Published in final edited form as:

Plast Reconstr Surg. 2009 February ; 123(2 Suppl): 45S–55S. doi:10.1097/PRS.0b013e318191be4a.

Mesenchymal Stem Cells Can Participate in Ischemic Neovascularization

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Abstract

Background—Cells from the bone marrow contribute to ischemic neovascularization, but the identity of these cells remains unclear. The authors identify mesenchymal stem cells as a bone marrow–derived progenitor population that is able to engraft into peripheral tissue in response to ischemia.

Methods—A murine model of skin ischemia was used. Bone marrow, blood, and skin were harvested at different time points and subjected to flow cytometric analysis for mesenchymal and hematopoietic markers ($n = 3$ to 7 per time point). Using a parabiotic model pairing donor green fluorescent protein (GFP)–positive with recipient wild-type mice, progenitor cell engraftment was examined in ischemic tissue by fluorescence microscopy, and engrafted cells were analyzed by flow cytometry for endothelial and mesenchymal markers. In vitro, the ability of both bone marrow– and adipose-derived mesenchymal stem cells to adopt endothelial characteristics was examined by analyzing (1) the ability of mesenchymal stem cells to take up DiI-acetylated low-density lipoprotein and Alexa Fluor lectin, and (2) phenotypic changes of mesenchymal stem cells co-cultured with GFP-labeled endothelial cells or under hypoxic/vascular endothelial growth factor stimulation.

Results—In vivo, the bone marrow mesenchymal stem cell population decreased significantly immediately after surgery, with subsequent engraftment of these cells in ischemic tissue. Engrafted cells lacked the panhematopoietic antigen CD45, consistent with a mesenchymal origin. In vitro, bone marrow– and adipose-derived mesenchymal stem cells took up DiI-acetylated low-density lipoprotein and Alexa Fluor lectin, and expressed endothelial markers under hypoxic conditions.

Conclusions—The authors' data suggest that mesenchymal precursor cells can give rise to endothelial progenitors. Consequently, cell-based therapies augmenting the mesenchymal stem cell population could represent powerful alternatives to current therapies for ischemic vascular disease.

In adults, new blood vessel formation (neovascularization) is essential for the recovery of ischemic tissue. Originally, it was believed that the mechanism for development of new vascular networks in the setting of growth or tissue ischemia was angiogenesis (i.e., the sprouting of microvessels from a preexisting capillary bed).^{1,2} Recent evidence suggests that

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Presented at the 52nd Annual Meeting of the Plastic Surgery Research Council, in Stanford, California, June 20 through 23, 2007.

Disclosure: None of the authors has any commercial associations or financial disclosures that might pose or create a conflict of interest with information presented in this article.

adult bone marrow is a source of cells that can also participate in new vessel growth through the recruitment of bone marrow–derived endothelial progenitor cells in response to signals generated by damaged tissue (postnatal vasculogenesis).^{3–5} Ischemia is one of the most potent stimuli for neovascularization because it induces formation of new blood vessels to restore adequate oxygen delivery to affected tissues.⁶ Although it is known that endothelial progenitor cells contribute to ischemic neovascularization through vasculogenesis,² characterizing the ontogeny and lineage of these cells has been difficult.

Vasculogenesis-mediated blood vessel development is altered in adults in many pathological conditions, such as peripheral vascular disease,⁴ myocardial ischemia and infarction,⁷ stroke,^{8,9} wound healing,⁴ retinopathy,¹⁰ and tumor growth.⁴ Endothelial progenitor cells offer tremendous promise as potential cell-based therapies for such disease states.^{4,11} Characterizing these cells and their precise function following ischemic injury is critical to developing a practical approach to endothelial progenitor cell–based therapies.

In the present study, we evaluated the potential of different populations of bone marrow–derived cells to serve as a source of endothelial cells. This study demonstrates the ability of both bone marrow– and adipose-derived mesenchymal stem cells to give rise to endothelial progenitors, which mobilize, migrate, and differentiate into mature endothelium in response to ischemia. Thus, mesenchymal stem cells may constitute an ideal source for cell-based therapies to treat vascular disease and its sequelae. For plastic surgeons, adipose tissue presents a particularly attractive source of mesenchymal stem cells, given the ease of cellular harvest and culture.

