Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation

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Abstract Recent research findings postulate that adipocytes and endothelial cells (EC) may share a common progenitor. However, the interlinking pathways between adipose tissue and endothelium, and the differentiation potential of cells to convert from one tissue into the other via progenitor cells have not been elucidated and are therefore the focus of this study. Stromal vascular fraction (SVF) cells were isolated from liposuction aspirates or excised adipose tissue and separated into CD31\(^+\) and CD31\(^-\)/S100\(^+\) populations by magnet-assisted cell sorting. Differentiation to fat tissue was induced in both CD31 fractions after expansion by insulin, dexamethasone, isobutylmethylxanthine, triiodothyronine, pioglitazone, and transferrin. Differentiation was assayed enzymatically and by cell counting. Maturation to endothelium was performed with vascular endothelial growth factor (VEGF), insulin-like growth factor-1 plus 2% fetal calf serum, and confirmed by flow cytometry and tube formation assays on Matrigel\(\text{TM}\). Our results show that the SVF contains a CD31\(^+\), S100\(^+\) cell type that can differentiate into adipocytes and EC. The SVF also comprises CD31\(^+\) cells that, although they have an endothelial phenotype, can be converted into mature adipocytes. These findings demonstrate the potency of SVF cells to perform both adipogenic and endothelial differentiation. Further, they reveal the plasticity of mature cells of mesenchymal origin to undergo conversion from endothelium to adipose tissue and vice versa.

Key words human adipose SVF · progenitor cell plasticity · preadipocytes

Introduction

The stromal vascular fraction (SVF) of adipose tissue remains a cell population that is very hard to characterize. Many different terms refer to this cell mixture isolated from adipose tissue by collagenase digestion. Most often, SVF cells are called preadipocytes, but human adipose tissue-derived stem cells (Miranville et al., 2004), or human adipose lineage cells (Planat-Benard et al., 2004) have been used to describe this population as well. These cells have the capacity to differentiate into multiple cell phenotypes including adipose, cartilage, osteogenic, smooth muscle, and neuronal lineage (Zuk et al., 2001; Erickson et al., 2002; Zuk et al., 2002; Abderrahim-Ferkoune et al., 2003; Ogawa et al., 2004). Adding to the remarkable differentiation capacity of SVF cells, they have recently been differentiated into an endothelial phenotype (Miranville et al., 2004; Planat-Benard et al., 2004).
Taking the broad mixture and combinations of cell surface CD markers, it is rather likely that the SVF is not a unique cell population, but rather a pool of various progenitor cells including mature endothelial cells (EC). This is supported by the fact that the cells are isolated from highly vascularized adipose tissue by liposuction or excision and subsequent collagenase digestion. As long as no further purification is applied, a cell mixture remains with a pool of mature EC, potent preadipocytes, and other cell types. However, a close evolutionary relation between preadipocytes and EC can be assumed as preadipocytes have been demonstrated to influence proliferation and reduce apoptosis in mature EC (Rehman et al., 2004). Furthermore, preadipocytes are able to secrete and increase the amount of vascular endothelial growth factor (VEGF) under hypoxia (Rehman et al., 2004) and during fat pad development (Neels et al., 2004). Reciprocally, EC promote adipose tissue-derived precursor cell proliferation in vitro in conditioned medium (Hutley et al., 2001), and fat tissue proliferation is dependent on angiogenesis (Rupnick et al., 2002). Anti-angiogenic agents leading to weight loss in mice as demonstrated by different obesity models (Rupnick et al., 2002) is further proof of the close relationship between EC and preadipocytes. Not surprisingly, Planat-Benard et al. (2004) demonstrated a common origin for adipose tissue and endothelium. They postulated that adipocytes and EC have a common progenitor; however, the interlinking pathways between adipose tissue and endothelium and the differentiation potential from one tissue into the other via progenitor cells have not been analyzed and are the focus of the present study. As SVF cells have been shown to be able to perform metaplasia from mature fat cells into EC (Planat-Benard et al., 2004), here we investigated to what extent the reverse differentiation pathway is possible. In order to better characterize the cell populations in liposuction material and excised fat, we first compared (i) freshly isolated SVF cells from lipoaspirates, (ii) from excised adipose tissue, as well as (iii) cultured SVF cells in terms of their morphological and surface profile differences. The cell surface markers CD31, CD34, CD51, and KDR were analyzed. CD31, a marker for mature EC, was used to separate SVF cells into CD31+ and CD31− populations by magnet-assisted cell sorting. Differentiation toward endothelium was induced in CD31− cells and maturation toward adipose tissue in both CD31+ and CD31− cells.

