Plasticity of Human Adipose Lineage Cells Toward Endothelial Cells
Physiological and Therapeutic Perspectives

Valérie Planat-Benard, PhD*; Jean-Sébastien Silvestre, PhD*; Béatrice Cousin, PhD; Mireille André, MS; Maryse Nibbelink, PhD; Radia Tamarat, PhD; Michel Clergue, BSc; Carole Manneville, MS; Corinne Saillan-Barreau, PhD; Micheline Duriez, BSc; Alain Tedgui, PhD; Bernard Levy, MD, PhD; Luc Pénicaud, PhD; Louis Casteilla, PhD

Background—Adipose tissue development and remodeling are closely associated with the growth of vascular network. We hypothesized that adipose tissue may contain progenitor cells with angiogenic potential and that therapy based on adipose tissue-derived progenitor cells administration may constitute a promising cell therapy in patients with ischemic disease.

Methods and Results—In mice, cultured stromal-vascular fraction (SVF) cells from adipose tissue have a great proangiogenic potential, comparable to that of bone marrow mononuclear cells in the mouse ischemic hindlimb model. Similarly, cultured human SVF cells differentiate into endothelial cells, incorporate into vessels, and promote both posts ischemic neovascularization in nude mice and vessel-like structure formation in Matrigel plug. In vitro, these cells represent a homogeneous population of CD34- and CD13-positive cells, which can spontaneously express the endothelial cell markers CD31 and von Willebrand factor when cultured in semisolid medium. Interestingly, dedifferentiated mature human adipocytes have the potential to rapidly acquire the endothelial phenotype in vitro and to promote neovascularization in ischemic tissue and vessel-like structure formation in Matrigel plug, suggesting that cells of endothelial and adipocyte phenotypes may have a common precursor.

Conclusions—This study demonstrates, for the first time, that adipocytes and endothelial cells have a common progenitor. Such adipose lineage cells participate in vascular-like structure formation in Matrigel plug and enhance the neovascularization reaction in ischemic tissue. These results also highlight the concept that adipose lineage cells represent a suitable new cell source for therapeutic angiogenesis in ischemic disease. (Circulation. 2004;109:656-663.)

Key Words: angiogenesis ■ ischemia ■ adipose tissue ■ therapy, cell ■ progenitor cells

White adipose tissue has long attracted attention because of its great and reversible capacity for expansion, which appears to be permanent throughout adult life.1 Adipose tissue enlargement is the result of adipocyte hypertrophy and the recruitment and differentiation of regenerative precursors located in the stromal-vascular fraction (SVF). However, development of the capillary network is also required to ensure adipose tissue remodeling.2,3 Indeed, a crucial link exists between adipose cells and the capillary network. First, the vascular pattern during embryonic development indicates that the formation of capillary convolutions is a decisive and specific phase in the development of fat lobules.4 Second, extensive vascularization is necessary for optimal functioning of adipose tissue as a metabolic and endocrine tissue. Third, adipose lineage cells have been shown to release potent angiogenic factors such as monobutyrin, vascular endothelial growth factor (VEGF), and leptin.5–8 Finally, antiangiogenic agents promote adipose tissue loss, underlining the functional interaction between adipogenesis and blood vessel formation.9

SVF represents a heterogeneous cell population surrounding adipocytes in fat tissue, including mature microvascular endothelial cells.10,11 Its culture was classically used to investigate preadipocyte differentiation into mature adipocytes. However, this fraction was also reported to be a very convenient and nonrestrictive source of pluripotent cells such as adipose tissue-derived precursor cells.2,3,12

Received November 7, 2003; accepted December 1, 2003.
From Unité Mixte de Recherche 5018 Centre National de la Recherche Scientifique, Université Paul Sabatier, Centre Hospitalier Universitaire Rangueil, Toulouse (V.P.-B, B.C., M.A., M.N., C.M., C.S-B., L.P., L.C.), and Institut National de la Santé Et de la Recherche Médicale Unité 541, Hôpital Lariboisière, Paris (J-S.S., R.T., M.C., M.D., A.T., B.L.), France.
*These authors contributed equally to this study.
This article originally appeared Online on January 20, 2004 (Circulation. 2004;109:r23–r30).
Correspondence to Louis Casteilla, PhD, UMR 5018 CNRS UPS, IFR31, CHU Rangueil, 1 avenue J. Poulhès, 31054 Toulouse, France. E-mail casteill@toulouse.inserm.fr
© 2004 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000114532.38265.61
as hematopoietic progenitors and spare mesodermal stem cells able to differentiate into osteogenic, chondrogenic, and myogenic lineages.12–14 Recently, the spectrum of differentiation potential was even extended to the neurogenic phenotype.15 We hypothesized that adipose tissue and especially SVF may also contain progenitor cells with angiogenic potential able to differentiate into mature endothelial cells and to participate in blood vessel formation.

