**Derivation of Late Outgrowth Endothelial Progenitor Cells from Adult Human Peripheral Blood Mononuclear Cells**

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**Short Abstract:**

Late outgrowth EPCs have the capacity for clonal growth, and tolerate passaging in culture. These features facilitate the use of late outgrowth EPCs to study the biology of the endothelium in various disease states. The following article describes the methods for their derivation and characterization from adult human subjects.

**Long Abstract:**

Coronary artery disease is the leading cause of death and heart failure in the United States. Endothelial cells play several critical roles in the pathogenesis of coronary disease: 1) Surface molecules of endothelial cells prevent thrombus formation within the vessel lumen, 2) Endothelial cells regulate local blood flow within the vessel by secretion of substances that regulate vascular tone. Understanding the mechanisms that control the development of endothelial dysfunction and the pathways involved in stimulation of new vessel formation in humans is essential to development of new therapies for coronary artery disease and reversal of heart failure in ischemic heart disease. Endothelial progenitor cells (EPCs) provide a model system to understand the biology of human endothelium under normal conditions and in disease.

EPCs are defined as blood borne cells that form endothelial cells or contribute to new blood vessel formation *in vivo*. Late outgrowth EPCs are distinguished from early outgrowth EPCs by their growth in culture as colonies over ten to thirty days on collagen coated surfaces. Late outgrowth EPCs will proliferate as colonies when plated in limiting dilution, indicating they have the capacity for clonogenic growth, and also form new blood vessels *de novo* when transplanted *in vivo*. Late outgrowth EPCs also have a distinct transcriptional profile when compared with early outgrowth EPCs and blood monocytes, and have a high degree of overlap with authentic endothelial cells.

Given that late outgrowth EPCs have features of bonafide endothelial cells, they provide an important model system to understand the biology of endothelial cells from humans, can be conveniently obtained through a peripheral venous blood draw, and facilitate studies of how different disease states modify their biology in humans. These features provide an important model system in humans to develop new approaches to normalize dysfunction and growth in disease.

**Introduction**

Coronary artery disease is the leading cause of heart failure and death in the United States. Endothelial cells play several critical roles in the pathogenesis of coronary disease: 1) surface molecules of endothelial cells prevent thrombus formation within the vessel lumen, 2) endothelial cells regulate local blood flow within the vessel by secretion of substances that regulate vascular tone. Endothelium becomes damaged, dysfunctional, and senescent when exposed to toxins including substances in tobacco smoke, elevated or oxidized lipoproteins, and exposure to elevated shear stress in hypertension (1). These factors impair the normal functions of endothelium resulting in formation of atheromatous plaque and in some cases occlusion of the vessel lumen due to thrombus formation. Once a vessel is occluded by thrombus or atheromatous plaque, the cardiac tissue surrounding the blocked blood vessel will lose oxygen supply, and contractile function, resulting in heart failure symptoms. Understanding the mechanisms that control the development of endothelial dysfunction and the pathways involved in stimulation of new vessel formation in humans is essential to development of new therapies for coronary artery disease and reversal of heart failure in ischemic heart disease. Endothelial progenitor cells (EPCs) provide a model system to understand the biology of human endothelium under normal conditions and in disease.

EPCs are rare (0.0001% of blood mononuclear cells), blood borne cells that form endothelial cells or contribute to new blood vessel formation *in vivo* (2). Ashahara and co-workers (3) initially described EPCs as a population of cells present in the mononuclear cell fraction of blood that bears features of endothelium when cultured under permissive conditions, and show perivascular incorporation *in vivo*. Subsequent work showed that there are at least two distinct types of endothelial progenitor cells, defined by their growth properties in culture (4). Early outgrowth EPCs are defined by formation of colonies in culture after less than seven days in culture on fibronectin coated surfaces (5), and cell surface expression of the antigens CD45, CD133 and VEGFR2. Recent evidence showed that early outgrowth EPCs contribute to the formation of new vessels by facilitating angiogenesis but cannot form *de novo* blood vessels *in vivo* (6). Additionally, early outgrowth EPCs have marker expression and biologic activities consistent with a monocyte lineage hematopoietic stem cell (7) including the ability to phagocytose bacteria (6).

