Characterization of endothelial-like cells derived from human mesenchymal stem cells


Background: Blood-derived endothelial progenitor cells (EPC) have been used to treat ischemic disease. However, the number of EPC that can be obtained from adult blood is limited. Objective: To characterize endothelial-like cells obtained from human bone marrow and determine their ability to stimulate new blood vessel formation in vivo. Methods: Mononuclear cells (MNC) were isolated from human bone marrow or umbilical cord blood and cultured in endothelial growth medium (EGM-2). Mesenchymal stem cells (MSC) were isolated from bone marrow and induced to differentiate into endothelial-like cells (MSCE), or adipocytes or osteocytes by growth in EGM-2, adipogenic or osteogenic medium. Results: Cells obtained by culturing bone marrow MNC in EGM-2 formed cord- or tube-like structures when grown on MatrigelTM and expressed several endothelial marker proteins. However, cell morphology and the profile of endothelial marker protein expression were different from those of cord blood-derived EPC (cbEPC). Cells with a similar phenotype were obtained by differentiation of MSC into MSCE, which was accompanied by an increase of endothelial marker proteins and a diminished capacity to differentiate into adipocytes. Subcutaneous implantation of MSCE in collagen plugs in non-obese diabetic severe-combined immunodeficient (NOD-SCID) mice resulted in formation of functional blood vessels that had incorporated the MSCE. Conclusions: Our results show that MSCE and cbEPC are different cell types. The formation of functional blood vessels by MSCE, combined with high yields and a reduced capacity to differentiate into other cell types compared with MSC, makes these cells potentially useful for autologous therapy of ischemic disease.

Keywords: endothelial cell, mesenchymal stem cells, vasculogenesis.

Introduction

Endothelial progenitor cells (EPC) have been isolated from human blood and may contribute to the repair of damaged endothelium and the formation of new blood vessels [1–4]. Blood EPC are derived from hematopoietic/angioblast stem cells residing in the bone marrow. The therapeutic potential of ex vivo expanded EPC has been demonstrated in animal models of ischemia. Preliminary clinical studies suggested that infusion of blood-derived EPC may improve myocardial perfusion and contractile function in patients with myocardial infarction [5] and that infusion of crude bone marrow may improve limb perfusion in patients with peripheral arterial disease [6]. However, a recent study using intracoronary infusion of bone marrow cells after myocardial infarction suggested that such improvements are only transient [7]. Therefore, the therapeutic benefit of cell-based therapy of cardiovascular disease, as well as the optimal cell source, dosage and infusion method remain to be determined for each clinical syndrome (for a discussion see [8,9]).

An obstacle to the therapeutic use of blood-derived EPC is the difficulty in obtaining enough cells. Indeed, EPC derived from adult blood have a limited proliferation potential compared with cord blood-derived EPC (cbEPC) [10]. As an alternative, the utility of mesenchymal stem cells (MSC) for vascular repair has been explored. MSC express a wide spectrum of angiogenic growth factors and may stimulate collateral vessel formation by paracrine mechanisms [11]. Also, some of the injected MSC are incorporated in new blood vessels [12]. When cultured in the presence of endothelial

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growth supplements, MSC start to express endothelial markers [13]. This suggests that MSC can differentiate into endothelial-like cells (MSCE). An advantage of MSC, compared with blood-derived EPC, is the greater number of cells that can be obtained from adult sources [14,15]. However, the capacity of MSC to differentiate into unwanted daughter cells, such as adipocytes, chondrocytes, osteocytes, muscle cells or nerve cells [15,16] remains a cause of concern.

We investigated the phenotype of cells obtained by culturing bone marrow cells in the presence of endothelial growth supplements. The endothelial-like cells thus obtained were different from blood-derived EPC and similar to endothelial-like cells obtained by differentiation of MSC. Differentiation of MSC into MSCE reduced their ability to differentiate into adipocytes. The MSCE induced the generation of functional

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Fig. 1. Comparative analysis of cord blood-derived endothelial progenitor cells (cbEPC) and endothelial-like cells obtained by directly cultivating human bone marrow mononuclear cells (MNC) on gelatin-coated plates in endothelial growth medium (EGM-2). (A) Phase contrast analysis revealed a cobble stone morphology for cbEPC and a spindle shape morphology for the bone marrow-derived cells. Flow cytometry histograms for Ulex europaeus lectin (UEA-1) binding or the indicated antigens are shown by the open peaks, whereas the filled peaks represent results with isotype-matched antibody controls. The histograms are representative for three independent analyses. (B) Analysis of formation of cord- or tube-like structures on Matrigel™ of early outgrowth EPC (1) (note the absence of such structures), cbEPC (2, 5 and 6) and bone marrow-derived cells (3, 4, 7 and 8). Panels 1 to 3 show phase contrast microscopy images, panel 4 confocal microscopy analysis of PKH26-prelabeled bone marrow cells and panels 5, 6 and 7, 8 staining of consecutive cryosections for UEA-1 binding (green) and 4′-6-diamidino-2-phenylindole (DAPI) nuclear staining (blue). The scale bars represent 50 μm.
blood vessels in vivo and were incorporated into these blood vessels.

