturing).

Differentiation into endothelial cells

To check the angiogenic properties of SVF progenitors, cells were incubated 24 h in angiogenic medium (endothelial basal medium – EBM with 2% of foetal bovine serum). Then, tests verifying the pro-angiogenic properties of SVF cells were performed using this medium for further cell suspension (see below).

Differentiation into adipocytes

To differentiate into adipocytes, confluent SVF cells were stimulated with mixture of dexamethasone, IBMX and insulin (MDI) mixture (Table 1) (Hauner *et al.* 1989). After 2 days, MDI mixture was removed and cells were cultured in adipogenic medium (Table 1) for up to 26 days.

Gene expression

The trisol method was used for isolation of total RNA from SVF cells at day 1 after isolation from adipose tissue, or from cells incubated 24 h in the angiogenic medium with 2% serum (pro-angiogenic), or from SVF cells incubated 24 h with adipogenic medium (after activation of pre-adipocyte differentiation by MDI mixture added for 48 h). One microgram total RNA was reverse transcribed with using a reverse transcription kit (SuperScript; Promega, Madison, WI, USA) with random hexamers. Quantitative real-time polymerase chain reaction (PCR) was performed with the QuantiTect SYBR Green PCR kit, using primers listed in Table 2. Amplification was performed using the continuous fluorescence detection system DNA Engine Opticon (MJ Research Inc., Waltham, MA, USA)

Data were obtained in a form of sigmoid amplification plots in which fluorescence was plotted against the number of cycles. The threshold cycle (C_T) served as a measure of the starting template amount in each sample. Calculation was performed using the Calculation Matrix for PCR Efficiency program (Pfaffl 2001). Expression rates were calculated as normalized

Table 2. Sequences of primers used in real-time PCR experiments

Gene	Forward	Reverse
18S rRNA	5'-CGGCTACCATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'
CD34	5'-ATGAAAAAGCACCAATCTGACC-3'	5'-CAATCAGGGTCTTTTGGAATA-3'
CD31	5'-GGAAAGCTGTCCCTGATGC-3'	5'-CATCTGGCCTTGCTGTGCTAA-3'
eNOS	5'-CATCACCAGGAAGAAGACCT-3'	5'-TCACTCGCTTCGCCATCA-3'
vWF	5'-GATGGAGTCCAGCACCAGTT-3'	5'-GCTACTTCACACAGGCCACA-3'
Jagged	5'-AAGGAGTGGATGTCACCAGGTCTT-3'	5'-GCGGAAACATTCTTCAAAATATCA-3'
CXCR4	5'-CACCGCATCTGGAGAACCA-3'	5'-TCCTCGGTGTAGTTATCTGAAGTGTATATAC-3'
CXCR12	5'-AGCCAACGTCAAGCATCTCAA-3'	5'-TCTTCAGCCGGGCTACAATC-3'
Notch4	5'-ACCTAGGCGAGGACAGCATTG-3'	5'-CAACTCCATCCTCATCAACTTCTG-3'
LPL	5'-CAGCAAAACCTTCATGGTGAT-3'	5'-CAAGTTTGGCACCCAAGTC-3'
PPAR γ 1	5'-GTGGCCGACAGAAATGACC-3'	5'-CCACGGAGCTGATCCCAA-3'
PPAR γ 2	5'-GATACTGTCTGCAAACATATCACAA-3'	5'-CCACGGAGCTGATCCCAA-3'
C/EBP α	5'-AAGAAGTCGGTGGACAAGAACAG-3'	5'-TGCGCACCAGCATGT-3'
ap2	5'-GCTTTGCCACCAGGAAAGTG-3'	5'-ATGGACGCATTCCACCACCA-3'
adiponectin	5'-GAGATGGCACCCTGGTGA-3'	5'-CCCTTAGGACCAATAAGACCTGG-3'
leptin	5'-GTGCGGATTCTTGTGGCTTT-3'	5'-GGAATGAAGTCCAAACCG-3'
CD36	5'-ACAGATGCAGCAGCCTCATTTCC-3'	5'-CGTCGGATTCAAATACAGCA-3'

C_T difference between the control probe and the sample with adjustment for amplification efficiency. As a control, expression of the reference 18S rRNA gene was used. Electrophoresis of PCR products was performed in 2% agarose gel stained with ethidium bromide.