MATERIALS AND METHODS

Isolation of Cells from Bone Marrow

All experiments followed protocols approved by the Administrative Panel on Laboratory Animal Care at the Stanford University School of Medicine. C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, Mass.). Six-week-old C57BL/6 mice were euthanized by means of carbon dioxide asphyxiation and both femurs from each mouse were harvested. Marrow plugs were flushed with phosphate-buffered saline and incubated in red blood cell lysis buffer (0.83% ammonium chloride/0.1% potassium bicarbonate in water) for 3 to 5 minutes at 4°C. Ten volumes of cell culture media [Dulbecco's Modified Eagle's Medium (1.0 g/ml glucose), 10% fetal bovine serum, 1 mM glutamine, 1% penicillin/streptomycin; GIBCO, Grand Island, N.Y.] was added and the cells were centrifuged under standard conditions (for 5 minutes at 300 g and 4°C).

Flow Cytometric Analysis of Bone Marrow and Circulating Cells

Unless otherwise noted, all antibodies were purchased from Becton Dickinson (Franklin Lakes, N.J.). Samples were incubated with a lineage negative (lin—) antibody cocktail (Ter119/CD4/CD8a/Gr-1/CD45R/CD11b) followed by phycoerythrin-Cy5 anti-rat secondary antibody (Caltag, Buckingham, United Kingdom). For mesenchymal stem cell analysis, conjugated antibodies were added against CD45 (phycoerythrin-Cy7) and Sca-1 (fluorescein isothiocyanate). For hematopoietic stem cell analysis, conjugated antibodies were added against CD45 (phycoerythrin-Cy7), c-Kit (phycoerythrin), and Sca-1 (fluorescein isothiocyanate). Samples were incubated with antibodies for 30 minutes and then washed by adding 5 volumes of 2% fetal bovine serum in phosphate-buffered saline with 2 mM ethylenediaminetetraacetic acid. Cells were centrifuged and then re-suspended in propidium iodide for 1 minute at 4°C. Samples were analyzed using an LSR Flow Cytometer (Becton Dickinson, Franklin Lakes, N.J.). Using CellQuest software (Becton Dickinson), samples were

gated for lin⁻/Sca-1⁺/CD45⁻ to define mesenchymal stem cells and for lin⁻/Sca-1⁺/c-Kit⁺/CD45⁺ to define hematopoietic stem cells (Fig. 1).

For circulating cells, 1 ml of blood was collected in a chilled ethylenediaminetetraacetic acid-coated tube. After three rounds of red blood cell lysis (as detailed above), samples were treated with the same staining and analysis protocols as described for bone marrow.

Murine Parabiosis and Ischemia Models

For green fluorescent protein (GFP)/wild-type parabiosis, mice were anesthetized to full muscle relaxation and joined by a modification of the technique of Bunster and Meyer.^{13,14} A model of graded soft-tissue ischemia was created on the dorsum of mice as described previously.¹⁵ Ischemia is graded from proximal to distal, and this was confirmed by tissue oximetry and color laser Doppler analysis.

Endothelial Cell Harvest from Ischemic Skin and Flow Cytometric Analysis

Dorsal ischemic skin flaps were harvested, cut into 1-mm² sections, and incubated in a 0.15% dispase/0.075% collagenase solution at 37°C for 1 hour. After incubation, remaining tissue pieces were filtered and the effluent was treated with red blood cell lysis buffer. After centrifugation, samples were stained with a lineage antibody cocktail as described above. Next, CD31 antibody was added followed by Alexa Fluor 647 anti-rat secondary antibody (Molecular Probes, Eugene, Ore.). Phycoerythrin-Cy7-conjugated anti-CD45 antibody was incubated last. Samples were prepared and analyzed as described above.

