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Plasticity of human dedifferentiated adipocytes toward endothelial cells

Antonella Poloni[a], Giulia Maurizi[a], Sara Anastasi[a], Eleonora Mondini[b], Domenico Mattiucci[a], Giancarlo Discepoli[c], Fabiola Tiberi[c], Stefania Mancini[a], Stefano Partelli[d], Angela Maurizi[d], Saverio Cinti[b], Attilio Olivieri[a], and Pietro Leoni[a]

[a]*Clinica di Ematologia, Dipartimento di Scienze Cliniche e Molecolari, Università Politecnica delle Marche, Ancona, Italy;* [b]*Dipartimento di Medicina Sperimentale e Clinica, Università Politecnica delle Marche, Ancona, Italy;* [c]*Laboratorio di Citogenetica e Genetica Molecolare, Clinica di Pediatria, Università Politecnica delle Marche, Ancona, Italy;* [d]*Clinica Chirurgia del Pancreas, Università Politecnica delle Marche, Ospedali Riuniti, Ancona, Italy*

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The process of cellular differentiation in terminally differentiated cells is thought to be irreversible, and these cells are thought to be incapable of differentiating into distinct cell lineages. Our previous study showed that mature adipocytes represent an alternative source of mesenchymal stem cells. Here, results showed the capacity of mature adipocytes to differentiate into endothelial-like cells, using the ability of these cells to revert into an immature phase without any relievable chromosomal alterations. Mature adipocytes were isolated from human omental and subcutaneous fat and were dedifferentiated in vitro. The resulting cells were subcultivated for endothelial differentiation and were analyzed for their expression of specific genes and proteins. Endothelial-like cells were harvested from the differentiation medium and were traditionally cultured to evaluate the endothelial markers and the karyotype. Cells cultured in specific medium formed tube-like structures and expressed several endothelial marker genes and proteins. The endothelial-like cells expressed significantly higher levels of vascular endothelium growth factor receptor 2, vascular endothelial cadherin, Von Willebrand factor, and CD133 than the untreated cells. These cells were positively stained for CD31 and vascular endothelial cadherin, markers of mature endothelial cells. Moreover, the low-density lipoprotein–uptake assay demonstrated a functionally endothelial differentiation of these cells. When these cells were harvested and reseeded in basal medium, they lost the endothelial markers and reacquired the typical mesenchymal stem cell markers and the ability to expand in a short time period. Moreover, karyotype analysis showed that these cells reverted into an immature phase without any karyotype alterations. In conclusion, the results showed that adipocytes exhibited a great plasticity toward the endothelial lineage, suggesting their possible use in cell therapy applications for vascular disease.

Endothelial progenitor cells (EPCs) have been described as a rare population of nonhematopoietic cells that reside in the bone marrow and support the integrity of the vascular endothelium [1–3]. Endothelial progenitor cells have been isolated from human blood and may contribute to the formation of new vessels and the repair of damaged endothelium [4–7]. Even if a large number of articles describing early endothelial progenitor cells has been published [8–10], most of these cells were identified to be monocytes/macrophages. It is now conceivable that only a small population of outgrowth cells cultivated for several passages from human blood could be late endothelial progenitor cells. Although the nature and functions of endothelial progenitors are controversial, it is likely that a population of blood cells plays a role in promoting angiogenesis or endothelial repair [11].

Moreover, revascularization of tissue following a cardiac infarct is one of the aims of conventional therapy, and EPCs have been widely studied as a potential source of cell-based therapy for several cardiovascular disorders [12–17]. However, the number of EPCs that can be obtained from adult blood is limited.

Several studies have demonstrated that mesenchymal stem cells (MSCs) from various tissues have the potential

AP and GM contributed equally to this work.

Offprint requests to: Dr. Antonella Poloni, Department of Hematology, Università Politecnica delle Marche, Via Tronto 10, Ancona 60020, Italy; E-mail: a.poloni@univpm.it

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to differentiate into other cell types, suggesting that precommitted and committed cells have plasticity in cell fate determination [18].

Mesenchymal stem cells can be isolated from a variety of adult tissues and organs, and adipose tissue is abundant and easily accessible at most ages. Mature adipocytes are numerous in adipose tissue, and they are easily isolated without painful procedures or donor site injuries. We previously demonstrated that mature adipocytes isolated from fat tissue can dedifferentiate into MSCs using an easy in vitro dedifferentiation strategy and a small amount of adipose tissue. Thus, white mature adipocytes gave rise to dedifferentiated cells when they lose their fat in culture. During this process, gene reprogramming events occur, leading to changes in the epigenetic status of the cells, which allow them to acquire morphologic and functional stem cell properties [19]. Therefore, dedifferentiated adipocytes may be an attractive source of stem cells for regenerative medicine and other cell-based therapies.

The aim of the present study was to examine whether dedifferentiated fat cells could be induced into the endothelial lineage, using the ability of these cells to revert into an immature phase without any relievable karyotype alterations.