This study completes the present knowledge on the differentiation capacity of SVF cells by demonstrating endothelial and adipogenic maturation potential in the same cell type. This might have relevant implications for atherosclerosis and its pathogenesis, which seems to involve EC displaying adipocyte properties, such as lipid uptake (van Hinsbergh et al., 1983; Tokunaga et al., 2002). Furthermore, our findings reveal that even some mature cells of mesenchymal origin have a remarkable potency to perform transdifferentiation between endothelium and adipose tissue.

Material and Methods

Reagents

Collagenase solution type 1, Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 (F12), fibronectin, and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), Iscove’s Modified Dulbecco’s Medium (IMDM) with GlutaMAX from Invitrogen (Karlsruhe, Germany), Endothelial Cell Growth Medium (ECGM) with supplement mix from PromoCell (Heidelberg, Germany), Endothelial Growth Medium 2 Microvascular (EGM-2 MV) with supplement mix from Cambrex Bio Science (Walkersville, MD), HEPES, triiodo-t-thyronine, transferrin, dexamethasone, and isobutylmethylthiykanthine (IBMX) from Sigma (Deisenhofen, Germany), and pioglitazone from Takeda Pharmaceuticals Co. Ltd. (Lincolnshire, IL). Basic fibroblast growth factor (bFGF), VEGF, insulin-like growth factor 1 (IGF-1), and stem cell growth factor β (SCGF-β) were from Peprotech (London, England, β-mercaptoethanol from Fluka (Deisenhofen, Germany), bovine serum albumin (BSA), trypsin, and penicillin/streptomycin from PAA Laboratories (Colbe, Germany). Matrigel™, growth factor reduced, was from BD Biosciences (Bedford, MA). The 250 µm-nylon sieve was from Verseseidag Techfab GmbH (Geldern, Germany). Anti-CD31 paramagnetic Dynabeads and MPC-1 magnetic rack were from Dynal Biotech GmbH (Hamburg, Germany), the anti-CD31 MicroBead Kit was from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), and glycercol-3-phosphate dehydrogenase (GPDH) assay kits from TaKaRa (Kyoto, Japan). S100 A1 and von-Willebrand-Factor (vWF) antibodies were from DAKO GmbH (Hamburg, Germany), mouse antihuman CD31, CD34, CD51/61, KDR, and secondary FITC antibodies from Dianova GmbH (Hamburg, Germany), anti-human CD105 FITC-conjugated antibody and isotype controls from ImmunoTools GmbH (Friesoythe, Germany), the CD31 APC-conjugated antibody was from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), and acLDL cell tracker from Cell Systems (St. Katharinen, Germany). All other materials were of the best quality and purchased from diverse conventional suppliers.

Isolation of SVF cells

Preadipocytes were isolated from freshly excised human subcutaneous abdominal or mammary fat tissue at the Department of Plastic Surgery and Hand Surgery—Burn Center from patients who underwent elective operations (e.g., abdominoplasty, breast reduction, liposuction). Harvesting of adipose tissue by liposuction was performed according to Sydney Coleman (manually applied negative pressure using a 10cc-syringe with a blunt tip cannula, no addition of any solution) from the abdomen and breast (Coleman, 1997). Adipose tissue from aspiration was digested by collagenase treatment after washing with 0.9% NaCl. Fat lobules from excised adipose tissue were prepared by carefully removing capillaries and connective tissue. After mincing into small pieces of 2–6 mm, adipose tissue was washed with 0.9% NaCl and also digested. Digestion of liposuctioned and excised fat tissue was performed with collagenase CLS type 1 (0.494 U/ml) and 1.5% BSA dissolved in collagenase buffer (100 mM HEPES, 120 mM NaCl, 50 mM KCl, 1 mM CaCl2, 50 mM glucose, pH 7.4) for 60 min at 37°C under constant shaking. The gained suspension was filtered through a 250 µm filter and centrifuged at 700 × g for 7 min at room temperature. SVF cells were separated from mature adipocytes by discharging the fat layer on top. For separation of CD31+ and CD31− cells, the cell pellet was resuspended in EGM-2 MV culture medium in order to enhance EC numbers and to maintain EC characteristics of CD31-positive cells in the SVF. For plain flow...
CD31 Dynabead separation of SVF cells