Late outgrowth EPCs are distinguished from early outgrowth EPCs by their growth in culture as colonies over ten to thirty days (depending on the age of the donor’s mononuclear cells) (6) on collagen coated surfaces. Late outgrowth EPCs will proliferate as colonies when plated in limiting dilution, indicating they have the capacity for clonogenic growth, and will form new blood vessels de novo when transplanted *in vivo* (6). Very few specific molecular markers distinguish early and late outgrowth EPCs from each other, and there is overlapping expression of endothelial cell surface molecules in both cell types. Late outgrowth EPCs can be purified from blood mononuclear cells by sorting for positive expression of CD34 and CD146, and the absence of CD45 (8). However, late outgrowth EPCs have a distinct transcriptional profile when compared with early outgrowth EPCs and blood monocytes, indicating their biologic features are clearly different and have a high degree of overlap with authentic endothelial cells (9).

Due to their relatively recent discovery and characterization, the biology of late outgrowth EPCs, and their responses to normal and abnormal physiologic stimuli remain to be defined. While the effects of cardiovascular disease risk factors on the quantity of early outgrowth EPCs (10, 11) have been established, similar experiments have not been performed to determine how CVD risk factors affect the quantity and angiogenic activity of both early and late outgrowth EPCs. The interrelationship of the early and late outgrowth EPCs in disease remains to be determined in detail, and how the interplay of these two cell types may be altered to either regulate vessel formation, or be impaired in disease. For example, the effects of moderate to high concentrations of high-density lipoprotein on cell viability and senescence has been explored using both early and late outgrowth EPCs in culture (12). In another example, the quantity of both CD34+ CD133+ VEGFR2+ EPCs and late outgrowth EPCs were quantified in the blood of patients with rheumatoid arthritis, and the quantity of both cell types correlated with disease activity (13). The culture of late outgrowth EPCs may also hold promise to identify patients at increased risk for thrombosis in myeloproliferative neoplasms. In a retrospective study, increased late outgrowth EPCs correlated with patients that had a history of blood clotting disorders (14). While a prospective test of this concept is required, the finding suggests that the biology of the late outgrowth EPC may provide clinically useful information that may be used to alter therapy for patients to prevent disease. While late outgrowth EPCs are rare in adult human blood and challenging to isolate in culture, a particular advantage of the late outgrowth EPC is that the cells can be passaged while the early outgrowth EPCs cannot, facilitating characterization of the effects of disease on endothelial cells in disease. Evaluation of the biology of both early and late outgrowth EPCs will be important to understand the biology of human endothelium and regeneration in disease states.

Given that late outgrowth EPCs have features of bonafide endothelial cells, they provide an important model system to understand the biology of endothelial cells from humans, can be conveniently obtained through a peripheral venous blood draw, and facilitate studies of how different disease states modify their biology in humans. These features provide an important model system in humans to develop new approaches to normalize dysfunctional endothelial cells in disease, and regulate their growth in ischemic and malignant disease states. The following article describes the methods for isolation and characterization of late outgrowth endothelial progenitor cells from adult humans and in a large animal model of cardiovascular disease using pigs (15). Importantly, our goal is to standardize the approach to derivation of late outgrowth EPCs, and place our approach with adult human blood in the context of other leaders in the field of late outgrowth EPC biology to provide insights into our approach that may be compared and contrasted with the approach of others in the field (16, 17).

**Protocol Text:**

**1.) Pre Study Considerations**

1.1) Obtain approval for human subjects studies from the local institutional review board, and obtain informed consent from study subjects. The results shown were obtained under an approved protocol with the University at Buffalo Health Sciences IRB (MED6690710E). For studies of EPCs involving animals, obtain approval to obtain blood from animals from your institutional IACUC panel.

**2) Preparation of Culture Medium and Coating of Cultureware**

2.1) Prepare the EGM-2 culture medium with all growth factor supplements provided for growth of late outgrowth EPCs. Remove 50 mL of the culture medium from the bottle and replace with 40 mL of fetal bovine serum for a final concentration of 10% FBS. Batch testing of fetal bovine serum may be required as not all lots of FBS support growth of late outgrowth EPCs. Add the growth factor supplements to the vial of FBS provided, then add directly to the medium. The EGM-2 medium should be prepared at least monthly for optimal results.

2.2) Prepare rat tail collagen solution. Add glacial acetic acid (0.575 mL) to sterile, endotoxin free water (495 mL) and sterile filter. Rat tail collagen solution is added to the diluted acetic acid solution to obtain a final concentration of 50 μg/mL. Consult the spec sheet for your specific lot of rat tail collagen to determine its concentration. The collagen solution is then added to culture dishes (2mL per well of a six well dish). Incubate dishes with collagen solution for 24 hours prior to plating of mononuclear cells in a humidified incubator.

**3.) Preparation of Blood Mononuclear Cells**

3.1) Blood mononuclear cells are obtained using standard venipuncture methods.