In this study, we demonstrate for the first time that adipocytes and endothelial cells have a common progenitor. Such adipose lineage cells participate in vascular-like structure formation in Matrigel plug and enhance the neovascularization reaction in ischemic tissue.

Methods

Animals and Tissue Sampling

Seven-week-old male C57BL/6 or nu/nu mice (Harlan, France) were housed in a controlled environment (12 hours light/dark cycle at 21°C) with free access to water and standard chow diet. At the end of the experiments, mice were killed by cervical dislocation under CO2 anesthesia. Inguinal adipose tissue or muscle was rapidly removed and processed for analyses as described below. All procedures were performed according to SELASA norms.

We obtained samples of human abdominal subcutaneous fat from patients undergoing abdominal dermolipectomy or nephrectomy in the Department of Plastic Surgery and Urology of the Hospital Rangueil Hospital (Toulouse, France). The patients gave their written informed consent and the Ethics Committee of the Hospital approved the study.

Cell Isolation and Culture

Mouse bone marrow cells were obtained by flushing the tibias and femurs. Low-density mononuclear cells were isolated by density gradient centrifugation with Ficoll, as previously described.16 Cells were isolated from adipose tissues according to Bjornstorpe et al17 with minor modifications. Inguinal adipose tissue excised from mice and human adipose tissue from dermolipectomy were digested at 37°C in PBS containing 2% BSA and 2 mg/mL collagenase (Sigma) for 45 minutes. After elimination by filtration through 25 μm filters of undigested fragments, mature adipocytes were separated from pellets of SVF cells by centrifugation (600g, 10 minutes). SVF cells were plated (30 000 cells/cm2) in DMEM-F12 10% newborn calf serum (NCS) medium. Six hours after plating, all nonadherent cells were removed and processed for analyses as described below. SVF cells isolated either from mice or human tissue. On day 14, the angiogenic potential of SVF derived from adipose tissue was first assessed with cultured mouse SVF cells and was analyzed in 3 different sites (gastrocnemius, gracilis, and quadriceps muscles, respectively, 25 μL per injection) of the ischemic leg. Functional analyses were performed 15 days after the onset of ischemia.

Quantification of Angiogenesis

Vessel density was evaluated by high-definition microangiography at the end of the treatment period, as previously described.20 The angiographic score was expressed as a percentage of pixels per image occupied by vessels in the quantification area. Microangiographic analysis was completed by assessment of capillary density by using antibody against total fibronectin.20 Capillary density was analyzed in 3 different sections of gastrocnemius muscle. Five different fields were used in each section (Histolab software, Microvision Instruments). Capillary/myocyte ratios were calculated, and the results were then expressed according to ischemic/nonischemic ratios.

To provide functional evidence for ischemia-induced changes in vascularization, laser Doppler perfusion imaging experiments were performed in mice, as previously described.20

Angiogenesis Assay Using Matrigel Plug

Mice received 0.5 mL subcutaneous injection with Matrigel+1×106 SVF cells isolated either from mice or human tissue. On day 14, the mice were euthanized and analyses were performed as previously described.21

Statistical Analyses

Results were expressed as mean±SEM. One-way ANOVA was used to compare each parameter. Post hoc Bonferroni t test comparisons were then performed to identify which group differences account for the significant overall ANOVA.