Cells were grown to subconfluence, trypsinized after washing, resuspended in PBS/0.1% BSA and 2 mM EDTA (buffer), and passed through a 70 μm nylon mesh. Twenty microliter FcR Blocking reagent was added to a maximum concentration of 1 × 10^6 cells in 60 μl buffer. The gained suspension was vortexed thoroughly, and 20 μl of CD31 MicroBeads were added. Cells were incubated for 15 min at 4°C, washed with 5 ml medium, and transferred onto MACS columns. CD31^+ cells were collected after passing through the column without attachment, centrifuged, washed, and seeded onto fresh culture flasks. CD31^+ cells from SVF cells with Dynabeads attached were resuspended in fresh DMEM/F12 (1:1). For attachment to culture flasks, both cell types were cultivated in DMEM/F12 (1:1) supplemented with 10% FCS overnight.

CD31 MicroBead separation of SVF cells

Cells were grown to subconfluence, trypsinized after washing, resuspended in PBS/0.1% BSA and 2 mM EDTA (buffer), and passed through a 70 μm nylon mesh. Twenty microliter FcR Blocking reagent was added to a maximum concentration of 1 × 10^6 cells in 60 μl buffer. The gained suspension was vortexed thoroughly, and 20 μl of CD31 MicroBeads were added. Cells were incubated for 15 min at 4°C, washed with 5 ml medium, and transferred onto MACS columns. CD31^+ cells were collected after passing through the column without attachment, centrifuged, washed, and seeded onto fresh culture flasks. CD31^+ SVF cells, which attached to the columns, were detached and resuspended in fresh DMEM/F12 (1:1). Both CD31^+ and CD31^- SVF cells were cultivated in DMEM/F12 (1:1) supplemented with 10% FCS overnight for adherence to culture dishes.

Culturing of SVF cells

Culture media were changed for both CD31^- and CD31^+ to either endothelial or adipogenic differentiation medium 12 hr after separation with Dynabeads or MicroBeads, respectively. To evaluate differentiation plasticity toward endothelium, two types of differentiation media were evaluated. The first culture medium (CM-1) tested was a hematopoietic and endothelial progenitor cell medium for peripheral blood progenitor cells according to Gehling et al. (2000), containing IMDM, 50 ng/ml VEGF, 100 ng/ml SCGF-β, 30% FCS, 1% BSA, and 10^-4 mol/l β-mercaptoethanol. The second medium, an endothelial differentiation medium (CM-2), was ECGM supplemented with 50 ng/ml VEGF, 20 ng/ml IGF-1, and 2% FCS, according to Miranville et al. (2004), with minor modifications. At confluence, cells were trypsinized, split into 1:2, and transferred to fresh culture flasks. To perform adipogenic differentiation, cells were cultured in DMEM/F12 (1:1) supplemented with bFGF (10 ng/ml) and 10% FCS (referred to as proliferation medium) to obtain cell confluence again. Lipid droplet development was then promoted for 21 days by changing medium to DMEM/F12 (1:1) without serum addition, supplemented with 66 nM insulin, 100 nM dexamethasone, 0.5 mM IBMX, 0.1 μg/ml pioglitazone, 1 mM triiodo-L-thyronine, and 10 μg/ml human transferrin. After 5 days of incubation, medium was used as before but without IBMX and pioglitazone for 16 more days. Culture media were changed every 3 days.

Flow cytometry

For performing flow cytometry analyses, freshly isolated cells were incubated for 15 min with erythrocye lysis buffer at 4°C. For staining with DiI, cells were washed with PBS, and incubated with DiI-ac-LDL (acLDL) (10 μg/ml) for 1 hr at 37°C. Afterwards, cells were washed twice with plain medium, once with PBS, and fixed with 4% paraformaldehyde (PFA). Next, cells were centrifuged at 400 × g for 10 min and resuspended in PBS containing 0.2% FCS and 2 mM EDTA. For incubation with primary anti-human CD31, CD34, KDR, CD51.61, vWF, S100, and CD105-FITC cells were also fixed with 4% PFA, resuspended in PBS containing 0.2% FCS and 2 mM EDTA, and maintained at 4°C for 30 min. Washing and incubation with secondary FITC-conjugated antibodies was performed for 30 min at 4°C with all samples, except for the already FITC-labeled CD105. For additional staining with CD31 APC-conjugated antibodies to generate double labeling, cells were incubated with CD31-APC for 10 min at 4°C, washed, and resuspended in PBS containing 0.2% FCS and 2 mM EDTA. Labeled cells were analyzed by flow cytometry using a FACS Calibur apparatus (BD Biosciences, San Jose, CA) and CellQuest Pro analysis. Isotype controls for IgG1 and IgG2a were used as recommended by the supplier to exclude non-specific binding of antibodies and were always negative. Each labeling with FITC-CD markers was compared with control cells exclusively stained with the secondary FITC-conjugated fluorescence antibody. The percentage of positively stained cells was determined after correction for the percentage of cells reacting with the secondary FITC antibody. Staining of CD31^- as well as CD31^+ SVF cells was performed on the day of adipogenic and endothelial differentiation induction.

