Improvement of Postnatal Neovascularization by Human Adipose Tissue–Derived Stem Cells

A. Miranville, MSc; C. Heeschen, MD; C. Sengenès, PhD; C.A. Curat, PhD; R. Busse, MD, PhD; A. Bouloumié, PhD

Background—Several studies have suggested that stem cells are present in the stroma-vascular fraction (SVF) of adipose tissue (AT).

Methods and Results—To characterize the cell populations that compose the SVF of human AT originating from subcutaneous and visceral depots, fluorescence-activated cell sorter analysis was performed by use of fluorescent antibodies directed against the endothelial and stem cell markers CD31, CD34, CD133, and ABCG2. The freshly harvested SVF contained large numbers of CD34− cells as well as cells expressing CD133 and ABCG2. Further analysis of the CD34+ cells revealed 2 CD34+ cell populations with differential expression of the endothelial cell marker CD31. Selection of the CD34+/CD31− cells by use of magnetic microbeads, followed by cell culture, demonstrated that this cell population could differentiate under appropriate conditions into endothelial cells. Moreover, in mouse ischemic hindlimb, intravenous injection of CD34+/CD31− cells was associated with an increase in the blood flow and the capillary density and an incorporation of the cells in the leg vasculature.

Conclusions—Our data indicate the presence of a cell population within the SVF of human AT characterized as CD34+/CD31− exhibiting characteristics of endothelial progenitor cells. Therefore, human AT might represent a source of stem/progenitor cells useful for cell therapy to improve vasculogenesis in adults. (Circulation. 2004;110:349-355.)

Key Words: vasculogenesis • endothelium • obesity • ischemia

Vasculogenesis and angiogenesis are the 2 major processes contributing to neovascularization. Vasculogenesis requires the recruitment of stem cells/endothelial cell (EC) progenitors, whereas angiogenesis involves the quiescent ECs of the preexisting vessels.1,2 Until recently, it was accepted that vasculogenesis was restricted to embryogenesis. However, the characterization of a population of circulating progenitor cells (CPCs) in adult tissues such as bone marrow and peripheral blood and the observations that transplanted CPCs could incorporate into sites of angiogenesis after tissue ischemia in the limb,3 retina, and myocardium4,5 had suggested that CPCs may contribute to neovascularization in adults. CPCs are believed to originate from the common precursor of hematopoietic cells and ECs and are characterized by the expression of markers such as CD34 and CD133.6-8 The concept of CPC-triggered vasculogenesis has recently evolved on the basis of findings that circulating angiogenic cells originating from the monocyte/macrophage lineage improved the neovascularization to an extent similar to purified CPCs. The latter effects probably involved the release of proangiogenic factors rather than their functional incorporation into vessels.9

The stroma-vascular fraction (SVF) of human adipose tissue (AT) is reported to contain multipotent mesenchymal stem cells that possess the ability of differentiating, in vitro, into cells of mesenchymal lineage, including adipocytes, osteoblasts, chondrocytes, and myoblasts.10 Moreover, in rodents, the presence of hematopoietic stem cells in the AT-derived SVF has also been suggested.11 In the present study, we demonstrate the presence of a cell population expressing the stem cell marker CD34 in the SVF from subcutaneous and visceral human AT. The purification of the CD34+/CD31− cells from human AT-derived SVF revealed that this population contained cells that differentiate, in vitro, into ECs and participate, in vivo, in the revascularization of the ischemic hindlimb in nude mice.

Methods

Materials

Collagenase was obtained from Biochrom AG. A selection kit for human CD34+ cells was from CellSystems (easysep, CellSystems), and magnetic microbeads coupled with anti-CD31 or anti-CD14 antibodies were from Dynal Biotech. EC culture media...
were from Promocell. For the fluorescence-activated cell sorter (FACS) analyses, the mouse IgG antibodies (FITC, perCP, and PE) and the anti-human CD14 (PE), CD34 (perCP), and CD45 (PerCP) antibodies were from BD Biosciences. The anti-CD31 (FITC) was purchased from Cymbius Biotechnology, the anti-CD133 (PE) from Miltenyi Biotech, and the anti-ABCG2 (FITC) from Chemicon. For the immunocytochemistry analyses, the primary mouse monoclonal antibodies directed against von Willebrand factor (vWF) and CD31 (platelet and endothelial cell adhesion molecule) were provided by Dako. Secondary antirabbit and anti-mouse conjugated with Alexa Fluor 488 were purchased from Molecular Probes.