Results

Mouse SVF Cells Promote Angiogenesis in Ischemic Muscle

The angiogenic potential of SVF derived from adipose tissue was first assessed with cultured mouse SVF cells and was compared with the proangiogenic potential of bone marrow–derived mononuclear cells (BM-MNC). SVF cells were...
prepared from inguinal adipose tissue and cultured for a limited culture of 3 days with no successive plating. Intra-muscular transplantation of $1 \times 10^6$ freshly isolated SVF cells resulted in only a small increase in neovascularization (data not shown). After limited culture for 3 days, transplantation of $1 \times 10^6$ cultured SVF cells markedly improved tissue neovascularization in ischemic hindlimbs as revealed by a 2.6-fold increase in angiography score (Figure 1a and 1d, $P<0.01$), a 1.6-fold rise in Doppler tissue perfusion score (Figure 1b and 1e, $P<0.001$) and a 2.3-fold increase in capillary density (Figure 1c and 1f, $P<0.01$). The SVF-induced increase in ischemic/nonischemic capillary density ratio was confirmed by CD31 immunostaining (0.71±0.09 versus 1.34±0.18 in control and SVF-treated mice, respec-

Figure 1. Cultured mouse SVF cells exhibit angiogenic properties after their injection in ischemic hindlimbs; comparison with BM-MNC. 
a and d, Representative microangiography of the right ischemic and left nonischemic hindlimbs 15 days after femoral artery occlusion. Red arrows indicate ligated ends of femoral arteries. 
b and e, Hindlimb blood flow monitored in vivo by laser Doppler perfusion imaging, 15 days after femoral artery occlusion. In color-coded images, normal perfusion is depicted in red, and marked reduction in blood flow of ischemic hindlimb is depicted in blue. 
c and f, Representative photomicrographs of ischemic muscle sections 15 days after femoral artery occlusion, hybridized with antibody directed against total fibronectin. Capillaries appear in white and myocytes in black. 
Values are mean±SEM, n=6 per group. PBS indicates PBS-treated mice with hindlimb ischemia; 3T3, mice with hindlimb ischemia receiving 3T3 cells; SVF, SVF-treated animals with hindlimb ischemia; and BM-MNC, mice with hindlimb ischemia receiving BM-MNC. 
**$P<0.01$, ***$P<0.001$ vs PBS-treated mice.
tively, \( P<0.01 \)). This demonstrated that the SVF cells that emerged after a limited culture had a higher proangiogenic potential than freshly isolated SVF cells. Interestingly, administration of \( 1 \times 10^8 \) cultured SVF cells enhanced the neovascularization process to a similar extent as that of \( 1 \times 10^6 \) BM-MNC (Figure 1a through 1c). It is noteworthy that 3T3 administration did not modulate the neovascularization reaction (Figure 1). In addition, experiments with SVF cells purified from brown adipose tissue, well known to be more highly vascularized than white adipose tissue, were unsuccessful. Indeed, the ischemic/nonischemic ratio for angiographic score was 0.51 \( \pm 0.07 \) versus 0.49 \( \pm 0.06 \) (\( P=0.91 \)) and that of blood flow was 0.66 \( \pm 0.05 \) versus 0.63 \( \pm 0.06 \) (\( P=0.69 \)) in mice treated with SVF cells derived from brown adipose tissue compared with control animals, respectively (data not shown). This suggests that the high proangiogenic potential of cultured white adipose tissue SVF is not due to circulating endothelial precursors, but rather to cells resident in the fat tissue.

Phenotypic Characterization of Freshly Isolated SVF Cells and of SVF Cells Cultured for 72 Hours

We next analyzed the cellular phenotype of freshly isolated and cultured SVF cells in murine and human species by flow cytometry. As similar results were obtained in both species, we pursued this study with human cells. In freshly prepared SVF cells from human subcutaneous adipose tissue, we detected several cell populations as shown on forward scatter (Figure 2a). Culture of these SVF cells for 3 days in previously described standard conditions led to the selection of the single cell population with the highest forward scatter (Figure 2b). The antigenic phenotype was consistent with this analysis; the great majority of the population (90\% to 99\%) was composed of undifferentiated cells expressing CD34, CD13, and HLA-ABC and negative for monocyte/macrophage, hematopoietic (CD45 and CD14), and differentiated endothelial cell markers (CD144 and CD31) (Figure 2c and 2d). Hence, an in vitro cell culture of 3 days appeared to increase the proportion of a unique population of cells with no surface markers of any differentiated cells and some surface antigens characteristic of immature cells with proangiogenic potential such as CD34-positive cells.

**Figure 2.** Culture of a heterogeneous population of freshly prepared human SVF promotes a homogeneous cell population with a high forward scatter. a and b, Scattergrams of freshly prepared and cultured human SVF cells. c and d, Panhematopoietic (CD45), monocyte/macrophage (CD14) and mature endothelial (CD144) antigens were identified in a heterogeneous population of freshly prepared SVF (c, open bars) markers and not in cultured SVF cells (d, filled bars), which expressed CD34, CD13, and HLA-ABC–positive markers.