Immuno-staining with vWF

Cells were analyzed for vWF by immunofluorescent staining. Glass slides with adherent cells were fixed in acetone for 20 min at 4°C and incubated with primary anti-vWF antibodies for 1 hr at room temperature. After washing, slides were stained with FITC-conjugated secondary antibodies for 1 hr at 37°C. Detection of vWF was performed by fluorescence microscopy. vWF staining of CD31^+ and CD31^- SVF cells was performed on the day of adipogenic and endothelial differentiation induction.

Determination of GPDH activity

To evaluate preadipocyte differentiation on a molecular level, the activity of GPDH was measured with a kit system from TaKaRa. GPDH is a molecular key marker of adipogenic conversion (Rosen and Spiegelman, 2000), which has been used for decades to measure preadipocyte differentiation (Pairault and Green, 1979; Spiegelman et al., 1983). Cells were washed twice with PBS and cell lysis was induced by adding “Enzyme Extraction Buffer.” Afterwards, the lysate was diluted to fourfold by adding “Dilution Buffer.” GPDH activity was determined by measuring absorbance at 340 nm. Enzyme activity was calculated with the following formula supplied by TaKaRa: GPDH activity (units/ml) = AOD340 × 2.06 × dilution ratio of the sample. Cellular protein content was measured according to a method by Bradford (1976) to normalize GPDH levels to the number of cells.

Oil Red O Staining

Monolayer cultures were washed with PBS and fixed with cold 10% PFA and incubated for 3 hr at 4°C. Oil Red O working solution was added to culture flasks for 2 hr at room temperature. After washing, stained cells were kept in 10% PFA and examined by light microscopy.
Statistical evaluation

Data of expression of surface markers and GPDH activity were expressed as mean value ± SD. The significance of differences between CD31<sup>-</sup> and CD31<sup>+</sup> cell differentiation was evaluated by the t-test. Differences at p<0.05 were considered significant and p<0.01 highly significant.

**Results**

Characterization and phenotype of native SVF—comparison of excised fat tissue with liposuction material

The surface expression of freshly isolated SVF cells from liposuction and excision was characterized by flow cytometry without performing any previous purification. SVF cells harvested from excised fat tissue show strong labeling for CD34, similar to cells positive for CD34, and a less intensive staining rate (Fig. 1B) are positive for CD34, similar to cells from excised tissue (compare Table 1). CD31 and CD51/61 fluorescent labeling was stronger in liposuction from excised tissue (compare Table 1). Cultured under endothelial differentiation conditions in the presence of VEGF and IGF-1, these SVF cells develop tubular structures on plastic without Matrigel<sup>™</sup>. This was demonstrated by vWF-immunofluorescence staining after culturing for 2 weeks (Fig. 2B). Following separation into CD31<sup>-</sup> and CD31<sup>+</sup> cells by Dynabeads or MicroBeads, respectively, CD31<sup>-</sup> SVF cells show no expression of vWF or CD31 (Figs. 2C,2E), either after 1 or after 2 weeks of culturing in proliferation medium. Furthermore, CD31<sup>-</sup> cells do not reveal endothelial network formation on plastic culture dishes (Fig. 2C). Analyzing the expression of S100, an early marker of adipogenesis, these cells are found to be almost 100% positive in flow cytometry (Figs. 2D,2E). CD31<sup>+</sup> cells, in contrast, demonstrate strong vWF expression (97% ± 2% stained cells) and CD105 staining after 1 week. The Dynabead isolation procedure was demonstrated to be effective as 95% ± 5% of the cells carry Dynabeads, confirming that they are positive for CD31 (Figs. 2F–2I,3E). Cells isolated with CD31 MicroBeads show strong staining for CD31 in flow cytometry (Figs. 2L,2N,2P). Furthermore, CD31<sup>+</sup> MicroBead-isolated cells reveal the same surface expression characteristics for CD105 and vWF as after isolation with Dynabeads. During proliferation, magnetic beads initially remain bound to the cells and resist washing steps; however, cell proliferation reduces bead numbers continuously.