Methods

Isolation of EPC from human bone marrow or cord blood

Bone marrow and cord blood were obtained after approval by the institutional ethics committee of the University Hospital of Geneva, in accordance with the Helsinki declaration. Written consent was obtained from each bone marrow donor or a parent of each cord blood donor. Mononuclear cells (MNC) from human bone marrow or cord blood were obtained by density-gradient centrifugation and cultured in endothelial growth medium (EGM-2; Cambrex, Verviers, Belgium) [17], which contains hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, hydrocortisone and heparin and 2% fetal bovine serum (FBS). Early outgrowth EPC were obtained after 7 days in culture and late outgrowth after more than 4 weeks in culture.

Isolation and differentiation of human MSC

Bone marrow MNC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS (Invitrogen, Carlsbad, CA, USA). Differentiation of the MSC into endothelial-like cells, adipocytes or osteocytes was initiated by further incubation of the cells for 3 weeks in EGM-2 or in medium with adipogenic or osteogenic supplements (StemCell Technologies, Reinach, Switzerland), respectively. Adipocytes or osteocytes were analyzed according to the supplier’s instructions.

Antibodies

Murine monoclonal antibodies to the following human proteins were used: CD31 (JC/70A) from Dako (Dako A/S, Glostrup, Denmark); FITC-labeled antihuman CD31 (which cross-reacts with mouse CD31) and CD144 (VE-cadherin; TEA1/31) from Coulter/Immunotech (Beckman coulter, Fullerton, CA, USA); eNOS from Abcam (Abcam, Cambridge, UK); CD45 (MEM-28) from Alexis Biochemicals (Axoora, Lausen, Switzerland); CD105 and CD73 from Serotech (Serotech, Düsseldorf, Germany); CD34 and CD90 from Miltenyi Biotec (Miltenyi biotec, Bergisch-Gladbach, Germany); KDR from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); CD146 from Biocytex (Biocytex, Marseille, France). Rat antimouse CD31 (390) was from BioLegend (Biolegend, San Diego, CA, USA). This antibody does not recognize human CD31. Antibodies against smooth muscle cell alpha-actin and desmin were a gift from Dr Bochaton-Piallat at Geneva University Medical Centre. Isotype-matched control antibodies were from Dako. FITC- or phycoerythrin-conjugated goat IgG against mouse IgG were from Sigma (Sigma-Aldrich, St Louis, MO, USA) or Molecular Probes (Invitrogen). Rhodamine-conjugated goat IgG against rat IgG was from Abcam. FITC-labeled Ulex europaeus agglutinin (FITC-UEA-1) was from Sigma.

Flow cytometry analysis

Cells were washed in phosphate buffered saline (PBS), trypsinized, washed in PBS-5% FBS and incubated with primary antibodies (10 μg mL⁻¹ in PBS-0.5% bovine serum albumin) for 1 h, washed and incubated with conjugated secondary antibodies for 1 h. For eNOS, cells were fixed for 10 min using 2.5% paraformaldehyde and permeabilized with 0.1% saponin before adding primary antibody. All incubations were performed at 4 °C. Cells were washed, fixed in 2.5% paraformaldehyde, 2% glucose in PBS and analyzed on a FACSscan instrument (Becton Dickinson) using CELQuest software (Becton Dickinson). Isotype-matched murine antibodies were used as negative controls.

In vitro Matrigel assay

Matrigel™ (BD Biosciences) was added to a 24-well plate (500 μL well⁻¹) and allowed to solidify for 30 min at 37 °C.

Fig. 2. (A) The proliferation rate of cells directly isolated from human bone marrow using endothelial growth medium (EGM-2). The figure shows the results of three different batches. (B) Proliferation of human mesenchymal stem cells (MSC) before (squares) and after their differentiation into endothelial-like cells (MSC-E) (circles). Note that the proliferation potential of MSC is maintained throughout the expansion period and that the proliferation capacity of MSC-E or cells directly derived from bone marrow start to decline within a few passages.