A Homogeneous Human SVF Cell Population Induces Neovascularization in Mouse Ischemic Muscle and Differentiates Into Endothelial Cells

We next assessed the efficiency of injected cultured human SVF cells on revascularization in immunodeficient nude...
mice. As with mouse cells, the injection of $1 \times 10^6$ cultured human SVF cells after 15 days of hindlimb ischemia significantly improved angiographic score and cutaneous blood flow (by 1.6- and 1.5-fold, respectively, when compared with untreated ischemic nude mice; $P<0.01$) (Figure 3a and 3b). Two potential but nonexclusive mechanisms could explain this proangiogenic effect, as follows: either the release by SVF cells of angiogenic growth factors and/or a direct contribution of injected cells by their incorporation into regenerative vessels. Subsequently, we easily detected VEGF by radioimmunoassay, a potent angiogenic factor in the culture supernatant compared with cell-free medium ($31\pm8$ versus $9\pm6$ ng/mL, respectively, $P<0.01$, $n=6$). Then, to assess the ability of SVF-derived cells to incorporate new blood vessels, we performed immunohistochemistry experiments using an antibody specific to human CD31, which did not cross-react with mouse tissue. Numerous CD31-positive cells lining regenerated vessels were revealed in SVF-injected hindlimbs (Figure 3c). No CD31+ cells were detected in noninjected contralateral hindlimbs. The detection of human CD31+ cells in mice ischemic leg strongly suggested that, in vivo, human SVF-derived cells can differentiate into endothelial cells and directly contribute to vascular regeneration.

Cultured Human SVF Cells Spontaneously Exhibit Endothelial Markers In Vitro and Differentiate Into Endothelial Cells in Subcutaneously Implanted Matrigel Plug

CD34 antigen expression in cultured SVF cells, associated with their efficient in vivo transformation into endothelial cells, raised the question of their differentiation potential. As previously described, cultured human SVF cells easily differentiated into adipocytes in an adipogenic medium (Figure 4a). To test their in vitro endothelial differentiation potential, we first cultured these cells in a semisolid medium known to reveal endothelial potential in progenitors.18 In these conditions, cultured human SVF cells formed a network of branched tubelike structures (Figure 4b), and most of these cells were strongly stained by antibodies directed against CD31 and vWF (Figure 4c and 4d). To definitively determine the endothelial potential of cultured SVF cells, we subcutaneously injected these cells mixed with Matrigel.21 Within the Matrigel plug, cultured SVF cells formed numerous tubelike structures, and the presence of erythrocytes in the lumen demonstrated the existence of a functional vascular structure (Figure 4e and 4f). vWF antibodies positively stained the cells that lined these vessel-like structures (Figure 4g and 4h). Overall, these data demonstrated that cultured SVF cells could spontaneously differentiate into adipocytes and endothelial cells.

Unique Capacity of Mature Human Adipocytes to Dedifferentiate in Culture

The efficient emergence of endothelial as well as adipocyte cells from cultured SVF cells raised the question of the existence of a common precursor for both phenotypes. To address this issue, we used another approach previously described to obtain a homogeneous population of adipocyte precursor cells from adipose tissue.22–24 We isolated human mature adipocytes from tissue, obtaining a $>99\%$ pure floating population of cells. When cultured under the described conditions, adipocytes first lost their fatty acids and changed their morphology to a preadipocyte-like then to a fibroblast-like type of cell that could attach to the coverslip. This morphological change was associated with
The main result of our study is that adipose lineage cells have the potential to differentiate into an endothelial cell phenotype and to participate in blood vessel formation in vivo.