**Isolation of the SVF From Human AT**

Subcutaneous gluteal AT was obtained from 19 individuals (mean body mass index [BMI], 25.01±1.08) undergoing abdominal surgery. Subcutaneous abdominal AT was obtained from 7 normal or moderately overweight individuals (mean BMI, 25.25±0.52) undergoing plastic surgery (liposuction). Visceral abdominal AT was obtained from 22 individuals (mean BMI, 26.32±0.82) undergoing abdominal surgery. The study was approved by the ethical committee of the University Hospital/Frankfurt am Main. The AT was digested by use of collagenase (300 U/mL in PBS, 2% BSA) for 45 minutes (gluteal) or 90 minutes (visceral and subcutaneous abdominal) under constant shaking. After removal of the floating mature adipocytes, the pellets were resuspended in erythrocyte lysis shaking. After incubation (4°C, 20 minutes), the cell suspension containing the beads, suspended in 10 mL PBS/0.1% BSA, was exposed to the magnet for 1 minute. The magnetic bead-free fraction, CD34+/CD31+ cells, was collected, centrifuged (200g, 10 minutes), and resuspended in PBS/0.1% BSA. The CD34+ cells were depleted of CD14+ cells by use of CD14-coupled magnetic microbeads and then CD31+ cells by the approach described above.

**FACS Analysis**

FACS analysis was performed with freshly harvested SVF or CD34+/CD31+ cells from human AT. At least 50 000 cells (in 100 μL PBS/0.5% BSA/2 mmol/L EDTA) were incubated with fluorescence-labeled monoclonal antibodies or the respective isotype control (1/20 diluted, 4°C, 30 minutes). After washing steps, labeled cells were analyzed by flow cytometry by use of a FACSCalibur flow cytometer and the CellQuest Pro software (BD Biosciences).

**Immunocytochemistry**

Immunocytochemistry was performed on CD34+/CD31+ cells plated on fibronectin-coated 48-well plates for 1 to 10 days in different culture media (20 000 cells/cm²). Cells were fixed with 4% paraformaldehyde, blocked with PBS/2% BSA (1 hour, 24°C), and incubated with the primary antibody (anti-CD31, 1/10, and anti-vWF, 1/50, 1 hour, 24°C). After washing steps and 1 hour of incubation time with the corresponding secondary antibody (1/200 diluted), DAPI (4',6-diamino-2-phenylindole, dihydrochloride) (Vectorshied, Vector Laboratories) staining was performed and the cells were observed under fluorescence microscopy.

**Bone Marrow Mononuclear Cells**

Bone marrow aspirate (50 mL) was obtained from healthy individuals, and bone marrow–derived mononuclear cells (BM-
MNCs) were isolated by density gradient centrifugation. After washing steps, cells were resuspended in 10 mL X-vivo 10 medium (Cambrex).

**Hindlimb Ischemia Model**
The neovascularization capacity of AT-derived CD34+/CD31− cells was investigated in a murine model of hindlimb ischemia in 8- to 10-week-old (18 to 22 g) athymic NMRI nude mice (Jackson Laboratory, Bar Harbor, Me). The proximal portion of the femoral artery including the superficial and the deep branches and the distal portion of the saphenous artery were ligated with 7-0 silk suture. All arterial branches between the ligation were obliterated by use of an electrical coagulator. The overlying skin was closed by use of 3 surgical staples. After 24 hours, 200 μL of PBS/2% FCS containing 500,000 freshly isolated AT-derived CD34+ cells was injected intravenously. Identical numbers of freshly harvested BM-MNCs and AT-derived CD34+ cells were used as positive and negative controls, respectively.

After 2 weeks, ischemic (right)/normal (left) limb blood flow ratio was measured by use of a laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System, moorLDI-Mark 2, Moor Instruments). Before scanning was initiated, mice were placed on a heating plate at 37°C. After laser Doppler color images had been recorded twice, the average perfusion of the nonischemic limbs was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, perfusion is expressed as the ratio of the ischemic to the nonischemic hindlimb.

**Histological Evaluation**
Tissue vascularization was determined in 5-μm frozen sections of the adductor and semimembranous muscles from the ischemic and the nonischemic limbs. ECs were stained with FITC-labeled monoclonal antibody directed against CD31 (Chemicon). Capillary density is expressed as the number of capillaries per myocyte relative to the individual nonischemic limb. Incorporation of injected human ATs was verified by costaining for HLA class I-APC (BD Pharmingen) and CD146-FITC.

**Statistical Analysis**
Comparisons between groups were analyzed by use of a t test (2-sided) or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with Bonferroni adjustment. Probability values of P<0.05 were considered statistically significant.