Fluorescence Microscopy of Ischemic Tissue

Fluorescence microscopy for GFP⁺ cells in ischemic tissue was performed by first adding 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, St. Louis, Mo.) nuclear stain to 10-μm sections of dorsal skin flaps frozen in optimal cutting temperature compound. Cells were then examined using an eGFP filter in a Leica MZ16F microscope (Leica Microsystems, Bannockburn, Ill.).

Bone Marrow Mesenchymal Stem Cell Harvest and Co-Culture with Endothelial Cells

Murine bone marrow plugs were isolated and processed as described above. Lineage-positive cells were depleted using a magnetic bead lineage depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Depleted cells were incubated with phycoerythrin-conjugated anti-CD45 and fluorescein isothiocyanate-conjugated anti-Sca-1 (conditions as described above) and sorted on the Vanford flow cytometer (Stanford FACS facility, Stanford, Calif.).

Murine bEnd.3 cells were obtained from the American Type Tissue Culture Collection (catalogue CRL-2299; Manassas, Va.). GFP⁺ bEnd.3 endothelial cells were made through infection of said cells with the supernatant from 293T transformed human kidney cells producing a GFP-expressing lentivirus. After 3 days of infection, GFP⁺ bEnd.3 cells were sorted on the Vanford flow cytometer. The lentiviral preparation was a kind gift from Dr. Daniel Kraft of the Irving L. Weissman laboratory (Stanford University). GFP⁺ bEnd.3 cells were grown to 50 percent confluence in endothelial growth medium (Cambrex, Walkersville, Md.). Harvested and sorted mesenchymal stem cells were added to these cultures, and plates were incubated to 90 percent confluence in fully supplemented endothelial growth medium. Cultured cells were harvested by 0.05% trypsin digestion and suspended in buffer (phosphate-buffered saline, 2% fetal bovine serum, 2 mM ethylenediaminetetraacetic acid) for GFP and CD31 flow cytometric analysis as described above. GFP⁻/CD31⁺ cells were considered mature endothelial cells differentiated from harvested mesenchymal stem cells.

Diioctadecyl-Tetramethylindocarbocyanine Perchlorate–Labeled Acetylated Low-Density Lipoprotein Uptake and Fluorescently Labeled Lectin Staining

Endothelial cells can be identified by their uptake of a diioctadecyl-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-acLDL) probe, in addition to binding to *Griffonia simplicifolia* lectin II (GS-II), which has affinity for certain endothelial-specific surface glycosylation residues.^{16–18} The dual ability to take up DiI-acLDL and bind GS-II suggests an endothelial or vascular progenitor phenotype.¹⁹

DiI-acLDL (Invitrogen Molecular Probes, Carlsbad, Calif.) was added to mesenchymal stem cell cultures to a final concentration of 10 µg/µl for a period of 4 hours at 37°C. After washing with phosphate-buffered saline, cells were incubated with 10 µg/µl of GS-II, Alexa Fluor 488 conjugate (Alexa Fluor lectin) for 1 hour at 37°C. DAPI (Sigma Aldrich) was added to visualize cell nuclei according to the manufacturer's protocol. Cell staining was examined using a Zeiss Axioplan 2 direct fluorescent microscope (Zeiss, Oberkochen, Germany).

Adipose-Derived Mesenchymal Stem Cell Harvest and Culture

Inguinal fat pads were excised from 6-week-old C57BL/6 mice and washed in serial dilutions of Betadine (Purdue Frederick Co., Broomfield, Col.) and then minced finely. Tissue was digested with 0.075% type II collagenase (Sigma Aldrich) dissolved in Hank's Balanced Salt Solution for 30 minutes at 37°C. Collagenase was inactivated by adding two volumes of cell culture media and the solution was subsequently centrifuged at 300g for 5 minutes to separate mature adipocytes from the remaining stromal fraction. The supernatant was discarded and the pellet resuspended and filtered through a 100-µm cell strainer to remove undigested tissue fragments. The cells were pelleted, resuspended in cell culture media [Dulbecco's Modified Eagle Medium (4.5 g/ml glucose), 10% fetal bovine serum, 1 mM glutamine, 1% penicillin/streptomycin (Invitrogen GIBCO, Carlsbad, Calif.)] and plated at 3×10^6 cells per 100-cm² culture plate. Exposure to vascular endothelial growth factor (VEGF) was achieved by adding 50 ng/ml of VEGF (R&D Systems, Inc., Minneapolis, Minn.) immediately after plating the cells and during every-other-day media changes. Cells were placed under normoxic (20% oxygen) or hypoxic (2.5% oxygen) (X Vivo Hypoxia Chamber; Bio-Spherix, Lacona, N.Y.) conditions for 5 days. Cultured cells were harvested by 0.05% trypsin digestion and suspended in buffer with conjugated antibodies to Flk-1 (fluorescein isothiocyanate) and CD31 (phycoerythrin) for flow cytometric analysis as described above. Flk-1 + /CD31 + cells were considered mature endothelial cells differentiated from adipose-derived mesenchymal stem cells.