Several hypotheses might be proposed to explain the presence of cells with proangiogenic potential in adipose tissue. These cells obtained from cultured adipose-derived cells may arise from circulating endothelial progenitor cells, which have been shown to mainly derive from monocyte/macrophages. A monocyte/macrophage origin can here be excluded because SVF cells are clearly negative for CD45 and CD14, panhematopoietic and macrophage antigens, respectively. One can also hypothesize that differentiation of endothelial cells from SVF could arise from multipotent adult stem cells (MAPC) present in various tissues. Our culture conditions with limited cell culture and no successive plating do not support the selection of such MAPC and exclude this possibility. Therefore, it is likely that the SVF cells may be bipotent and can be considered as preadipocytes as well as endothelial progenitors. Several findings definitively support this hypothesis. Culture conditions of SVF cells lead to the emergence of a homogeneous CD34+ cell population (Gronthos et al and present FACS analysis). This population of cells, traditionally used to study adipocyte differentiation, is able to spontaneously differentiate into endothelial cells in semisolid medium. In addition, human mature adipocytes that can dedifferentiate into immature cells have the potential to redifferentiate into mature adipocyte and, in both in vivo and in vitro assays, to rapidly acquire endothelial phenotype (expression of CD34, CD31, and vWF antigens) and functions. This suggests the existence of a common precursor for both the endothelial and adipocyte phenotypes (Figure 6). Identification of the pathways that regulate these plastic processes could lead to the development of new therapeutic strategies for control of adipose tissue development.

The ability of adipocyte lineage cells to differentiate into vascular cells is very consistent with histological examination and with recent reports, which suggest that adipose tissue vessels are in a relatively immature state compared with other organs that display a lower plasticity. This statement is also in line with the cellular antigenic phenotype of cultured SVF cells or dedifferentiated adipocytes, expressing the hemangioblast marker CD34 but not
other characteristic markers of endothelial progenitor cells such as CD144. This could be interpreted as a noncommitted status for adipocyte progenitors or dedifferentiated adipocytes, which is favored by the culture conditions.

Therapeutic enhancement of neovascularization is one of the most important strategies needed to limit the complications of postischemic injury. Considerable efforts have focused on the development of therapeutic strategies designed to increase vessel growth in the setting of ischemia. Particularly, transplantation of BM-MNC has been shown to stimulate neovascularization after experimental ischemic injury, resulting in long-term salvage and survival of viable tissue. The use of BM-MNC is now under intense investigation in humans, and the results of early small and uncontrolled studies point to a great potential for such therapy to limit disease progression. In the present work, we showed that the administration of adipose lineage cells can potentially affect revascularization to a similar extent as BM-MNC administration. As with BM-MNC, the proangiogenic properties of adipose lineage cells are consistent with their ability to differentiate into endothelial cells and to secrete growth factors such as VEGF and also their macrophage-like properties.

However, we cannot rule out that part of the SVF-induced vessel growth might be related to the proangiogenic effect of resident microvascular endothelial cells. The fundamental scarcity of endothelial progenitors, combined with their putative functional impairment in pathological conditions, constitutes a major limitation of an endothelial progenitor–based strategy. Thus, the identification of suitable sources of angiogenic cells represents a challenge for therapeutic angiogenesis of ischemic tissues. The concept that adipose lineage cells might be used or recruited in the context of ischemia to induce neovessel formation is an attractive one, especially because of the ready availability of adipose tissue and of the capacity of these cells to expand ex vivo.

In conclusion, our results indicate that adipose lineage cells can function as bipotent progenitors for endothelial cells and adipocytes. This opens new perspectives on adipose tissue development and plasticity and highlights the way for angiogenic therapy based on the administration of adipose tissue-derived stem cells in the treatment of cardiovascular disease.

Acknowledgments
This work was supported by grants from Association Française contre les Myopathies/INSERM (4CS01F, to L.C.), AFM (affectation 132, to L.C.), and the “Programme concerté de thérapie génique et cellulaire en Midi-Pyrénées” (1412924.00). We thank D. Calisse for his surgical expertise, Dr. Y. Barreira for management of the zootechnical facility, and N. Crowte for carefully reading the manuscript.
Mature adipocytes can dedifferentiate and reverse to a bipotent phenotype. Identification of the pathways that regulate these processes could lead to the development of new therapeutic strategies for control of adipose tissue development and therapeutic angiogenesis.

**References**

Plasticity of Human Adipose Lineage Cells Toward Endothelial Cells: Physiological and Therapeutic Perspectives

Valérie Planat-Benard, Jean-Sébastien Silvestre, Béatrice Cousin, Mireille André, Maryse Nibbelink, Radia Tamarat, Michel Clergue, Carole Manneville, Corinne Saillan-Barreau, Micheline Duriez, Alain Tedgui, Bernard Levy, Luc Pénicaud and Louis Casteilla

_Circulation_. 2004;109:656-663; originally published online January 20, 2004; doi: 10.1161/01.CIR.0000114522.38265.61

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circ.ahajournals.org/content/109/5/656

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/