Materials and methods

Isolation and culture of adipocytes

In accordance with the guidelines of the local ethical committee (300/DG), omental and subcutaneous fat tissue (5–10 g) were obtained from patients ($n = 10 + 10$, 53–81 years old) at the time of abdominal surgery. The patients were not obese and were undergoing surgery for pancreatic diseases that were localized and had not enlarged to the omental and subcutaneous fat tissues. Adipose tissue was promptly washed with Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Milan, Italy), and the visible blood vessels were removed. The samples were minced into smaller pieces and treated with 3 mg/mL type I collagenase (Gibco, Milan, Italy) at 37°C for 2 hours.

To obtain isolated mature adipocytes free of stromal-vascular elements, after collagenase digestion, the disrupted tissues were filtered through a 200-μm nylon sieve. The filtered cells were washed four times with DMEM and centrifuged at 250 *g* for 5 min. Only the floating top layer was collected after each centrifugation step, which allowed for the isolation of a pure fraction of floating adipocytes and a pellet containing the stromal vascular fraction (SVF) cells. After the last centrifugation step, the fatty layer was transferred to an inverted 25-cm^2 cell culture flask that was completely filled with DMEM supplemented with 20% fetal bovine serum (Stem Cell Technologies, Vancouver, Canada), and the cells were seeded for ceiling culture, where the bottom of the flask is on top [5]. Only mature adipocytes free of any detectable contamination of stromal-vascular elements were allowed to adhere to the top layer of the flask in a 5% CO_2 incubator (37°C), and the cultures were monitored daily for cell attachment. When the cells lost their spherical shape, they were trypsinized and subcultivated at an initial seeding density of 2×10^3 cells/cm^2.

Quantitative cytokine assay

When the cells reached confluence, the culture supernatants of the dedifferentiated adipocytes were collected and frozen at −20°C. Multiplex human cytokine, chemokine, and growth factor detection assays (BioPlex, BioRad, Milan, Italy) were utilized to measure the levels of interleukin (IL) 6, IL-8, basic fibroblast growth factor (FGF-β), basic granulocyte colony-stimulating factor (G-CSF), interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and vascular endothelium growth factor (VEGF) in the culture supernatants.

Endothelial differentiation of dedifferentiated adipocytes

Matrigel (BD Biosciences, Milan, Italy) was added to a 24-well plate (200 μL well^{-1}) and allowed to solidify for 30 min at 37°C. Cells (0.1×10^6) were suspended in 500 μL of DMEM and were placed on top of the Matrigel. After 24-hour incubation, the formation of cord- or tube-like networks was examined and recorded using a phase contrast microscope. Early outgrowth cells were obtained after 7 days in culture and were analyzed for the expression of several endothelial markers. The cells were harvested and studied by flow cytometry, real-time polymerase chain reaction (PCR), and immunocytochemistry.

Huvec cell line and SVF-derived mesenchymal stem cell (cultured in Matrigel) were analyzed in all the experiments as positive controls.

Immunophenotype analysis

The dedifferentiated adipocytes were characterized by flow cytometry before and after the endothelial differentiation procedure. To harvest the cells from Matrigel, a step of collagenase digestion was performed. The cells were stained with fluorescein isothiocyanate–, phycoerythrin- or PerCP-conjugated antibodies against CD31 (platelet endothelial cell adhesion molecule), CD34 (hematopoietic progenitor cell antigen, BD Biosciences), CD45 (leukocyte common antigen, BD Biosciences), CD90 (Thy-1, BD Pharmingen) and CD73 (ecto-5′-nucleotidase).

We used fluorescein isothiocyanate, phycoerythrin (Dako, Glostrup, Denmark), and PerCP (Becton Dickinson Pharmingen, Milan, Italy) negative isotypes as control antibodies. The cells were incubated with the primary antibodies at 4°C for 30 min. Thereafter, cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (Becton Dickinson Pharmingen). The data were analyzed using the CellQuest software (Milan, Italy).

Real-time analysis for endothelial differentiation markers

To harvest the cells from Matrigel, a step of collagenase digestion was performed. Total RNA was extracted using the RNeasy Plus Micro Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. The purity of the RNA was confirmed by determining the 260 nm/280 nm absorbance ratio (>1.8). For each sample, 1 μL of total RNA was reverse-transcribed in a 20 μL reaction containing 5 × reaction buffer (Invitrogen, Milan, Italy), 100 mmol/L dNTPs (Biotech, Milan, Italy), 50 mmol/L $MgCl_2$ (Promega, Milan, Italy), 3 μg/μL random hexamers (Invitrogen), 100 mmol/L dithiothreitol (DTT; Invitrogen), 40 U/μL RNase inhibitor (Takara, Shiga, Japan), and 200 U/μL Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). The reactions proceeded for 10 min at 70°C, 10 min at 20°C, 45 min at 42°C, and 3 min at 99°C.

Real-time PCR analysis of the dedifferentiated adipocytes and SVF-derived MSCs were performed to quantify the expression of