Statistical Analysis

Analysis was performed using the *t* test or with one-way analysis of variance, depending on the experiment. Results were considered significant for values of $p < 0.05$. All data were analyzed with Prism software (Prism, Irvine, Calif.). Unless otherwise stated, all measurements were done at least in triplicate from a sample size pool of $n = 3$ to 7 and expressed as mean $\pm$ SD.

RESULTS

Mesenchymal Stem Cells Mobilize from Bone Marrow to Sites of Ischemia

To evaluate the response of bone marrow resident mesenchymal stem cells to a peripheral ischemic stimulus, we used a previously described model of graded soft-tissue ischemia in which a peninsular flap is created on the dorsum of mice.²⁰ Cells were harvested from bone marrow, the circulation, and homogenized skin at different time points after ischemic induction

and analyzed for the presence of mesenchymal stem cells by flow cytometry. Mesenchymal stem cells were defined as $\text{lin}^-/\text{Sca-1}^+/\text{CD45}^-$ cells (Fig. 1).^{21,22}

At baseline, mesenchymal stem cells constituted 0.06 to 0.08 percent of the total bone marrow population. After the onset of ischemia, these cells showed a marked decrease in their concentration within the bone marrow, with a fourfold reduction by day 3 and a subsequent recovery to near baseline levels by day 14 (Fig. 2, *above, left*). Within the circulation, the mesenchymal stem cell population increased sixfold 7 days after ischemic injury and returned to baseline level after 20 days (Fig. 2, *above, right*). Ischemic tissue demonstrated a significant increase in mesenchymal stem cell density immediately after surgery, with a greater than 10-fold increase after 2 days (Fig. 2, *below, left*). This increase plateaued on day 7 but ultimately returned to baseline by day 14. These observations suggest that, following an ischemic insult, bone marrow–derived mesenchymal stem cells are recruited from the circulation to sites of ischemia, where they subsequently differentiate and lose mesenchymal stem cell phenotype.

Response of bone marrow resident hematopoietic stem cells (defined as $\text{lin}^-/\text{Sca-1}^+/\text{c-Kit}^+/\text{CD45}^+$ cells²³) (Fig. 1) to peripheral ischemia was also examined, as this is the stem cell population purported to give rise to endothelial progenitors (Fig. 2, *below, right*). In contrast to mesenchymal stem cells, the hematopoietic stem cell population in the bone marrow showed a modest and delayed response following ischemia, with a maximal decrease within the bone marrow of only 40 percent at 7 days after surgery. We observed similarly small changes in circulating and tissue-specific hematopoietic stem cells (data not shown), all of which were minor compared with the marked changes observed with bone marrow mesenchymal stem cells.