**In vitro** differentiation of SVF cells under adipogenic medium conditions

Unseparated SVF cells from excised tissue differentiate under adipogenic culture conditions to almost 90% into adipocytes showing large lipid vacuoles after 21 days in adipogenic medium (data published elsewhere). Regarding the separated SVF cell fractions, the CD31<sup>-</sup> cells could differentiate into mature adipocytes by nearly 100%, as demonstrated by light microscopy and Oil Red O staining in Figures 3A–3D. Interestingly, purified CD31<sup>-</sup> mature EC from the SVF also convert into adipocytes after 21 days under adipogenic culture conditions. Undergoing differentiation, cells are viable and healthy with no signs of apoptosis present. From microscopical cell counting, approximately 65% of CD31<sup>+</sup> cells presented accumulation of lipid vacuoles equivalent to an adipocyte phenotype. As can be seen from Figures 3E–3G, CD31<sup>+</sup> cells have CD31 Dynabeads attached to their cell membranes, which confirms their CD31 surface expression. On comparing the GPDH activity of both CD31 subsets, CD31<sup>-</sup> cells showed a GPDH activity of 64% ± 11% compared with GPDH levels of CD31<sup>-</sup> cells set 100% (Fig. 4). Lipid droplets appeared approximately 2–3 days later during differentiation in CD31<sup>+</sup> than in CD31<sup>-</sup> cells. Applying flow cytometry, the proliferating CD31<sup>+</sup> cell fraction was screened for the presence of the surface

Splitting and purity of CD31<sup>-</sup> and CD31<sup>+</sup> SVF cells

To further characterize the unpurified SVF population, vWF immunofluorescence staining was performed 12 hr after cell isolation and culture in DMEM/F12%, 10% FCS. Freshly harvested SVF cells from excised fat tissue contain a small amount of approximately 10% of vWF-positive (Fig. 2A) and CD31-positive cells, as revealed by microscopical counting and FACS analyses (compare Table 1). Cultured under endothelial differenti-
Fig. 1 Characterization of stromal vascular fraction (SVF) cells from excised versus liposuctioned adipose tissue. SVF cells were isolated from excised (A) and liposuctioned (B) adipose tissue from the same patient by collagenase digestion and immediately analyzed by FACS analysis without any previous purification or culturing. Cells were screened for the expression of CD31, CD34, CD51/61, and KDR. IgG1 and IgG2a were used as isotype controls. Also, staining with secondary FITC-conjugated fluorescence antibodies (FITC control) only was monitored.
Table 1 Expression of surface markers on adipose tissue-derived SVF cells

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<th>Excision day 0 (%)</th>
<th>Excision day 14 (%)</th>
<th>Liposuction day 0 (%)</th>
<th>Liposuction day 14 (%)</th>
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<tr>
<td>CD31+</td>
<td>10 ± 2</td>
<td>6 ± 3</td>
<td>24 ± 15</td>
<td>12 ± 5</td>
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<tr>
<td>CD34+</td>
<td>57 ± 3</td>
<td>36 ± 10</td>
<td>57 ± 20</td>
<td>57 ± 4</td>
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<tr>
<td>CD51/61+</td>
<td>4 ± 1</td>
<td>5 ± 2</td>
<td>11 ± 8</td>
<td>7 ± 3</td>
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<tr>
<td>KDR+</td>
<td>14 ± 5</td>
<td>52 ± 8</td>
<td>43 ± 23</td>
<td>23 ± 7</td>
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SVF cells were isolated from excised adipose tissue or liposuction material, respectively, as described in Figure 1, and cultured in DMEM/F12 plus 10% FCS for 14 days, followed by flow cytometry analysis. Given is the expression of the surface markers CD31, CD34, CD51/61, and KDR in percentage. Fibroblasts were used as negative, and HUVEC as positive control. Data are derived from three individual experiments.