**Results**

**Expression of Stem Cell Markers in the SVF From Human AT**
CD34, CD133, and ABCG2 expression was assessed in freshly harvested SVFs by FACS analysis. Whatever the anatomic source of AT, the highest fluorescence signal was detected for CD34 (Figure 1A) and the lowest for CD133 (Table). Among the ATs, subcutaneous abdominal AT-derived SVF exhibited the highest expression of all 3 markers (2.3 times more CD34 and 4.5 times more ABCG2 than in the SVF from visceral AT, P<0.05; Table).

To characterize the CD34+ cell population, double-color FACS analyses were performed by use of fluorescence-labeled monoclonal antibodies directed against CD34 and the EC marker CD31. As depicted in Figure 1B, FACS analysis clearly showed that the CD34+ cell subset was composed of 2 cell populations: the CD34+/CD31+ cells and the CD34−/CD31− cells. The profile of the CD34+ cell population differed according to the source of AT (Figure 1B). Indeed, the percentage of CD34+/CD31+ cells was higher in the SVFs from subcutaneous ATs (gluteal and abdominal) than in visceral AT (4- to 7-fold increase in the gluteal and abdominal SVF, respectively, compared with the visceral SVF; P<0.05).

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Influence of BMI on CD34+ subsets in SVF of human subcutaneous/gluteal AT. Percentages of CD34+/CD31+ cells (A) and CD34−/CD31− cells (B) within SVF of gluteal AT from patients with BMI <30 (n=16) were determined by double-color FACS analysis.
To determine whether CD34\(^+\) subsets (CD31\(^-\) and CD31\(^+\)) in the gluteal SVFs could be affected by the development of fat mass, the influence of the BMI on the proportion of both CD34\(^+\) subsets was analyzed. To avoid the confounding effects of obesity-associated pathological conditions, the analysis was restricted to normal and slightly overweight individuals (BMI <30). The percentage of CD34\(^+\)/CD31\(^-\) cells was not modified by the BMI (Figure 2A), whereas the number of CD34\(^+\)/CD31\(^+\) cells in the gluteal SVF was positively correlated with the BMI (Figure 2B, \(P<0.01, r^2=0.4496\)).

Isolation and Characterization of the CD34\(^+\)/CD31\(^-\) Cells From SVF

Double-color FACS analysis of the freshly isolated cells demonstrated the efficiency of the extraction protocol. Indeed, FACS analysis showed that all the cells recovered were CD34\(^+\)/CD31\(^-\) (Figure 3A). Moreover, the isolated CD34\(^+\)/CD31\(^-\) population expressed neither the leukocyte marker CD45 nor the monocyte/macrophage marker CD14 (Figure 3B). Only a few cells coexpressed CD133 (1.4±0.2%, Figure 3C), whereas 18±4% coexpressed ABCG2 (Figure 3D).

In Vitro Proliferation and Differentiation of the CD34\(^+\)/CD31\(^-\) Cells Into Mature ECs and Their Effect on Revascularization

The CD34\(^+\)/CD31\(^-\) cells exhibited a high proliferative capacity in culture (doubling time of 33 hours in basal medium/10% FCS), further enhanced in EC growth medium (GM) supplemented with vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) (doubling time of 30.5 hours; Figure 4A; \(P<0.01\)). When plated at high density and cultured for 10 days, spindle-shaped cells developed in GM, whereas cobblestone area appeared spontaneously in BM/10% FCS (Figure 4B). Immunocytochemical analyses showed the appearance of specific labeling for CD31 (Figure 4B) and vWF (data not shown) in cells cultured in GM, which was increased in the presence of VEGF and IGF (26±11% and 14±5% of cells positive for CD31 and vWF, respectively).

To determine whether CD34\(^+\)/CD31\(^-\) cells could participate in the revascularization of ischemic tissues in vivo, freshly isolated CD34\(^+\)/CD31\(^-\) cells and CD34\(^-\)/CD31\(^+\) cells from SVF were injected into the tail vein of nude mice 24 hours after induction of ischemia by the ligation of the left femoral artery. The quantitative analysis of the laser Doppler imaging revealed a time-dependent increase in blood flow after the injection of CD34\(^+\)/CD31\(^-\) cells in the ischemic hindlimb (data not shown) that was maximal after 2 weeks (2-fold increase, \(P<0.05\)) and equivalent to that observed after the injection of BM-MNCs (Figure 5). Morphologically, histological analysis evidenced that the capillary density in the semimembranous and adductor muscles of the ischemic hindlimb was increased after the injection of CD34\(^+\)/CD31\(^-\) cells, to an extent similar to that observed after injection of BM-MNCs (Figure 6A). Moreover, the CD34\(^+\)/CD31\(^-\) cells incorporated into the leg, as evidenced by the presence of HLA-positive vessels within the mouse vasculature (Figure 6B).