Analysis of surface antigens by flow cytometry

For monitoring phenotype changes of SVF cells after 5, 7, 30, 47, 53 and 68 days of culture in adaptation medium (AM, Table 1), cells were collected (10^5 cells per sample) using cell dissociation solution (Sigma-Aldrich), washed with phosphate-buffered saline (PBS) and centrifuged (700 g, 10 min, room temperature). They were then re-suspended in PBS containing 10% bovine serum albumin and were incubated with primary monoclonal antibodies against CD45(FITC)/CD14(R-PE), VE-cadherin/FITC, CD34/PE, CD133/PE and CD31/PE diluted 1 : 20 for 30 min unlabeled cells were used as a negative controls. After immunolabelling, cell suspensions were diluted with 20 volumes of PBS and washed. Fluorescence was directly measured using a FACS Calibur Flow Cytometer (Becton Dickinson). Forward light scatter and side scatter were recorded with linear amplification. Fluorescence of FITC was recorded in channel FL1, PE-signal in channel FL2, with compensation between channels FL1 and FL2 set at 26%.

Angiogenic properties of SVF cells and HUVECs

To determine pro-angiogenic properties of SVF cells and HUVECs, tests of function were performed as follows.

Cell proliferation

Bromodeoxyuridine (BrdU) incorporation was used (Colorimetric Cell Proliferation ELISA kit, Roche) to check effects of investigated compounds on proliferation of SVF cells. The cells were incubated for 24 h in two types of medium (angiogenic or adipogenic medium, Table 1) with or without factors [0.2 nM VEGF, 0.5 nM basic fibroblast growth factor (bFGF), 500 nM sphingosine-1-phosphate (S1P), 30 nM of interleukin (IL)-8]. BrdU solution was added at the beginning of the experiment according to the protocol of the ELISA kit and after incubation, cells were fixed and stained according to the same protocol.

Migration assay

To analyse migration properties of SVF cells and HUVECs, cell culture inserts (3 μ m, Falcon) were used. These were placed in the upper two rows of a sterile 24-well cell culture plate to form a migration chamber (insert in the upper chamber and plate in the lower chamber). The SVF cells (up to two passages) or HUVECs (three to six passages) were trypsinized (0.5% trypsin, 5 min, 37 °C), washed in medium, harvested (10^6 cell/mL) and re-suspended in fresh medium (angiogenic or adipogenic medium) to yield a final cell concentration of approximately 10^6 cells/mL 200 μ L cell suspension was added to each cell culture insert (upper chamber). Next, 600 μ L of each medium containing chemoattractant (0.2 nM VEGF, or 0.5 nM bFGF, or 500 nM S1P, or 30 nM IL-8) or control sample without activator were inserted into wells in the lower chamber plate by carefully pipetting factor solution between the walls of the upper and lower chambers. Cells were incubated 24 h at 37 °C in a CO₂ culture incubator. Calcein-AM (2.5 μ M) solution was added to the lower chamber 2 h before the end of the incubation. Migration of cells was quantified in a standard fluorescence microplate reader. Chemotactic activity of cells was expressed as the chemotaxis index, which represents ratio of compound-stimulated number of migrating cells to that of random, non-stimulated number of migrating cells in a control sample (without factors). As a reference, HUVEC migration in the same conditions was used.

Formation of capillaries in three-dimensional MatrigelTM model of angiogenesis

Tubule formation by SVF cells was assessed using MatrigelTM (consisting of extracellular proteins: fibronectin, vitronectin, collagen and fibrinogen, Calbiochem). After trypsinization (0.5% trypsin, 5 min, 37 °C) and washing in medium (angiogenic or adipogenic medium), harvested cells (three to six passages, 10⁶ cell/mL) were re-suspended directly in MatrigelTM and were applied to cell culture dishes which were placed in a humidified CO₂ incubator at 37 °C for 30 min angiogenic or adipogenic medium supplemented or not with VEGF (10 ng/mL – 0.2 nM) was subsequently added to cover each MatrigelTM with cells and the whole was incubated for the next 72 h at 37 °C. Formation of tubules was then investigated using an Olympus light microscope (×10 magnification) and was photographed in the presence of a 1-mm length scale. Three images per well were prepared and analysed. Results are provided as average of total tubule length, measured independently by two experienced investigators.

Incorporation of SVF cells in formation of neocapillaries

To confirm angiogenic properties of SVF cells, the co-culture of the green fluorescent protein (GFP)-transfected SVF cells with unlabelled HUVEC was performed.