In vitro differentiation of CD31− cells under endothelial medium conditions

To evaluate the endothelial differentiation potential of CD31− SVF cells morphologically, this cell fraction was seeded onto Growth Factor Reduced Matrigel™ in CM-1 or CM-2 culture medium, respectively, and tube formation was assayed. Cells form three-dimensional tube-like structures in both media while CM-2 medium also allows formation of extensive intercellular tube networks (Figs. 5A, 5B). HUVECs, which were used as a positive control, also formed networks but were not able to grow three dimensionally (compare Fig. 5C).

Next, endothelial differentiation of CD31− cells grown in CM-1 or CM-2, respectively, was analyzed by flow cytometry to determine the quantitative expression of the surface markers CD31, KDR, and acLDL (compare Table 2). In CM-1 medium, CD31− SVF cells express CD31 by up to 46% and KDR up to 90%, demonstrating the capacity of this medium to enhance stem cell characteristics of SVF cells (Fig. 6A). In CM-2 medium, CD34 and KDR expression is significantly lower (p = 0.003 for CD34, p = 0.006 for KDR; Table 2, Fig. 6B). In both media, cells were positively stained for acLDL, confirming differentiation toward an endothelial phenotype (Fig. 6).

Discussion

The SVF of adipose tissue is a heterogenous cell population and therefore very hard to characterize. Although there are similar findings about the surface protein expression of SVF cells, much controversy remains. The cell fractions that have been used for surface analyses and differentiation assays cannot be compared as some research groups apply liposuction aspirates (Gronthos et al., 2001; Cao et al., 2005; Martinez-Estrada et al., 2005) or excised fat tissue (Miravalle et al., 2004; Planat-Benard et al., 2004), others use more purified, cell-sorted fractions (Miravalle et al., 2004; Sengenes et al., 2005), while still others identify cell surface structures from cells not before culturing for several passages (Cao et al., 2005). This leads to cell fractions with different surface characteristics and different proliferation and differentiation behaviors. In order (i) to illustrate the differences between SVF cells isolated from excised versus liposuction material, (ii) to determine the relative amount of endothelial and hematopoietic progenitor cells, and (iii) to be aware of potential cellular contaminations, SVF cells from liposapire and excised fat were screened for the presence of CD34, KDR, CD31, and CD51/61. Additionally, cells were cultured for 2 weeks in SVF/preadipocyte medium to promote proliferation of the desired phenotype before analysis by flow cytometry.

CD31 (PECAM-1) is commonly used for labeling EC but is also expressed by monocytes, platelets, lymphocytes, and granulocytes. CD34 is found on hematopoietic stem cells and EC. VEGF signaling is performed through KDR, a receptor tyrosine kinase. KDR is expressed during vasculogenesis and hematopoiesis and can be found on circulating endothelial progenitor cells, mature EC, and HUVECs. CD51/61 labels various types of EC while vWF is expressed by EC and platelets.

Flow cytometry of freshly isolated SVF cells from excised as well as liposuctioned adipose tissue revealed a significant population of progenitor cells as shown by strong expression of CD34 and KDR (Fig. 1). However, the SVF also includes a population of CD31−, CD51/61−, and vWF-positive cells, demonstrating a contamination with mature EC. CD31 and CD51/61 expression was stronger in liposuction SVF cells, with up to 40% CD31-positive cells, emphasizing the higher amount of contaminating EC in liposapire than in excised fat (5%–10% EC, Table 1). The vWF expression of 10% for excised tissue as determined by microscopical cell counting (Fig. 2A) completely matches the findings on CD31 and CD51/61 expression in flow cytometry (compare Table 1).

After cell culture in DMEM/F12 with 10% FCS for 2 weeks, cells from liposapire remain positive for CD34 to nearly the same percentage as freshly isolated cells, while cells from excised tissue show a significant reduction (p < 0.05, Table 1). The missing reduction in CD34 after cell adherence in liposuction material is surprising and the reason for this is under investigation. Endothelial surface characteristics, such as CD31 and CD51/61, however, decrease during culturing (Table 1) under
all conditions, which might be due to selection effects from culture medium as well as the lower proliferation capacity of CD31<sup>+</sup> SVF cells. CD31 expression in Figure 1A appears rather homogenous compared with Figure 1B; however, this is due to the two different preparation methods. In Figure 1A, excised fat lobules
were carefully isolated to only gain preadipocytes. As this is macroscopically not possible, there is a very small but homogenous fraction of EC displaying CD31 characteristics. The presence of these two cell fractions causes a double-peaked flow cytometry curve. In Figure 1B, liposuction material was used, which contains a much higher amount of endothelial cells due to its origin and the method of yield, which generates the two-peak appearance of the flow cytometry.