Mesenchymal Stem Cells Engraft within Blood Vessels at Sites of Ischemia

Using a GFP/wild-type parabiotic mouse model, we evaluated the participation of circulating mesenchymal stem cells in new blood vessel development within ischemic tissue (Fig. 3). In a parabiotic model, a transgenic mouse is surgically joined with a wild-type animal. Parabiotic partners share all major histocompatibility antigens and thus are free of immunologic barriers to cell migration and neovascularization.²⁴ This model facilitates tracking of all progenitor cells incorporated into areas of neovascularization. An ischemic flap was created on the dorsum of the wild-type (“recipient”) mouse. At 14 days after surgery, the flap was harvested, sectioned, and evaluated by fluorescence microscopy for the presence of GFP+ cells (Fig. 4). GFP+ cells were noted throughout the flap, suggesting that circulating cells derived from the GFP+ (“donor”) mouse had engrafted within ischemic tissue. To characterize the origin and ultimate fate of these cells, skin flaps were enzymatically digested and the resident cells evaluated by flow cytometry (Fig. 5). Endothelial cells constituted 6 percent of the lineage-negative cell population and were identified by the presence of CD31. Of this fraction of cells, 12 percent were GFP+, thus documenting that bone marrow–derived progenitor cells had engrafted within blood vessels in ischemic tissue and differentiated into endothelial cells. Assuming an approximately 50 percent level of chimerism within the circulation of each parabiotic partner, this suggests that up to 24 percent of new blood vessels formed at the site of ischemia are the result of differentiation of circulating vascular progenitors into mature endothelial cells. Eighty-two percent of these cells were negative for the panhematopoietic antigen CD45, suggesting that most of these cells were not of hematopoietic lineage.

Bone Marrow Mesenchymal Stem Cells Are Capable of Adopting an Endothelial Phenotype In Vitro

To validate these in vivo data, we evaluated the ability of mesenchymal stem cells to differentiate into endothelial cells in vitro. Bone marrow mesenchymal stem cells were co-cultured with GFP+ bEnd.3 endothelial cells. After 5 days of co-culture, mesenchymal stem

cells adopted a phenotype grossly similar to bEnd.3 cells cultured alone (Fig. 6). Flow cytometric analysis of GFP⁺ cells (i.e., mesenchymal stem cells or mesenchymal stem cell derivatives) demonstrated that this gross phenotypic change was paralleled by mesenchymal stem cell up-regulation of the endothelial marker CD31 (Fig. 7). Specifically, more than two times as many GFP⁺ cells expressed CD31 by day 5 compared with day 1 (16.6 ± 0.33 percent at day 5 versus 7.2 ± 0.97 percent at day 1, $p < 0.05$). Finally, these cultured bone marrow-derived mesenchymal stem cells uniformly took up DiI-acLDL, a functional characteristic typical of endothelial cells (Fig. 8). In aggregate, these results suggest that, under appropriate in vitro conditions, mesenchymal stem cells are capable of differentiating into endothelial cells.

Adipose-Derived Mesenchymal Stem Cells Are Capable of Adopting an Endothelial Phenotype In Vitro

Adipose tissue presents an attractive source of mesenchymal stem cells because, whereas isolation of bone marrow mesenchymal stem cells requires a fairly invasive process, adipose-derived mesenchymal stem cells may be harvested by removal of subcutaneous fat tissue, a procedure commonly performed by plastic surgeons. We thus sought to evaluate the potential of adipose-derived mesenchymal stem cells to adopt endothelial characteristics when subjected to in vitro stimuli.

Unlike the bone marrow niche, subcutaneous fat is believed to be relatively normoxic, which may prevent endothelial differentiation.²⁰ Thus, to investigate the ability of adipose-derived mesenchymal stem cells to differentiate into endothelial cells, we tested the combined influence of hypoxia and VEGF on the in vitro behavior of this cell population. Cells were housed in a hypoxic incubator at 2.5% oxygen and exposed to 50 ng/ml of VEGF. After 5 days, cultured cells were evaluated for their ability to take up DiI-acLDL and bind Alexa Fluor lectin, in vitro characteristics typically displayed by vascular progenitors and endothelial cells.¹⁹ A large number of cells stained doubly positive for DiI-acLDL and Alexa Fluor lectin, suggesting that adipose-derived mesenchymal stem cells are capable of differentiating into endothelial progenitor cells (Fig. 9).