Construction of vector containing a GFP gene

An adenoviral vector containing two reporter genes (enhanced green fluorescent protein and β-galactosidase) under the control of two separate human CMV promoters (Ad-CMVβgal-CMVGFP) was constructed as previously described (Konopka *et al.* 2005). Plasmid pUHG16-3 (Gossen & Bujard 1992) was cleaved with enzymes BamHI and XbaI, to obtain an insert containing the β-galactosidase encoding gene, which was subcloned into adenoviral shuttle vector pTrack-CMV (He *et al.* 1998). The adenoviral vector was prepared by recombination of pTrack-CMVβgal-CMVGFP and Ad-Easy 1 in the electrocompetent BJ 5183 bacterial strain. Viral vector Ad-CMVβgal-CMVGFP was produced in the 293 packaging cell line, according to the procedure described earlier (He *et al.* 1998).

Transfection

Transfection of SVF cells by adenoviral vector-containing GFP gene was performed by incubation of 10⁶ cells in angiogenic medium containing viruses (400 MOI). After 24 h incubation in a humidified CO₂ incubator at 37 °C, angiogenic medium was changed to fresh. Fluorescence was observed using an Olympus light microscope (×10 magnification) containing an ultraviolet (UV) light power supply (lamphouse 50 W Mercury, Olympus, Tokyo, Japan). Transfection efficiency was characterized by flow cytometry with the instrument set for fluorescein detection. After transfection, 10⁵ cells were trypsinized, centrifuged (400 g, 10 min, room temperature) and were fixed in 500 µL of 2% paraformaldehyde in PBS for flow cytometry.

Co-culture of SVF cells labelled with GFP with non-labelled HUVECs, in a three-dimensional MatrigelTM model of angiogenesis

Transfected SVF cells as well as HUVECs were trypsinized (0.5% trypsin, 5 min, 37 °C), washed in angiogenic medium (10⁶ cell/mL), harvested (400 g, 10 min, room temperature) and re-suspended immediately in MatrigelTM (SVF cells alone, HUVECs alone, SVF cells mixed with HUVECs in 1 : 1 ratio). Cells in MatrigelTM were then immediately applied to the cell culture dishes and placed in the humidified CO₂ incubator at 37 °C for 30 min angiogenic medium, supplemented or non-supplemented, with VEGF (0.2 nM) was subsequently added to cover cells and MatrigelTM and samples were incubated for the next 72 h at 37 °C. Formation of tubules was investigated using the Olympus light microscope (×10 magnification) after being photographed

in the presence of the 1 mm length scale. Three images per well were captured and analysed. Results are provided as an average of total tubule length measured independently by two experienced investigators. GFP illumination from the same samples was also photographed in UV light (lamphouse 50 W Mercury connected to microscope).

Confirmation of SVF cell participation in formation of new capillaries by HUVEC

Cell staining

Stromal-vascular fraction cells were incubated with 2500 nM of calcein-AM (Fluka, Seelze, Germany) for 30 min and HUVECs were incubated with 100 nM of Mitotracker (Cambrex, Walkersville, MD, USA) for 30 min. After incubation cells were washed using fresh medium and fluorescence was checked.

Co-culture investigation

A co-culture experiment, as described earlier, was executed. Formation of tubules was investigated using the Olympus microscope ($\times 20$ magnification) and was photographed in the presence of the 1 mm length scale. Fluorescence of cells labelled with Calcein or Mitotracker were observed in UV light (lamphouse 50 W Mercury connected to microscope) using filter modules for green and blue excitation; these also were photographed.

Adipogenic properties of SVF cells

Oil red O staining was used to identify accumulation of lipid in droplets of SVF cells cultured in adipogenic medium (Table 1) (Hauner *et al.* 1989).

Statistical analysis

Results of continuous variables were expressed as averages $\pm$ SEM from three to five experiments. Before statistical analysis, normal distribution and homogeneity of variables were tested. Parameters that did not fulfil these tests were logarithmically transformed. Statistical comparisons were made by unpaired *t*-tests for comparisons of quantitative variables. Levels of statistical significance were set at $P < 0.05$. Statistical analyses were performed using Microsoft Excel (version 5.0, Microsoft Corporation, Redmond, WA, USA).

RESULTS

Analysis of isolated and cultured SVF cells

For cell inoculation and attachment, SVF cells isolated from fresh human adipose tissue were cultured in Dulbecco's modified Eagle's medium supplemented with serum (10%, inoculation medium) for 1 day (Hauner *et al.* 2001). Such SVF cells (Fig. 1a) expressed genes characteristic of endothelial cells (Fig. 1b) as well as those for adipocytes (Fig. 1c), indicating the heterogeneous nature of the cell population.