3.2) Collect 10 tubes of venous blood in Vacutainer CPT tubes. Gently mix the blood with the citrate fluid by inversion. DO NOT CHILL the collected blood in the CPT tubes. This will impair separation of red blood cells in the Ficoll density gradient.

**4.) Centrifugation of Blood Mononuclear Cells**

4.1) Spin the blood collection tubes at 1500 x g for 30 minutes at room temperature with no brake to prevent disturbing the separated layers of blood.

4.2) Blood collection tubes should be opened in a biosafety cabinet. Pipette the plasma layer from the collection tubes. Avoid disturbing the plasma monocyte/lymphocyte band. Use a 1000 μL pipettor removing 500μL with each aspiration. Dispense the plasma into a waste container using sterile technique.

**5.) Washing the Blood Mononuclear Cell Fraction**

5.1) Carefully collect the lymphocyte/monocyte fraction from the tube, avoiding any debris or particulates that may not have spun through the Ficoll cushion, and dispense the cells into a separate 50mL conical tube.

5.2) From ten tubes of blood, the volume of lymphocytes/monocytes is usually 10 mL. Slowly add EGM-2 medium with 10% FBS (pre-warmed to 37C) to the lymphocyte/monocyte fraction and mix the medium with the cells by gentle swirling of the tube. Bring the volume of medium up to 40 mL using additional EGM-2 medium. Pellet the lymphocytes/monocytes by centrifugation at 300 x g for 15 minutes. Gently resuspend the pellet again in 10 mL of EGM-2. Wash the cells three times.

5.3) Remove a small aliquot of the cell preparation for cell counting after the third wash of the cells. 10-50 x 106 mononuclear cells are obtained from ten tubes of venous blood.

**6) Plating of Mononuclear Cells for Culture of Late Outgrowth EPCs**

6.1) Remove the collagen solution from pre-coated culture dishes and wash the wells once with EGM-2 medium with 10% FBS to remove residual acetic acid.

6.2) Gently transfer 10 x 106 cells of the lymphocyte/monocyte fraction in a 2.5mL volume to one well of a six well plate. Distribute the cells throughout the culture well and return the dish to the culture incubator.

**7) Culture of Mononuclear Cells for Derivation of Late Outgrowth EPC Colonies**

7.1) After one day in culture, remove 80-90% of the culture medium by aspiration. Wash the well once with fresh EGM-2 + 10% FBS containing medium (2.5 mL), then resume culture of the cells at 37°C/5% CO2.

7.2) Change the medium daily for the first seven days of culture. Then medium changes can be switched to every other day until colonies are visualized.

7.3) Review the culture wells with a phase contrast microscope using the 2X or 4x objective. After three to four weeks in culture, colonies should appear.

**8) Subculture of Late Outgrowth Endothelial Progenitor Cells**

8.1) Wash the culture well with pre-warmed PBS three times. Cloning cylinders can be placed around the identified LOEPC colonies to assure only these cells are passaged. Add trypsin (0.2mL per cloning cylinder) for 3 minutes at 37C. Observe the cells to assure they have rounded up after trypsin treatment.

8.2) Add 0.2 mL of EGM-2 medium containing 10% FBS to the cloning cylinder, or 0.5 mL medium to the culture dish if cloning cylinders are not used. LOPEC colonies are aspirated directly off the culture dish using a pipettor. Collect the dissociated cells into a 15 mL conical tube. Pellet the cells by centrifugation at 300 x g for 7 minutes.

8.3) Carefully remove the supernatant and resuspend the cells in 4mL EGM-2 medium. Plate the cells into two collagen coated culture wells.

**9) Characterization of** **Late Outgrowth Endothelial Progenitor Cells by Microscopy**

9.1) Grow late outgrowth EPCs to near confluence on a 24 well dish as above.

9.2) Wash cells three times with PBS (pre-warmed to 37°C), then fix cells by incubation with undiluted methanol (500 μL per well, pre-chilled to -20°C) for 15 minutes. Incubate cells at 4°C during fixation period. Then carefully remove methanol and rehydrate cells in pre-warmed PBS.

9.3) Block non-specific antibody binding by incubation in 5% donkey serum in PBS (blocking solution) for 1 hour at 4°C.

9.4) Remove blocking solution and replace with PBS. Add antibodies (listed in Materials Table,1:100 dilution). Incubate at 4°C for at least two hours.

9.5) Remove primary antibody solution. Wash cells three times in pre-warmed PBS incubating 5 minutes per wash.

9.6) Add secondary antibodies (listed in Materials Table, 1:500 dilution). Incubate cells at 4°C for one hour. Then aspirate secondary antibody solution. Wash cells twice in pre-warmed PBS incubating 5 minutes per wash.

