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CD146 (MCAM) in human cs-DLK1^{-}/cs-CD34^{+} adipose stromal/progenitor cells

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ABSTRACT

To precisely characterize CD146 in adipose stromal/progenitor cells (ASCs) we sorted the stromal vascular faction (SVF) of human abdominal subcutaneous white adipose tissue (sWAT) according to cell surface (cs) expression of CD146, DLK1 and CD34. This test identified three main SVF cell populations: ~50% cs-DLK1^{-}/cs-CD34^{+}/cs-CD146^{-} ASCs, ~7.5% cs-DLK1^{+}/cs-CD34$^{dim/+}$/cs-CD146^{+} and ~7.5% cs-DLK1^{+}/cs-CD34$^{dim/+}$/cs-CD146^{-} cells. All cells contained intracellular CD146. Whole mount fluorescent IHC staining of small vessels detected CD146^{+} endothelial cells (CD31^{+}/CD34^{+}/CD146^{+}) and pericytes (CD31^{-}/CD34^{-}/CD146^{+} ASCs). The cells in the outer adventitial layer showed the typical ASC morphology, were strongly CD34^{+} and contained low amounts of intracellular CD146 protein (CD31^{-}/CD34^{+}/CD146^{+}). Additionally, we detected wavy CD34^{-}/CD146^{+} and CD34^{dim}/CD146^{+} cells. CD34^{dim}/CD146^{+} cells were slightly more bulky than CD34^{-}/CD146^{+} cells. Both CD34^{-}/CD146^{+} and CD34^{dim}/CD146^{+} cells were detached from the inner pericyte layer and protruded into the outer adventitial layer. Cultured early passage ASCs contained low levels of CD146 mRNA, which was expressed in two different splicing variants, at a relatively high amount of the CD146-long form and at a relatively low amount of the CD146-short form. ASCs contained low levels of CD146 protein, which consisted predominantly long form and a small amount of short form. The CD146 protein was highly stable, and the majority of the protein was localized in the Golgi apparatus. In conclusion, the present study contributes to a better understanding of the spatial localization of CD34^{+}/CD146^{+} and CD34^{-}/CD146^{+} cells in the adipose niche of sWAT and identifies CD146 as intracellular protein in cs-DLK1^{-}/cs-CD34^{+}/cs-CD146^{-} ASCs.

1. Introduction

Adipose stromal/progenitor cells (ASCs) are the major reservoir of adipocyte precursors and are crucial for adipose tissue renewal, homeostasis and expansion (Berry et al., 2016; Rodeheffer et al., 2008; Tang et al., 2008; Yamamoto et al., 2007). In white adipose tissues (WAT), these cells mainly reside in the vascular stroma around small blood vessels and can proliferate and differentiate into adipocytes (Tang et al., 2008; Zimmerlin et al., 2010; Zwierzina et al., 2015). The entire fraction of adipose stromal cells can be isolated from the stromal vascular fraction (SVF) of disaggregated adipose tissue according to their property to adhere to plastic surfaces. Adipose stromal cells are thought to consist of a heterogeneous cell population. ASCs are a subpopulation of these cells, and certain cell surface marker combinations are employed to better characterize them (Bourin et al., 2013; Cawthorn et al., 2012). Most prominently ASCs are positive for the stromal marker CD90 (Mitchell et al., 2006) and for CD34 (Sengenès et al., 2005), which is a marker for diverse stromal/progenitor cell types (Sidney et al., 2014), as well as negative for the endothelial cell marker CD31 and hematopoietic lineage marker CD45 (Bourin et al., 2013). Another interesting marker for human ASCs is Delta-like protein 1/Preadipocyte factor 1 (DLK1/Pref1). DLK1 is a functional marker of ASCs (Hudak and Sul, 2013; Traustadottir et al., 2013; Mitterberger et al., 2012; Zwierzina et al., 2015) and other stromal/progenitor cell types (Abdallah et al., 2015), which regulates both proliferation and terminal adipogenic differentiation in ASCs. Approximately 30% of native SVF-cells of human subcutaneous (s) WAT are cell surface (cs)-DLK1^{+}/cs-CD34^{-} or cs-DLK1^{+}/cs-CD34^{dim} and ~45% are cs-DLK1^{-}/cs-CD34^{+} (Zwierzina et al., 2015). Only the cs-DLK1^{-}/cs-CD34^{+} subpopulation possesses high proliferative and adipogenic potential. Thus, surface expression of DLK1 and CD34 contributes to a better distinction between adipose stromal/progenitor cells in the SVF.