Phenotyping of cultured SVF cells growing in adaptation medium (serum-free AM) by flow cytometry confirmed their heterogeneity (Fig. 2). Cells cultured in AM medium for multiplication demonstrated very high (about 80–90%) expression of CD34 characterizing undifferentiated cells (Verfaillie 1998; Huss 2000). It declined over time of cell culture confirming differentiation of SVF (Fig. 2). Expression of AC133, VE-cadherin and CD31/PECAM (characteristic of endothelium) as well as CD45 and CD14 surface antigens (characteristic of leucocyte/monocyte cells) was observed in adhering cells. The progenitor cell fraction, after 5–10 days of stabilization

Figure 1. Stromal-vascular fraction (SVF) cells 24 h after isolation from adipose tissue and cultured in AM pre-conditioning medium. (a) Light micrograph of culture without staining ($\times 10$ magnification). Electrophoresis of real-time PCR products in 2% agarose gel, stained with ethidium bromide. (b) Endothelial-specific genes and (c) genes characteristic for adipocytes.

Figure 2. Time-dependent expression of the cell surface antigens on adhering SVF cells cultured in AM medium, obtained by flow cytometry.

in adaptation medium (AM), was used for further culture in pro-angiogenic or pro-adipogenic medium.

Medium-dependent pro-angiogenic properties of SVF cells

Gene expression

The observed decrease in CD34 gene expression (Fig. 3a) in both adipogenic and angiogenic conditions argues for differentiation of cells in both types of medium. After 24 h incubation of

Figure 3. Modification of relative expression ratio of genes specific for endothelial cells (a) or adipocytes (b); after 24 h incubation of SVF cells in hormone-supplemented serum-free medium (adipogenic medium) or in serum-containing angiogenic (Angio) medium (pro-angiogenic medium). Gene expression was measured using real-time PCR and compared to that in SVF cells 24 h after isolation from adipose tissue. As a reference gene, the expression of 18S rRNA was used. Significance * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

SVF cells in pro-angiogenic medium, the increase in expression of *eNOS*, *vWF*, *Jagged*, *CXCR4*, *CXCR12* and *Notch4* genes, which are characteristic of primary endothelial cells (Sullivan & Bicknell 2003) was found (Fig. 3a). This was contrary to findings in adipogenic medium where significant down-regulation of these genes was observed (Fig. 3a). In addition, significant up-regulation of PECAM (CD31) argues for changes of phenotype towards endothelial cells in angiogenic medium (Fig. 3a). Expression of chemokine (SDF and IL-8) receptors, *CXCR4* and *CXCR12* (Fig. 3A) suggests activation of homing and promotion of chemotaxis under these conditions (Burman *et al.* 2005; Parker & Katz 2006), yet on the contrary, significant down-regulation

of the adipocyte-characteristic genes (*PPAR γ 1*, *PPAR γ 2*, *C/EBP α* , *ap2*, *leptin*, *LPL*, *CD36*) in SVF cultured in pro-angiogenic medium versus adipogenic medium was observed (Fig. 3b).

Effects of incubation in pro-angiogenic or adipogenic medium on SVF cell pro-angiogenic properties

Proliferation

The proliferation rate of SVF cells was higher in angiogenic medium in comparison with adipogenic medium after 24 h stimulation with pro-angiogenic factors, such as VEGF or IL-8, but not by bFGF or S1P (Fig. 4a). Activation of SVF cell proliferation by use of cytokines was found in cells cultured in adipogenic medium (Fig. 4a).

Migration

Stromal-vascular fraction cells incubated in pro-angiogenic medium but not adipogenic medium demonstrated chemotactic activity (data not shown). Activators used stimulated chemotaxis of angiogenic-cultured SVF cells as well as HUVECs with potency: IL-8 > S1P > VEGF (Fig. 5b). Basic FGF was inactive in this respect (Fig. 4b).

Capillary network formation by SVF cells cultured in pro-angiogenic medium

Spontaneous tubulogenic activity was observed in SVF cells cultured in angiogenic medium but not in adipogenic medium (Fig. 4c). VEGF potentially activated tubulogenic properties of angiogenic medium-incubated SVF cells. An insignificant tendency of capillary network formation by VEGF stimulation was also observed in adipogenic medium-cultured SVF cells (Fig. 4c).