KDR expression in liposuction material is initially 43% ± 23% and decreases to 23% ± 7%, perhaps due to the culturing conditions. This is in accordance with the reduction of endothelial markers. In excised adipose tissue, however, the findings of KDR expression remain

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**Fig. 2** Scheme of the application of CD31 Dynabead separation for stromal vascular fraction (SVF) cells. Freshly isolated SVF cells from excised tissue were analyzed by fluorescence microscopy after isolation and transferred to tissue culture dishes (A). Cells were then either cultured in endothelial cell (EC) medium without Dynabead treatment and analyzed again after 2 weeks (B) or separated by CD31 Dynabeads or MicroBeads into a CD31− (C–E) and a CD31+ fraction (F–P). To confirm the purity of the isolation procedure, flow cytometry was performed with CD31− cells double-stained for CD31 and S100 expression (D, E) and with CD31+ cells double-labeled for CD31 in combination with CD105, von-Willebrand-Factor (vWF), or CD34 (K–P). For fluorescence microscopy, cells were stained with vWF and DAPI. All pictures except for (F; vWF alone) and (I; bright field and vWF, merged) are vWF-DAPI merged images. (F–I, K, M, and O): Dynabead cell sorting, (L, N, and P): MicroBead cell sorting. Arrows in (H) and (I) indicate Dynabeads attached to cell surfaces. Magnification in A, B, C, F, G, and I is × 200, in H × 400.

**Fig. 3** Morphological analysis of in vitro differentiation of stromal vascular fraction (SVF) cells under adipogenic medium conditions. SVF cells were separated into CD31− (A–D) and CD31+ (E–H) by Dynabead treatment. Both fractions were independently differentiated toward mature adipocytes. All pictures, except for (D) and (H), which show oil red staining results, are bright-field images. Shown in the left upper corner of (G) is a high-power magnification of a differentiating cell with Dynabeads attached. The arrows in (E), (F), and (G) indicate Dynabeads attached to cell surfaces. Magnification in (A)–(D), (E), and (H) is × 100, in (F) and (G) is × 200.
controversial as KDR expression immediately after isolation is surprisingly low and its increase due to cultivation is not in agreement with liposuction findings.

To analyze CD31⁺ and CD31⁻ differentiation characteristics and surface markers independently, unpurified SVF cells from excised adipose tissue were split by CD31 Dynabeads. Alternatively, CD31 MicroBeads were applied for the cell separation if double-labeling was performed.

Whether or not Dynabead attachment to the cell surface influences the differentiation potential of preadipocytes has not been analyzed so far. In order to minimize any impact of the Dynabeads, the concentration of beads was kept low to allow proper attachment of cells to culture dishes, which is otherwise impaired. As Dynabeads might bind non-specifically to adherent and phagocytic cells, all steps with Dynabeads were carried out at 4°C. At this temperature, cell activity is low and non-specific binding reduced to a minimum. To further minimize non-specific binding, the bead-cell suspension was pipetted a few times after incubation (before separation on the magnet) to loosen any non-target cells adhering to the beads. All these procedures reduced non-specific binding of Dynabeads, which was finally evaluated by staining Dynabead-carrying cells for vWF to ensure the endothelial phenotype. Cell counting with fluorescence and light microscopy revealed that 95% ± 5% of the cells carried Dynabeads (Figs. 2F–2I). Of this fraction, 97% ± 2% were positive for vWF. This is supported by flow cytometry after MicroBead cell sorting (Figs. 2M,2N). These results reveal the beneficial effects of the above-shown technical modifications in Dynabead usage and show that 92% ± 4% of CD31⁺ cells are vWF positive. Together with the high expression of CD105 in CD31⁺ cells (Figs. 2K,2L), this confirms the mature EC phenotype and the uniqueness of the CD31⁺ fraction.