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Stromal cells of WAT are organized in two layers, including a perivascular inner ring and peripheral supra-adventitial ring (Zimmerlin et al., 2010; Zwierzina et al., 2015). ASCs are mainly localized in the supra-adventitial ring, and a current model suggests that ASCs could arise from pericytic progenitors in the perivascular inner ring (Zimmerlin et al., 2013). It was hypothesized that $CD34^-$/$CD146^+$ pericytes in the inner ring differentiate from inside out into ASCs in the supra-adventitial layer, which are $CD34^+$, but do not express CD146 ($CD34^+$/$CD146^-$) (Zimmerlin et al., 2010, 2013). Given the importance of DLK1 and CD34 as ASC markers as well as CD146 as a stem cell marker in bone marrow stromal cells (bMSCs) (Sacchetti et al., 2007; Sacchetti et al., 2016), it would be interesting to further characterize CD146 in cs-$DLK1^-$/cs-$CD34^+$ ASCs. CD146, which is also referred to as melanoma cell adhesion molecule (MCAM) or cell surface glycoprotein MUC18, is a 113-kDa transmembrane glycoprotein that belongs to the immunoglobulin superfamily (IgSF-CAM) and participates in intercellular adhesion (Wang and Yan, 2013). This protein exists in two isoforms, CD146 long (CD146-l) and CD146 short (CD146-s), which differ in the length of their intracellular domain (Wang and Yan, 2013; Alais et al., 2001) and function (Wang and Yan, 2013; Kebir et al., 2010). In tissues of adults, CD146 is expressed in vascular endothelial cells, smooth muscle cells, cord blood stromal cells and pericytes in bone marrow sinusoids, skeletal muscle and the periosteum (Zimmerlin et al., 2010; Wang and Yan, 2013; Russell et al., 2010; Sacchetti et al., 2016). Although its biological functions are not precisely understood, studies suggest that CD146 plays a role in signal transduction as well as regulates tissue development and homeostasis (Wang and Yan, 2013). The CD146 cell surface levels likely correlate positively with the multipotency of bone marrow stromal cells (BMSCs) (Russell et al., 2010; Sorrentino et al., 2008). The aim of the present study was to more precisely analyze CD146 in stromal/progenitor cells of human sWAT.

2. Material and methods

2.1. Isolation and cultivation of human adipose-derived stromal/progenitor cells

Human ASCs were isolated from abdominal sWAT samples obtained by incisions from female donors undergoing elective plastic abdominal surgery as described in Mitterberger et al. (2012). Briefly, adipose tissue biopsies were transferred into sterile serum-free ASC medium (DMEM/F-12 medium (1:1) with HEPES and L-glutamine (Gibco, Vienna, Austria) after surgery and supplemented with 33 μM biotin, 17 μM pantothenate and 12.5 μg/ml gentamicin. The samples were kept at 4 °C for 1–3 h before sterile processing in a laminar flow sterile work bench class II. After washing the cells with PBS, fibrous material and blood vessels were dissected. The tissue was cut into pieces and digested by collagenase in digestion buffer (PBS containing 200 U/ml collagenase (CLS Type I, Worthington Biochemical Corp., Lakewood, NJ) and 2% w/v BSA). The dispersed tissue was centrifuged, and floating adipocytes were aspirated. The sedimented cells were suspended in erythrocyte lysis buffer (0.155 M NH4CI, 5.7 mM K2HPO4, 0.1 mM EDTA, pH 7.3) and incubated for 10 min at room temperature (the colony-forming efficiency before and after erythrocyte lysis were not separately tested). Afterwards, the cells were purified through additional centrifugation and filtration steps. Isolated cells are referred to as the stromal vascular fraction (SVF). These cells were suspended in ASC medium (DMEM/F-12 medium (1:1) with HEPES and L-glutamine (Gibco, Vienna, Austria) containing 33 μM biotin, 17 μM pantothenate and 12.5 μg/ml gentamicin) supplemented with 10% FBS (Gibco, Vienna, Austria) and seeded into 6-well culture dishes at a density of 70,000 cells per cm^2. Following 16 h of attachment, cells were kept for 6 days in serum-free ASC medium, and non-adherent cells were continuously removed through washing. The remaining cell fraction was referred to as passage 1 (P1). These cells were trypsinized and stored in liquid N2. After thawing, cells were seeded overnight in ASC medium supplemented with 10% FBS, and then, adherent cells were washed and cultivated in PM4 medium (ASC medium supplemented with 2.5% FBS, 10 ng/ml EGF, 1 ng/ml bFGF, 500 ng/ml insulin). Note, in this step, significant numbers of cells that do not efficiently adhere are lost, and more cells are lost than in a standard thawing/freezing cycle. The cells were passaged at a ratio of 1:2 when they reached 70% confluence. For expansion of ASCs, cells were trypsinized and seeded in ASC medium supplemented with 10% FBS at a density of 5000/cm^2, followed by cultivation in PM4 medium after cell attachment. Cells were passaged at a ratio of 1:2 whenever they reached 70% confluence. In this study, SVF cells and ASCs from passages 1 to 7 (P1–P7) were used.