Incorporation of SVF cells into newly formed capillaries in Matrigel

To verify that angiogenic medium-cultured SVF cells could incorporate into the capillary network formed by more differentiated endothelial cells, the co-culture of HUVEC with angiogenic medium SVF cells transfected with GFP was used. Fluorescence of GFP was observed only in the transfected angiogenic medium SVF cells (Fig. 5d), whereas the non-transfected HUVECs had no fluorescence (Fig. 5b). Similarly to HUVECs (Fig. 5a), SVF cells transfected with GFP vector alone formed a capillary-like structure (Fig. 5c). When the same number of HUVECs mixed with SVF cells was used for the co-culture experiments, a potential for tubulogenic activity was observed (Fig. 5e,i). Incorporation of GFP-labelled angiogenic medium SVF cells into newly formed capillary network (by HUVECs) was observed (Fig. 5f). Presence of VEGF increased this effect (Fig. 5g–i).

To confirm that SVF cells indeed took part in formation of new capillaries together with HUVECs, the same co-culture experiment of those cells was prepared, and in order to distinguish between the two types of cells, fluorescence labelling was performed. SVF cells were incubated with calcein-AM, whereas HUVECs were labelled using Mitotracker. Fluorescence of the two probes was possible by using filter modules for green and blue excitation. Images were taken in UV light ('green filter', 'blue filter') as well as by visible light microscopy ('optical light') for better characterization of photographed structures. Capillaries composed of SVF cells in Matrigel (Fig. 6b) were seen only with the blue filter, where fluorescence of calcein could be observed, whereas capillaries made by HUVECs (Fig. 6a) were observed only with the green filter, where the fluorescence of Mitotracker could be seen. When SVF cells were mixed with HUVECs in the 1 : 1 ratio of the co-culture experiment, cooperation of both types of cell in formation of capillary network were observed both in UV light as well as by light microscopy (Fig. 6c) and this effect was augmented in the presence of VEGF (Fig. 6d).

Figure 4. Analysis of pro-angiogenic properties of SVF cells. Influence of VEGF (0.2 nM), bFGF (0.5 nM), IL-8 (30 nM) or S1P (500 nM) on proliferation (a), migration (b) and capillary network formation (three-dimensional model of angiogenesis in Matrigel) (c) SVF cells incubated with adipogenic (Adipo) or angiogenic (Angio) medium, K = control (cells without stimulation by adipogenic factors). Mean $\pm$ SEM, $n = 3-5$ experiments. Significance * $P < 0.05$.

Figure 5. Participation of SVF cells in capillary network formed by HUVECs. Tubulogenic activity of HUVEC and GFP transfected SVF alone or in co-culture was performed in pro-angiogenic (Angio) medium. (a) Representative picture of tubes formed by HUVECs in the presence of VEGF (0.2 nM), light microscopy. (b) Image as previous sample, from the same specimen as (a), UV light; (c) Representative image, tubules formed by GFP-transfected SVF cells in the presence of VEGF (0.2 nM) light microscopy. (d) Image as previous, same specimen as (c), UV light. (e) Representative image, capillaries formed by 1 : 1 ratio GFP-SVF and HUVECs in the absence of VEGF light microscopy. (f) Image as previous, same specimen as (e), UV light. (g) Representative picture of capillaries formed by 1 : 1 ratio GFP-SVF and HUVECs, in the presence of VEGF (0.2 nM) light microscopy. (h) Image as previous, same specimen as (g), UV light. (i) Comparison of capillary length formed in matrigel by HUVECs, SVF cells or mixture of these cells, in Angio medium with or without VEGF (0.2 nM) supplementation. Significance * $P < 0.05$. Mean $\pm$ SEM from $n = 3-5$ experiments.

Stromal-vascular fraction cell differentiation into adipocytes in serum-free adipogenic medium

Stimulation of the differentiation process of SVF cells was induced by MDI added for 48 h, followed by incubation in adipogenic medium (Hauner *et al.* 2001). The SVF cells differentiated to lipid accumulating adipocyte-like cells (Fig. 7d) with altered gene expression pattern (Fig. 3). Lower numbers of Oil red O-positive cells were found in angiogenic medium SVF cells after addition of pro-adipogenic MDI mixture for 48 h (Fig. 7c). Absence of MDI mixture failed to

Figure 6. Confirmation of SVF cells' participation in formation of new capillaries made by HUVECs. SVF cells, labelled with calcein (fluorescence observed with blue filter, Fig. 6b), as well as HUVECs, labelled with Mitotracker (fluorescence observed with blue filter, Fig. 6a) formed capillary-like structures in Matrigel alone. Co-culture experiment (SVF cells mixed with HUVECs in 1 : 1 ratio) showing both types of cell work together to form the capillary network (Fig. 6c), particularly in the presence of VEGF (Fig. 6d).

promote the adipogenic switch of SVF cells either cultured in angiogenic medium (Fig. 7b) or even in adipogenic medium (Fig. 7a).