Fig. 3. Differentiation of mesenchymal stem cells (MSC) into osteocytes. MSC were kept in Dulbecco’s modified Eagle’s medium (DMEM) (DMEM)-10% fetal bovine serum (left) or osteogenic medium (right) for 3 weeks in a six-well plate. Osteogenic induction was analyzed by von Kossa staining of calcium deposits. The changes in morphology of undifferentiated MSC are as a result of the von Kossa staining conditions. The scale bars represent 50 μm.

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Cells (2.5 x 10^5) were suspended in 500 l of EGM-2 and placed on top of the Matrigel™. After 3- or 24-h incubation the formation of a cord- or tube-like network was examined and recorded using a phase contrast microscope. PKH26 (Sigma) prelabeled MSCE seeded on the Matrigel™ were also analyzed by confocal microscopy. Cryosections were made from the Matrigel™ and stained with 4¢,6-diamidino-2-phenylindole (DAPI) and FITC-labeled UEA-1.

**Fig. 4.** Changes induced by differentiation of mesenchymal stem cells (MSC) into endothelial-like cells (MSCE). MSC were cultured for 3 weeks either in Dulbecco's modified Eagle's medium (DMEM)-10% fetal bovine serum (FBS) (MSC) or in the endothelial growth medium (EGM-2) (MSCE). (A) Changes in marker protein expression were analyzed by flow cytometry (open peaks). The filled peaks show the isotype-matched antibody controls. (B) Phase contrast image of MSC and MSCE. (C) Morphology of MSC or MSCE after cultivation in medium with adipogenic stimulatory supplements. Note the large lipid deposits in the differentiated MSC and the small lipid deposits in the MSCE-derived cells (white arrows). (D) The average number (± SD) of cells containing large or small lipid deposits observed per three random fields of vision. (E) Formation by MSCE of a cord- or tube-like network in Matrigel™ after 24 h. (F) Comparison of MSC and MSCE as to their ability to form a network after 3 h in the absence of VEGF. (G) Staining of cryosections of the cord- or tube-like network with FITC-UEA-1 lectin (green) and 4¢,6-diamidino-2-phenylindole (DAPI) (blue). The scale bar represents 50 μm.

Subcutaneous implantation of MSC and MSCE into NOD-SCID mice

Ten-week-old male non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice, weighing 25-30 g and maintained under pathogen-free conditions, were used. Experimental protocols were approved by the Ethics Committee for animal experiments of the Geneva University Medical Centre and by the Geneva veterinary authorities. All mice were
anesthetized with 1.5% isoflurane (Abbott, Cham, Switzerland) in 100% oxygen. MSC or MSCE were trapped in 150-µL collagen plugs (3.0 mg mL⁻¹; BD Biosciences, San Jose, CA, USA) before s.c. implantation in the back of recipient mice. For some experiments the cells had been labeled with PKH26. Cell-free collagen plugs of the same size were used as controls. Four weeks later, all mice were sacrificed and collagen plugs were recovered for examination of vessel formation.

Immunohistochemical staining and microscopy and vessel counting

The frozen collagen plugs were cryosectioned and stained with hematoxylin-eosin or immunostained for CD31, for smooth muscle cell actin or desmin or for binding of FITC-UEA-1. Images were acquired using a fluorescence microscope and a digital charge-coupled device (CCD) camera. For each collagen plug, blood vessel density was determined for three slices and three different positions per slice after staining with hematoxylin-eosin and for CD31, as described [18]. The results are expressed as number of vessels per mm².

Results

Isolation and analysis of human bone marrow-derived endothelial-like cells

Blood-derived EPC originate from stem cells residing in the bone marrow. As the proliferation capacity of EPC derived from adult blood is poor, we sought to directly isolate such cells from bone marrow. Bone marrow MNC were cultured on gelatin-coated tissue culture plates in EGM-2 medium. For comparison, human cbEPC were isolated using the same procedure.

After 4 weeks of culture, the bone marrow-derived cells exhibited a spindle-shaped morphology, which was distinct from the cobblestone morphology of cbEPC (Fig. 1). Both the bone marrow-derived cells and the cbEPC bound FITC-UEA-1 and were positive for eNOS, CD105, CD73 and CD34 (Fig. 1). cbEPC were positive for CD31, CD144, CD146 and KDR, while bone marrow-derived cells were negative or only weakly positive for these markers. EPC were negative and bone marrow-derived cells positive for CD90. Both cell types were negative for CD45. Both cbEPC and the bone marrow-derived cells formed cord- or tube-like structures in Matrigel™ (Fig. 1B).