9.7) Add bis-benzamide to counterstain nuclei (1:500 dilution); incubate at room temperature for 10 minutes. Remove bis-benzamide solution and replace with warm PBS.

9.8) Visualize with an immunofluorescence microscope.

**Representative Results:**

The number of late outgrowth EPC colonies derived from each subject varies between individuals, and between blood draws due to the fact that the cells are a rare event in the blood stream (0.0001% of mononuclear cells). Most subjects will derive one to two colonies from 80 mLs of blood. In our experience, we derive 1.9 ± 0.4 (mean ± SEM, n=10) colonies per subject from 80 mL of blood, with an efficiency of 36%. Late outgrowth EPC colonies are easily distinguished from the surrounding background of mononuclear cells as darker, flatter, and spindle shaped than the surrounding round, phase bright, mononuclear cells (Figure 1A and B). Figure 1A provides an example of the earliest stages of colony outgrowth of late outgrowth EPCs, and Figure 1B shows a more fully developed colony. Late outgrowth EPC colonies will appear in cultures after three to five weeks in culture. When late outgrowth EPC colonies are not obtained in culture only the background of phase bright mononuclear cells are obtained. A lack of late outgrowth EPC colony formation, or lack of detection of endothelial specific antigens may suggest a problem with reagents (growth medium, serum, or collagen solution). Troubleshooting steps for the technique are outlined in the Discussion section.

After late outgrowth EPC colonies are derived and expanded at least one passage they must be characterized to assure their endothelial cell type, and lack of leukocyte markers. Figure 2 shows representative immunofluorescence microscopy images of late outgrowth EPCs obtained from normal human subjects after one passage in culture. The cells should express the cell surface antigens PECAM, CD34, CD146 and VE-Cadherin as illustrated in Figure 2. Additionally, intracellular von Willebrand Factor can be visualized as intracellular needle like structures that are Weibel-Palade bodies containing the protein, and are a hallmark of endothelial cells (18). Cells that do not express these antigens may represent contamination of dermal fibroblasts, mesenchymal cells or monocytes. In our hands other cell types are not obtained using the culture conditions and colony focused passaging techniques described.

Flow cytometry should be used to assess the purity and quality of the late outgrowth EPCs derived after passaging of initial outgrowth colonies. Live cells are stained in suspension using previously described methods (19). Both unstained cells and cells incubated with mouse IgG controls (APC, FITC or PE) are used to determine background fluorescence of the cells (Figure 3, panel A). Figure 3, panel B shows representative results of flow cytometry analysis of late outgrowth EPC cultures stained for the endothelial cell antigens PECAM, VEGFR2, CD146, and CD34. The derived cells homogeneously express these endothelial cells antigens, while lacking expression of markers found in leukocytes or monocytes (CD45, CD133 or CD14) shown in Figure 3 panel C. Additional markers of mesenchymal cell types (CD90, Stro-1, and NG2-proteoglycan) were also negative (not shown).

**Table and Figure legends:**

**Figure 1-Representative Late Outgrowth Endothelial Progenitor Colonies Derived from Adult Human Peripheral Blood Mononuclear Cells.** Representative phase contrast images of late outgrowth EPC colonies after three (Panel A) and four (Panel B) weeks in culture from the same individual. Late outgrowth EPC colonies appear phase dark, flat and spindle shaped, and grow in clusters, while the surrounding monocytes are round, phase bright, and randomly scattered throughout the culture dish. Results are representative of findings from >5 adult human subjects. Scale bars indicate 200 μm.

**Figure 2-Representative Immunofluorescence Photomicrographs of Adult Human Late Outgrowth Endothelial Progenitor Cells.** Late outgrowth EPCs from an adult human subject stained with antibodies to the endothelial specific antigens PECAM, CD34, CD146, VE-Cadherin, and von Willebrand Factor (vWF)**.** The arrows denote vWF staining in a rod-like configuration consistent with Weibel-Palade bodies. Nuclei are stained with bis-benzamide. Results are representative of findings from >5 adult human subjects. Scale bars indicate 100 μm.

**Figure 3-Representative Flow Cytometry Analysis of Adult Human Late Outgrowth Endothelial Progenitor Cells.** Late outgrowth EPCs were harvested for flow cytometry analysis. Live cells were analyzed unstained, or with Mouse IgG-FITC, APC or PE conjugated antibodies (Panel A). Quadrants were set to background fluorescence levels. Panel B illustrates representative flow cytometry findings of LOEPCs stained with conjugated antibodies to typical endothelial cell antigens (PECAM-FITC, VEGFR2-APC, CD146-FITC, CD34-APC). Panel C importantly shows that LOEPCs lack the pan leukocyte antigen CD45, CD133, or the monocyte marker CD14. Results shown are representative of findings in >5 human subjects.