DISCUSSION

Our results have confirmed observations that changes in cell culture medium may switch differentiation of adipose tissue-derived SVF cells towards pro-endothelial-like cells. Presence of serum promotes such properties, inhibiting differentiation towards adipocytes.

Adipose tissue, for optimal functioning as an energy store and endocrine organ, needs plasticity of vascular networks. Adipocytes, endothelial cells as well as adipose tissue progenitors, secrete

Figure 7. Differentiation of SVF cells to adipocyte-like cells in adipogenic medium. Light microscopy ($\times 10$ magnification), Oil red O stained (a) SVF cells incubated in serum-free hormone-stimulated medium (adipogenic medium) without stimulation by 48 h incubation with mixture of differentiation factors (IBMX, dexamethasone, insulin), (b) SVF cells incubated in serum-containing medium (EBM) without stimulation by 48 h incubation with mixture of differentiation factors, (c) SVF cells incubated in serum-containing medium (EBM) with stimulation by 48 h incubation with mixture of differentiation factors, (d) SVF cells incubated in serum-free hormone-stimulated medium (adipogenic medium) with stimulation by 48 h incubation with mixture of differentiation factors.

numerous angiogenic and anti-apoptotic mediators that are involved in regulation of growth and differentiation of mesenchymal cells, including adipose progenitor SVF cells (He *et al.* 1998; Rehman *et al.* 2004). Angiogenesis or neovascularization, in the postnatal period is a process of creating new blood vessels as outgrowths of the existing network (Carmeliet 2003). The major cell type involved in this process is the endothelium cells of which line blood vessels and constitute virtually the entirety of capillaries of the body (Eichmann *et al.* 2005). Endothelial cells exhibit a number of unique cell-specific characteristics that define them. Primary endothelial cells generally express a high constitutive production of von Willebrand factor (vWF), platelet endothelial cell adhesion molecule (PECAM-1, CD31) and endothelial nitric oxide synthase (eNOS) (Unger *et al.* 2002), whereas CD34 and AC133 are characteristic of progenitor cells (Urbich & Dimmeler 2004). CD34 is a known transmembrane cell surface sialomucin, which is commonly expressed on haematopoietic progenitor cells (Verfaillie 1998; Huss 2000). AC133 (CD133, prominin) is a highly conserved antigen with unknown biological activity, which is expressed on haematopoietic stem cells but is absent from mature endothelial cells and monocytes

(Urbich & Dimmeler 2004). VE-cadherin (CD144, cadherin-5) belongs to the calcium-dependent adhesion molecules known to be involved in homophilic cell interactions, VE-cadherin is thought to play a role in vascular permeability and remodelling (Urbich & Dimmeler 2004). The CD14 receptor that distinguishes endothelial cells from the haematopoietic/monocytic lineage is a recognition molecule of the innate immune system, against exogenous and endogenous stress factors, and is also implicated in monocyte activation, leucocyte-endothelial cell interactions and regulation of apoptosis (Arroyo-Espliguero *et al.* 2004).

Here, the observed up-regulation of vWF, PECAM-1/CD31 and eNOS argue for differentiation of SVF cells to the endothelial lineage (Unger *et al.* 2002; Urbich & Dimmeler 2004). High expression of *Jagged*, *Notch 4*, *CXCR4* and *CXCR12* genes in SVF cells in cultured in angiogenic medium suggest maintenance of high pro-proliferation/low differentiation stage of these cells, which is important also for angiogenesis (Lindner *et al.* 2001; Sullivan & Bicknell 2003). To achieve new blood vessel formation, endothelial cells must first escape from their stable matrix location by first breaking through basement membranes (Carmeliet 2003). This is associated with dedifferentiation of mature endothelial cells, and is marked by up-regulated expression of Jagged-Notch signalling (Sullivan & Bicknell 2003). Expression of Notch-4 is characteristic of vessel wall cells (Lindner *et al.* 2001) and detection of Notch-4 points to the presence of non-definitive differentiated endothelial progenitor cells (Lindner *et al.* 2001; Sullivan & Bicknell 2003). CXCR4 is a specific receptor for CXCR12 (SDF-1). This pathway is important for chemotaxis and homing of progenitor cells in target tissue (Murdoch *et al.* 1999; Burman *et al.* 2005).