The bone marrow-derived endothelial-like cells could undergo on average seven passages (range 4–12), before they stopped proliferating; cell number expansion was seven- to twelvefold between passage 1 and 5 (Fig. 2A).

Differentiation of MSC into endothelial-like cells, adipocytes or osteocytes

Although cells isolated from bone marrow expressed several endothelial marker proteins, their morphology and low expression of CD31, CD34 and CD144 imply that they are distinct from cbEPC. This raises the question of whether these cells were derived from MSC. We isolated MSC from bone marrow and observed that they expressed CD105 and CD73 and were negative for CD45 and CD34 (data not shown) could differentiate into adipocytes or osteocytes (Figs 3 and 4). To establish whether these MSC could differentiate in response to endothelial growth factors they were further cultured in
EGM-2. As controls, we used MSC maintained in DMEM-10%FBS. The cells resulting from culture in EGM-2 are designated henceforth MSCE. After 3-weeks culture in their respective media, expression of CD31, CD144, KDR, CD105, CD34, eNOS and CD73, as well as FITC-UEA-1 binding, was increased in MSCE compared with MSC (Fig. 4A). No changes were observed in CD146 (Fig. 4A), VCAM-1, CD45 or CD90 (data not shown). The morphology of confluent MSCE was different from that of MSC but similar to that of the cells directly isolated from bone marrow in EGM-2 (Figs 1 and 4B). We investigated whether differentiation of MSC into MSCE had modified their ability to differentiate into adipocytes. MSC cultured for 3 weeks in adipocyte differentiation medium readily differentiated into adipocytes, whereas MSCE had a greatly reduced ability: both in the number of cells with an adipocyte phenotype and the amount of lipid accumulated in the vacuoles of the adipocyte-like cells (Fig. 4C,D). The differentiated MSCE could form a network of cord- or tube-like structures in a Matrigel invasion assay (Fig. 4E and G). A direct comparison of MSC and MSCE on Matrigel in the absence of VEGF revealed that after 3 h the MSCE had partially formed cord- or tube-like structures, whereas the MSC had not invaded the Matrigel (Fig. 4F). However, after 24 h the MSC also had formed cord- or tube-like structures (data not shown).

We compared the proliferation potential of MSC and MSCE. MSC could be maintained for at least 14 passages (two thousandfold expansion) without loss of proliferation potential. Transfer at passage 9 of MSC into EGM-2, resulted in an arrest of proliferation within three passages and a 4-fold expansion only (Fig. 2B).

**Effect of implanted MSC or MSCE on in vivo blood vessel formation**

To investigate whether MSCE can promote new blood vessel formation in vivo, we s.c. implanted collagen plugs containing MSC or MSCE as control or no cells onto the back of NOD-SCID mice. Thirty days after implantation we recovered the collagen plugs containing MSCE or MSC and observed for both the formation of functional red blood cell-containing vessels (Fig. 5A). Blood vessel density was higher with $10^6$ cells than with $3 \times 10^5$ cells (data not shown). No blood vessel formation was observed in cell free-collagen plugs (Fig. 5A). We quantified blood vessel density in the MSC- and MSCE-seeded plugs by counting CD31-positive vessels (Fig. 5B); average vessel density was $121 \pm 64$ and $73 \pm 21$ ($n = 4$ plugs each) vessels per mm² for MSCE- or MSC-seeded plugs, respectively. The difference was not statistically significant. We observed CD31-positive cells in vascular structures both for the MSCE and MSC-containing collagen plugs (Figs 5B and 6). In addition, we observed a weak CD31 staining within the lumen of the blood vessel. This most likely represents staining of murine leukocytes, because the UEA-1 lectin revealed only vascular structures (Fig. 6). The origin of the CD31- and UEA-1-positive vascular structures was determined using two approaches. First, we compared CD31 and UEA-1 staining of slices of MSCE-containing collagen plugs with that of a s.c. murine Lewis lung carcinoma grown in C57Bl/6 mice. Using mouse-specific anti-CD31 antibodies we stained blood vessels only in the murine tumor samples and not in the MSCE-collagen plug, whereas the UEA-1 marked the MSCE-collagen plug but not the murine tumor (Fig. 7A). As a second approach, we labeled MSCE with PKH26 prior to implantation. The same structures were labeled with both PKH26 and UEA-1 (Fig. 7B). These data show that the cells in the vascular structures were of human origin. An analysis for the presence of smooth muscle cells revealed that the CD31-positive cells surrounding the blood vessels were also positive for smooth muscle cell-actin and desmin (Fig. 8).
Discussion