Freshly harvested CD31⁻ SVF cells, in contrast to CD31⁺ cells, show no expression of vWF and CD31 as EC would do, either after 1 or after 2 weeks of culture in proliferation medium (data not shown). Furthermore, CD31⁻ cells do not present endothelial network formation on plastic culture dishes (Fig. 2C). Flow cytometry analyses reveal that this cell fraction is 100% positive for S100 (Figs. 2D,2E). Even though S100 labels nervous tissue, skeletal and cardiac muscle, malignant melanoma cells, and some other cell types, it is well
accepted as an early marker of adipogenesis (Atanassova, 2001). It can therefore be assumed that the CD31⁻ fraction consists of adipocytes in a precursor state, the original preadipocytes. Thus, separation of SVF cells by CD31 Dynabeads generates one cell fraction with preadipocyte characteristics (CD31⁻) and one with endothelial properties (CD31⁺). SVF cells have recently been shown to dedifferentiate from mature fat cells into a progenitor cell type and to differentiate from there into EC in CM-1 medium (Planat-Benard et al., 2004). This has high implications for angiogenesis-related and cardiovascular topics. In this study, we investigated whether the reverse pathway, i.e., from mature EC to adipocyte, is possible as well. A successful differentiation of EC to adipocytes might have a major impact on atherosclerosis-related issues as well as the pathogenesis might involve EC displaying adipocyte properties, such as lipid uptake. CD31⁺ and CD31⁻ were separately cultivated under adipogenic medium conditions to analyze their ability to convert into adipocytes. As expected, CD31⁻ cells were isolated from the SVF by Dynabeads and grown in CM-1 or CM-2, respectively, followed by quantitative analysis of the expression of the surface markers CD34, KDR, and acLDL (compare Fig. 6). Data are from three individual experiments.

SVF, stromal vascular fraction.

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<th>Table 2 Expression of surface markers on CD31⁻ SVF cells under endothelial medium conditions</th>
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<td>Culture medium</td>
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<tr>
<td>CM-1 (%)</td>
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<tr>
<td>CD34⁺</td>
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<td>KDR⁺</td>
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<td>acLDL</td>
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To further verify the theory on mesenchymal differentiation plasticity, we performed morphological studies on CD31⁻ SVF cells seeded onto growth factor-reduced Matrigel™ in two different culture media, CM-1 or CM-2, respectively. The first culture medium (CM-1) has been applied as a hematopoietic and endothelial progenitor cell medium for peripheral blood progenitors (Gehling et al., 2000). The second medium (CM-2) is a culture medium to induce differentiation toward endothelial lineage (Miranville et al., 2004). CD31⁻, which were initially 100% positive for the adipose tissue progenitor cell marker S100 (compare Figs. 2D,2E), were able to form three-dimensional tube-like structures in both media (Figs. 5A,5B). CM-2 medium additionally allowed the formation of extensive intercellular tube networks (Fig. 5B). HUVECs used as a positive control also formed networks but were not able to form three-dimensional capillary-like structures (compare Fig. 5C). Analyzing endothelial differentiation of CD31⁻ cells grown in CM-1 or CM-2 by flow cytometry, we found that in CM-1 medium CD31⁻ SVF cells express CD34 up to around 40% and KDR by 80% (Fig. 6A, Table 2). This medium therefore seems to support endothelial precursor characteristics in the CD31⁻ cells. In CM-2 medium, in contrast, CD34 and KDR expression is significantly lower (Fig. 6B, Table 2), demonstrating the capacity of this medium to enhance endothelial differentiation to mature EC and diminish endothelial precursor cell characteristics of SVF cells. In both media cells were positively stained for acLDL, confirming differentiation toward an endothelial phenotype (Fig. 6). acLDL labels endothelial progenitor cells, vascular EC, and macrophages.

This study demonstrates that the term preadipocytes can be misleading if purification by cell sorting or equivalent methods have not been applied. Depending on the method of yield, the amount of EC in the SVF varies between 10% ± 2% in excised fat and 24% ± 15% in lipoaspirate. The SVF is therefore not a unique cell fraction but a mixture of various cells with varying surface protein expressions, one of which is a...
CD31−, S100+ cell type that has the capacity to differentiate into adipocyte as well as endothelial precursor cell and mature EC, depending on the involved differentiation-inducing factors. In addition, the SVF further contains a CD31+, CD34+, S100− cell fraction with an endothelial phenotype which can be converted into adipose tissue under adequate differentiation conditions. These results encourage new approaches in adipose tissue engineering, as the use of purified multipotent SVF with adipogenic and angiogenic potential instead of applying the unpurified SVF cell pool might allow better vascularization and tissue growth. Furthermore, our findings might have relevant impact on the pathogenesis of atherosclerosis as the development and advancement of atherosclerotic lesions seems to involve EC displaying both endothelial and adipogenic properties.

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