Down-regulation of CD34 in angiogenic medium-cultured SVF cells indicates, however, parallel activation of differentiation (Verfaillie 1998; Huss 2000). Up-regulation of PECAM/CD31, eNOS and vWF argue for differentiation of these cells towards the endothelial lineage (Murdoch *et al.* 1999; Carmeliet 2003; Eichmann *et al.* 2005).

Activation of metalloproteinases released by surrounding tissue and macrophages plays an essential role in formation of new capillaries due to digestion of extracellular membranes and promotion of endothelial cell proliferation and chemotaxis (Eichmann *et al.* 2005). Once this is achieved, endothelial cells migrate towards an angiogenic stimulus due to activity of chemo-attractants such as IL-8, Gro- α , Gro- β , PF-4, IP-10 or VEGF. Such mediators are released from surrounding tissue or from infiltrating ischaemic tissue macrophages (Carmeliet 2003) acting on specific receptors for binding the chemokines (CXCRs) (Murdoch *et al.* 1999). When forming a new capillary, endothelial cells proliferate to provide the necessary number of cells for making it (Carmeliet 2003; Eichmann *et al.* 2005). Subsequent to proliferation, new branching outgrowths of endothelial cell buds are formed to reorganize into a three-dimensional tubular structure (Carmeliet 2003; Eichmann *et al.* 2005).

Previously, it has been found that progenitor cells isolated from mouse adipose tissue can differentiate to endothelial cells and hence could be used as a source for therapeutic angiogenesis in ischaemic tissue (Planat-Benard *et al.* 2004), our results confirm this observation. 24 h incubation in angiogenic medium supplemented with serum inhibits differentiation to adipocyte-like cells, strongly up-regulates gene expression characteristic for endothelium and promotes biological, pro-angiogenic properties of these cells.

In pro-angiogenic environment (angiogenic medium, supplemented with serum), human SVF cell proliferation and chemotaxis was activated by IL-8 and VEGF, which are factors crucial for angiogenesis and for primary capillary network formation (Carmeliet 2003; Eichmann *et al.* 2005). Chemotactic IL-8 is secreted by a variety of cell types including endothelium (Murdoch *et al.* 1999). VEGF and nitric oxide released by adipose tissue stromal cells are known to be potent activators of endothelial progenitor cell proliferation and chemotaxis with anti-apoptotic

properties (Kimura *et al.* 2004). Both factors are important for vasculogenesis and angiogenesis (Carmeliet 2003; Eichmann *et al.* 2005). We also observed that S1P, a potent activator of endothelial differentiation (Kimura *et al.* 2004), significantly stimulated migration of SVF cells cultured in pro-angiogenic medium. Angiogenic medium SVF cells spontaneously, as well as stimulated with VEGF, were able to form capillary-like structures in the Matrigel *in vitro* model of angiogenesis. Human adipose tissue-harvested SVFs are a heterogeneous population (Rodriguez *et al.* 2005, plus our results). Presence of several surface antigens measured by flow cytometry (such as CD45, CD14) points to existence of myelogenic cells in this cell type (Verfaillie 1998; Huss 2000; Lindner *et al.* 2001; Arroyo-Espliguero *et al.* 2004; Rehman *et al.* 2004; Urbich & Dimmeler 2004).

The Hauner group stressed the importance of usage of serum-free media for differentiation of SVF to adipocytes (Hauner *et al.* 1989, 2001). They proved that the presence of serum almost completely prevents their adipose differentiation in culture, in contrast to cell lines or cultured rodent adipose precursor cells (Hauner *et al.* 1989). These results are in agreement with our observation that human SVF cells could efficiently differentiate to adipocyte-like cells only in absence of serum and after stimulation with MDI mixture (Hauner *et al.* 1989). Such adipogenic medium-cultured SVF cells are characterized by the higher expression of the adipose tissue genes like those for transcription factors (PPARs, C/EBP α), fatty acid transporters (FAT/CD36, aP2/FABP4) as well as lipoprotein lipase (LPL), adiponectin (AdipoQ) and leptin, which are characteristic for differentiation of adipocytes (Gregoire *et al.* 1998; Rosen & Spiegelman 2000; Guerre-Millo 2004). Parallel down-regulation of genes characteristic for endothelium in adipogenic medium-cultured SVF cells was observed. We have shown that in serum-free medium our cells accumulate lipids. However, it is unknown whether the observed cells are adipocytes or Oil Red O-stained macrophages. To verify this, further functional studies will be prepared in the future. Inhibition of adipogenesis by the presence of serum may be connected with regulation by protein pockets involved in cell cycle (e.g. Rb protein) or linked to a mammalian target of rapamycin pathway (Farmer 2006). This hypothesis also will be studied in the future.