To quantify this, cells were evaluated by flow cytometry for the presence of the endothelial markers CD31 and Flk-1 (Fig. 10). A threefold increase in the combined expression of these markers was noted in hypoxic cells compared with control mesenchymal stem cells cultured under normoxic conditions. These results demonstrate that, similar to bone marrow mesenchymal stem cells, adipose-derived mesenchymal stem cells, when subjected to environmental stimuli known to be present at ischemic sites, exhibit phenotypic characteristics and behavior typically displayed by vascular progenitors and endothelial cells.

DISCUSSION

Since its initial discovery in 1997,³ questions have persisted regarding the origin and functional characteristics of endothelial progenitor cells.²⁵ For instance, even though circulating endothelial progenitor cells are traditionally regarded as originating from the bone marrow, some researchers cite extramedullary sources as contributors to these cells.^{26–28} There is some evidence to suggest functionally and phenotypically hierarchical subpopulations of endothelial progenitor cells.^{29–31} These subpopulations (often labeled as early versus late endothelial progenitor cells) are heterogeneous in proliferative, differentiative, and self-renewal potential. To complicate matters, the limited quantity of circulating endothelial progenitor cells has been a significant obstacle to clinical trials.¹² From a therapeutic perspective, finding an optimal population of endothelial progenitor cells requires a reliable and predictable tissue source from which endothelial progenitor cells can be easily harvested and expanded. Such clinical demands clearly conflict with our current knowledge regarding the biology of vasculogenesis.

This study represents an incremental step in clarifying the lineage of endothelial progenitor cells. Using a well-validated model of murine skin ischemia, we identify mesenchymal stem cells as a key progenitor subset mobilized from the bone marrow to migrate through the circulation and directed to affected tissue. Using a parabiosis model, we show that these cells engraft within newly forming blood vessels at the ischemic site, with vasculogenesis accounting for up to 24 percent of new blood vessel development. The eventual phenotypic manifestations typical of developing endothelial progenitor cells (acetylated low-density lipoprotein uptake and lectin binding) in the ischemic mobilization model suggest site-specific differentiation of mesenchymal stem cells into endothelial cells. In vitro, we observed that mesenchymal stem cells display phenotypic endothelial characteristics under appropriate environmental conditions. Thus, mesenchymal stem cells may be a convenient progenitor cell for the production of neovessels in ischemic tissue. From a practical standpoint, therapeutic use of bone marrow–derived mesenchymal stem cells is difficult because they are present in limited quantities. Other procedures for isolating bone marrow mesenchymal stem cells are invasive. Thus, an alternative source of these cells is required.

Recent work has focused on the utility of adipose-derived mesenchymal stem cells because of the ease with which these cells may be harvested in large numbers from patients by simple methods.^{32,33} Some studies have suggested the ability of adipose-derived mesenchymal stem cells to improve tissue ischemia in a mouse model of limb ischemia.³⁴ In our study, we confirm the ability of adipose-derived mesenchymal stem cells to adopt endothelial functions and characteristics such as acetylated low-density lipoprotein uptake and lectin binding. These observations suggest the potential of adipose-derived mesenchymal stem cells to differentiate into mature vascular endothelium during ischemia-induced neovascularization. These cells may hold considerable promise as cytologic therapy for the treatment of vascular disease.

What is the mechanism for mesenchymal stem cell—endothelial cell differentiation in ischemic tissue? It is known that hypoxia induces progenitor cell mobilization through HIF-1 α induction of SDF-1 and also controls subsequent differentiation into endothelial cells through HIF-1 α -regulated VEGF expression.^{20,35–37} Here, we observe that mesenchymal stem cells mobilize to sites of ischemia, and both circulating and adipose-derived mesenchymal stem cells adopt a partial endothelial phenotype when exposed to similar vasculogenic stimuli such as hypoxia. Mesenchymal stem cell recruitment and subsequent endothelial differentiation within ischemic tissue may indeed be driven through the HIF-1 α /SDF-1/VEGF pathway. There are implications to these findings. We believe that by identifying and studying the involvement of mesenchymal stem cells in the process of vasculogenesis, we may provide a new means for the development of cell-based therapies for vascular disease. Procedures and processes central to the practice of plastic surgery such as skin grafts, distraction osteogenesis, wound healing, and flap transfers all rely on blood vessel remodeling. Consequently, the characterization of accessible stem cell populations that promote neovascularization is of particular importance to plastic surgeons. In a clinical setting, the attainment of bone marrow–derived mesenchymal stem cells through invasive bone marrow aspirates may limit their practical utility. Adipose-derived mesenchymal stem cells are of great interest because their easy access could facilitate the translation of these new therapies from the bench to the bedside.