Discussion

The present study demonstrates that cells expressing the stem cell markers CD34, CD133, and ABCG2 are present within
the SVF of human AT. The isolated CD34+/CD31− population contains cells that differentiate, in vitro, into ECs and participate, in vivo, to the revascularization of the ischemic hindlimb in mice.

Human AT consists of adipocytes and cells composing the so-called SVF. On the basis of immunohistochemistry, the SVF from human mammary and visceral ATs has been reported to contain ECs as well as noncharacterized stromal cells, blood cells, and tissue macrophages. A recent study, applying FACS analysis to the cultured SVF derived from lipoaspirates, reported the presence of mesenchymal stem cells. To more thoroughly characterize the cells present in the SVF, we performed FACS analyses on freshly harvested SVFs from different types of human AT samples. Cells were assessed for the surface expression of CD34, commonly used as a stem cell marker; CD133, widely used to identify the CPCs when coexpressed with CD34; and ABCG2, recently described to identify primitive stem cells. Our results demonstrate that the SVF contains distinct cell populations expressing these 3 markers independently of the anatomic origin. Intriguingly, recent studies by Zuk et al described the absence or low level of CD34+ cells, whereas Planat-Benard et al reported more than 90% CD34+ cells in SVFs derived from AT. The most likely reason for the apparent discrepancies is that the latter investigations were performed by use of cultured SVFs, whereas we investigated the cellular makeup of freshly harvested SVFs.

Because CD34 is expressed on both stem cells and ECs, we selected CD34+/CD31− cells from the SVF so as to discriminate between ECs and putative stem cells. The data obtained with the isolated CD34+/CD31− cells clearly confirm their stem origin. Indeed, the cells exhibited a high proliferative capacity and an ability to change their morphology depending on the culture medium, and more specifically, to spontaneously form cobblestone area, a property attributed to macrocirculation-derived ECs and stem cells. Further characterization showed that the CD34+/CD31− cells lacked the hematopoietic lineage markers CD45 and CD14. Moreover, because 20% of the CD34+/CD31− cells also expressed the primitive cell marker ABCG2, it is suggested that the CD34+/CD31− cell population contains a stem cell subset not already committed into a specific lineage. It should be noted that the percentage of CD34+/CD31− cells within the SVF isolated from human AT was markedly higher than in the peripheral blood (at least 500 times higher; personal observations), suggesting that the presence of the CD34+/CD31− cell population is an intrinsic characteristic of the SVF of human AT.

To determine the fate of the CD34+/CD31− cells, and specifically their ability to participate in neovascularization, the isolated CD34+/CD31− cells were cultured in conditions described to promote the differentiation of CPCs. The appearance of the mature EC markers CD31 and vWF in spindle-shaped cells in the presence of VEGF and IGF demonstrated that the CD34+/CD31− population contains cells capable of differentiating into ECs in vitro. Finally, in vivo experiments showing a marked increase in the blood flow as well as in the capillary density in the ischemic hindlimb of nude mice strongly support the hypothesis that the CD34+/CD31− cell population from human AT contains cells that exhibit the classic features of progenitor cells. Moreover, it is suggested that the increased neovascularization observed with the CD34+/CD31− cells is a result of a direct incorporation of the cells into the ischemic leg, although other mechanisms cannot be ruled out, such as stimulation of proangiogenic factors, local stimulation of angiogenesis, or chemotraction of endogenous stem/progenitor cells.

Increasing numbers of reports highlight the possibility that adipogenesis and neovascularization are reciprocally regulated and tightly linked in rodents. Few studies have focused on the microcirculation within human AT,
and no data are available regarding the impact of the development of fat mass on the extension of the microcirculation. In the present study, we show that the percentage of CD34+/CD31+ cell population (a hallmark of capillary ECs)24 from the SVF remains constant whatever the BMI. The fact that the relative proportion of ECs was not altered shows that the development of AT in humans is associated with a concomitant increase in the microcirculatory network. Although the mechanisms involved remain to be determined, the presence of the CD34+ cells within the SVF of human ATs from different sources. Moreover, our results indicate that this cell population represents an easily accessible cell source that can be used therapeutically to improve postnatal neovascularization.

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References


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