2.2. Cultivation of human U2OS osteosarcoma cells and human foreskin fibroblasts (HFFs)

U2OS cells and HFFs were cultivated in Dulbecco modified Eagle medium (DMEM, Sigma Aldrich), 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

2.3. Flow cytometry analysis

To phenotypically characterize passage 5 from cultures, ASC fluorescence-activated cell sorting (FACS Canto II, BD Biosciences) was used (Ejaz et al., 2016). Cells were either used in an un-permeabilized form or fixed and permeabilized using a BD Cytofix/Cytoperm solution before they were simultaneously stained with directly conjugated mouse anti-human antibodies (CD31-FITC-A (WM59), CD34-PE-Cy7A (581), CD90-PE-A (5E10), CD105-PerCP-Cy5-5-A (266); BD Pharmingen) and a primary rat monoclonal anti-human DLK1/PREF1 antibody (Adipogen, San Diego) along with the anti-rat-APC-A antibody (BD Pharmingen). To analyze the CD146 protein in ASCs, HFFs and U2OS cells, two different staining protocols were applied. To detect cell-surface-tethered CD146, cells were exclusively stained without fixation and permeabilization. To detect intracellular and cell surface tethered CD146, cells were fixed and permeabilized. Three different monoclonal mouse anti-human CD146-antibodies were used, including two directly labeled anti-human CD146 antibodies (PE anti-human CD146 clone SHM-57, BioLegend and PE anti-human CD146 clone TEA 1/34, BeckmanCoulter) as well as one unconjugated anti-human CD146 antibody (clone 1/MCAM, BD Biosciences) combined with a FITC-labeled secondary antibody. Isotype matched non-immune immunoglobulins were used as controls. Cell sorting was conducted at the Tyrolean Cancer Research Institute, Innsbruck, Austria, under the supervision of Dr. Sieghart Sopper. Overall, 20×10^6 SVF cells were stained with anti-CD146 (clone SHM-57), anti-CD34 and anti-DLK1 (PREF-1) antibodies and sorted by FACSArIa (BD, Bioscience). The purity of the sorted fractions was analyzed by FACS before functional studies. For surface stainings, cells were stained without fixation, while cells were fixed and permeabilized for intracellular stainings.

2.4. Real-time quantitative PCR

Expression analysis with real-time quantitative PCR (qPCR) was performed as described (Mitterberger et al., 2014b). Briefly, total RNA was isolated by the RNeasy Micro Kit (Qiagen), and cDNA synthesis was carried out with a First Strand cDNA Synthesis Kit (Fermentas). Quantitative expression analysis was performed using SYBR Green I Master Mix (Roche) on a LightCycler 480 Real-Time PCR System (Roche, Austria), and mRNA quantification was based on normalization to the β-actin gene using the 2-ddCT method. The primer sequences used in this study are shown in Table 1.