Culture of SVF cells in adaptation medium (Hauner *et al.* 2001) is an additional step for clearance of the target population from cells also isolated from adipose tissue (e.g. blood cells and differentiated endothelial cells); thus, after 5–10 days of culture the population consist mostly of progenitor CD34⁺ cells, which was revealed by flow cytometry; in addition, these results are consist with the results of others (Festy *et al.* 2005). Expression of CD34⁺ decreases during long-term culture (seen in flow cytometry and real-time PCR), which seems to indicate the differentiation process (Huss 2000; Festy *et al.* 2005). Thus, it seems that SVF cells isolated from human adipose tissue, after adaptation, form a population of progenitor cells able to undergo differentiation to adipocytes as well as endothelial cells. This is consistent with findings that endothelial cells and pre-adipocytes have common progenitors (Planat-Benard *et al.* 2003). However, it is also possible that there is more than one cell population in the SVF isolated from adipose tissue and this hypothesis needs to be investigated further.

Many findings suggest that pre-adipocytes and endothelial cells could cooperate to form a capillary network. SVF cells cultured in endothelial basal medium secreted significant amounts of VEGF and TGF- β , which stimulate endothelial cell proliferation and survival, indicating that these two types of cell may cooperate to build efficient vasculature. Pre-adipocytes enhance endothelial cell proliferation and secrete VEGF under hypoxia (Rehman *et al.* 2004), on the other hand, endothelial cells increase adipose tissue progenitors' proliferation rate (Hutley *et al.* 2001). Furthermore, Wosnitza and co-workers have reported that adipose tissue contains a population of CD31⁻ as well as CD31⁺ cells, which could undergo transdifferentiation depending on their conditions of growth (Wosnitza *et al.* 2007).

We have shown that angiogenic medium SVF cells together with HUVECs incorporate into newly forming capillaries, which is in agreement with a suggestion that progenitor endothelial cells collaborate with endothelial cells to form capillary networks (Verfaillie 1998; Huss 2000; Lindner *et al.* 2001; Arroyo-Espiguero *et al.* 2004; Rehman *et al.* 2004; Urbich & Dimmeler 2004), thus providing a body of evidence pointing to a strong relationship between endothelial cells and pre-adipocytes. The phenomenon that SVF cells together with HUVECs could build a capillary network is consistent with the findings of other workers, who have shown the dynamic interaction between differentiating adipocytes, stromal cells and angiogenesis, in adipose tissue of live, obese mice (Nishimura *et al.* 2007). This cooperation could be an important step in angiogenesis during an adipose tissue mass extension process.

The discovery that adipose tissue possesses a fraction of progenitor cells able to undergo differentiation to endothelial cells as well as to adipocytes, that are successfully isolated, cultured and differentiated *in vitro*, could be an opportunity for tissue repair (Fraser *et al.* 2006; Gomillion & Burg 2006; Izadpanah *et al.* 2006; Klein *et al.* 2006). Several observations already confirm the therapeutic potential of adipose tissue progenitor cells for tissue repair (Planat-Benard 2003; Bacou *et al.* 2004; Parker & Katz 2006). Since the presence of serum in blood insulin, corticosteroids and cyclic adenosine monophosphate (cAMP)-elevating factors could stimulate progenitors (in our case SVF) to differentiate to endothelial cells instead of to adipocytes, but our results argue against simple direct intravenous or intra-arterial application of progenitor cells in progenitor cell-based tissue-regenerative therapy (Klein *et al.* 2006). Additionally, an observed phenomenon of pro-angiogenic properties of SVF cells could also add to understanding of adipose tissue mass extension by remodelling of vasculature (Rupnick *et al.* 2002; Hausman & Richardson 2004; Nishimura *et al.* 2007). This paper provides clarification of a cell culture model that could be applicable to investigation of changes in differentiation capacity of human adipose tissue SVF cells.

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