A major obstacle in the therapeutic use of EPC in patients with ischemic diseases is the limited number of autologous cells that can be obtained from adult blood. Therefore, we investigated whether EPC can be isolated directly by culturing adult bone marrow MNC in the presence of endothelial growth supplements. Using this approach a large number of cells expressing some endothelial marker proteins could be obtained. However, using cell morphology and marker protein expression, these cells differed from cbEPC. We could also obtain these cells by differentiating MSC in medium containing endothelial growth supplements, which led to an increased expression of endothelial markers such as CD31, CD144, KDR, CD105, CD34 and UEA-1 lectin binding. However, staining for these marker proteins was lower than in cbEPC. Both MSCE and cbEPC formed cord or tubule-like structures in Matrigel™. These results imply that the culture conditions, using endothelial cell growth supplements, are more conducive for growth from bone marrow of MSCE than of EPC.

Subcutaneous implantation of collagen plugs containing MSCE or MSC in NOD-SCID mice resulted in the formation of new blood 091ds. Collagen was chosen as cell carrier, instead of Matrigel™, because it is devoid of growth factors. Furthermore, in this model the signals stimulating blood 091d formation can only be generated by the implanted MSCE or MSC and not by host-derived cells. The new blood 091ds were functional and linked to the murine circulation as attested by the presence of red blood cells. Blood 091d density was dependent on the number of implanted cells and equivalent for MSCE and MSC. From this, we can conclude that differentiation of MSC into MSCE had not impaired or increased their ability to induce new blood 091ds. The blood 091ds were of human origin because PKH26 prelabeled MSCE were incorporated and positive for UEA-1 binding, which under our conditions labeled human but not mouse endothelium. In addition, the ve1ds were negative for immunostaining with a mouse-specific anti-CD31 antibody. We observed that the CD31 positive cells surrounding the blood 091ds were also positive for the smooth muscle cell markers alpha-actin and desmin. Our results suggest that in vivo MSCE express both endothelial markers and smooth muscle cell markers. The significance of this expression pattern remains to be investigated. A recent study reported for a rat hind limb ischemia model that transplanted rat MSC could differentiate into cells expressing the endothelial marker von Willebrand factor (VWF) or the smooth muscle cell marker alpha-actin [19].
However, it was not shown whether these markers were expressed by the same cell or by different cells.

We and others observed that EPC derived from cord blood can be expanded for more than twenty passages. However, the number of adult late outgrowth EPC in blood decreases with age and their proliferation potential is low compared with that of cbEPC [10]. So-called early outgrowth EPC can be obtained in relatively large numbers within a week. However, these cells are more likely derived from monocytes/macrophages and their capacity to further proliferate is limited [20]. MSCE have the advantage that they can be expanded from MSC, which are easily obtained in very large numbers [14,15]. Differentiation of MSC into MSCE markedly diminished their ability to differentiate into adipocytes. In the context of cell-based therapies, we can expect that this would reduce potential problems as a result of differentiation of MSC into undesired cell types such as adipocytes, chondrocytes or osteocytes.

MSC exhibit several characteristics that make them desirable for cell therapy. Among these is their migration to sites of tissue injury or ischemia, as observed in a rat acute myocardial infarction model [21]. This implies that the cells respond to homing signals from the infarcted region. In addition, the paracrine release by MSC – and most likely by MSCE – of angiogenic growth factors and chemokines may stimulate the influx and outgrowth of circulating EPC, which may further improve tissue perfusion as reported for a mouse model of hind limb ischemia [11]. Furthermore, MSC express immunosuppressive activities [22,23], which enable them to engraft and persist in mismatched allogeneic hosts, as shown in pig and rat models of myocardial infarction [12,24]. To what extent the immunosuppressive activity is maintained after differentiation of MSC into MSCE remains to be established. The possibility of using allogeneic cells would greatly facilitate MSCE-based therapies of acute ischemic diseases such as myocardial infarction and stroke.

In summary, our data show that MSCE stimulate the formation of new blood vessels in vivo. The characteristics of MSCE are clearly different from that of previously described late outgrowth blood-derived EPC. As MSCE are derived from MSC, which have a high proliferation potential, MSCE could be a useful source of autologous cells for cell-based therapies of ischemic diseases.

**Addendum**


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**Disclosure of Conflict of Interests**

The authors state that they have no conflict of interest.

**References**