Acknowledgments

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant R01-DK074095-04 (G.C.G.) and American Heart Association and Stanford University Dean's Postdoctoral Fellowships (H.T.)

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MSC Surface Markers	HSC Surface Markers
Lin- Sca-1+ CD45-	Lin- Sca-1+ c-Kit+ CD45+

Fig. 1.
Surface markers for mesenchymal and hematopoietic stem cells.

Fig. 2.

Changes in mesenchymal stem cell prevalence within the total cell population of (*above, left*) bone marrow, (*above, right*) circulation, and (*below, left*) the ischemic skin flap at days 0 to 20 after the ischemic insult were analyzed using flow cytometry. Bone marrow mesenchymal stem cells were identified as $\text{lin}^-/\text{Sca-1}^+/\text{CD45}^-$. Similar time-course studies were performed for (*below, right*) bone marrow-derived hematopoietic stem cells identified as $\text{lin}^-/\text{Sca-1}^+/\text{c-kit}^+/\text{CD45}^+$. The x axis units are expressed in terms of days postoperation (with the exception of a 12-hour time point that is provided for the mesenchymal and hematopoietic stem cell bone marrow analysis) and y axis units are expressed in terms of percentage of total cells for the studied tissue compartment.

Fig. 3.

We used a GFP+/wild-type parabolic model, where circulation is shared between paired mice permitting circulating and bone marrow-derived cells from the GFP+ donor to migrate and engraft within ischemic skin in the wild-type recipient. The ischemic flap was harvested at postoperative day 14.

Fig. 4. Sections of the flap were analyzed using fluorescence microscopy for the presence of GFP+ cells. GFP+ cells in ischemic recipient skin on day 14. Cells from the digested flap were also analyzed further by flow cytometry/fluorescence-activated cell sorting.

Fig. 5.

Cells harvested from ischemic skin were initially gated for lineage negativity and CD31 positivity (*left*). This population was further subgated for bone marrow-derived (GFP+) cells (*center*). Of the bone marrow-derived endothelial cells present within ischemic skin, the majority were CD45- (i.e., of nonhematopoietic origin) (*right*). For all flow cytometric plots, *x* axes are expressed in terms of arbitrary fluorescence units (log scale), whereas *y* axes are expressed in terms of arbitrary logarithmic side-scatter units.

Fig. 6. Morphology of unlabeled bone marrow–derived mesenchymal stem cells after 5 days of co-culture with GFP+ bEnd.3 cells (*left*). An image of GFP+ bEnd.3 endothelial cells after 5 days of culture alone (*right*) is provided for comparison.

Fig. 7.

After 1 and 5 days of mesenchymal stem cell/GFP+ bEnd.3 cell co-culture, cells were harvested and analyzed by flow cytometry to evaluate the fraction of cells expressing the endothelial marker CD31 that were derived from GFP- cells (i.e., mesenchymal stem cells or mesenchymal stem cell derivatives).

Fig. 8. Cultured bone marrow–derived mesenchymal stem cells were visualized by fluorescence microscopy after nuclear staining with DAPI (*left*) and assayed for DiI-acLDL uptake (*right*).

Fig. 9. Adipose-derived mesenchymal stem cells were visualized by fluorescence microscopy after nuclear staining with DAPI (*above*), assayed for DiI-acLDL uptake (*center*), and evaluated for Alexa Fluor lectin binding (*below*).

Fig. 10.

After 5 days of culture in hypoxic conditions with VEGF, expression of the endothelial markers Flk-1 and CD31 by adipose-derived mesenchymal stem cells was evaluated using flow cytometry. Values are expressed as means $\pm$ SEM.