**Discussion:**

The method for derivation, sub culturing, and characterization of adult human late outgrowth EPCs detailed provides a means to study the biology of human endothelial cells obtained by a simple venous blood draw. The important advantage of the late outgrowth EPC model versus early outgrowth EPCs includes the ability to passage the cells beyond initial derivation. Importantly, late outgrowth EPCs provide the only source of endothelial cells from blood that will form functional vessels when transplanted in vivo, and can be serially passaged, while early outgrowth EPCs do not possess these capacities (6).

In terms of the derivation of late outgrowth EPCs, there are several critical points to emphasize that may improve the reproducibility and reliability of the experimental model: 1) The age of the reagents used (less than one month) and screened lots of fetal bovine serum are critical to the success of this method in our hands. 2) The duration of culture needed to obtain late outgrowth EPC colonies generally depends on the age of the donor blood mononuclear cells. For umbilical cord blood mononuclear cells, colonies may be initially visualized after seven to ten days. Mononuclear cells from adult human donors require three to four weeks of culture before a clearly distinguishable colony can be identified. 3) Generally one small to medium size colony containing several hundred cells can be replated to one well of a six well culture dish without inducing cell cycle arrest. Smaller colonies may be replated to smaller culture vessels if needed. Generally late outgrowth EPCs obtained from adult humans will tolerate replating to passage 3 or 4 without becoming senescent in fetal bovine serum containing medium. We have had success passaging cells to passage 9 using EBM2 medium supplemented with human platelet lysate with no appreciable changes in the quality or character of the cells (20).

If no late outgrowth EPCs are obtained from a subject there are several troubleshooting steps to proceed through. 1) Assure that the EGM-2 medium is less than one month old. The medium contains recombinant growth factors that will degrade over time when diluted in medium. 2) Determine if the rat-tail collagen solution used to coat the culture dishes is less than 1 month old as this may decay in solution as well. 3) Some subjects have fewer numbers of late outgrowth EPCs per 80 mL of blood. It may require a larger volume of blood sampling to obtain late outgrowth EPC colonies. 4) Some lots of fetal bovine serum to not support the growth of late outgrowth EPCs. Two control experiments to perform lot testing on include deriving late outgrowth EPCs from a donor that has produced colonies previously, or derivation of the cells from umbilical cord blood mononuclear cells. Late outgrowth EPC colonies are more frequent and readily derived from this cell preparation. 5) Another possible outcome is derivation of large appearing endothelial cells containing vacuoles. This generally is observed when there are issues with the EGM-2 medium, either the medium is not fresh or the serum batch is not supportive of cell growth.

There are several potential limitations of the late outgrowth EPC model. While late outgrowth EPCs are a convenient model to study the biology of these circulating endothelial progenitors in culture, at this point it is unclear what their origin is, and if they contribute significantly to new vessel formation after injury in vivo.

How the biology of late outgrowth EPCs relates to endothelial cells in specific tissues of including coronary, aortic, pulmonary, or other organ arterial or venous endothelial cells remains to be determined. Tissue specific features of endothelium varies between organs and it is unknown if these features are acquired by the environment or intrinsic to the tissue type they are derived from. Future work to determine the origin of late outgrowth EPCs and their contribution to endothelial regeneration in adults will require cell type specific labeling methods that have not been developed to date. The model may prove to be clinically relevant to predict future cardiovascular disease risk based on the quantity of cells obtained, number of colonies obtained from initial outgrowth, or replating efficiency. Future applications of the cells may include a model to perform drug screening assays to identify novel pathways that may prevent or reverse the onset of senescence in human endothelium; a feature of the development of atherosclerosis in vivo. Additionally important clues regarding the stimuli that may augment or suppress their growth, and how age impacts the proliferative capacity of neonatal cord blood derived late outgrowth EPCs versus adult late outgrowth EPCs remain important questions to be answer about these cells, and provide important insights into the biology of human endothelium in aging and disease.

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**Disclosures (Conflict of Interest Statement):**

None

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| **Table of Equipment and Reagents** |  |  |
|  |  |  |
| **Name of Reagent/Material** | **Company** | **Catalog Number** |
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| Collagen I, rat tail | BD Biosciences | 354236 |
| EGM-2 Bullet Kit | Lonza | CC-3162 |
| BD Vacutainer CPT tube with sodium citrate | BD Biosciences | 362761 |
| 6 well culture plate | BD Biosciences | 353934 |
| Defined FBS | Hyclone | SH30070.01 |
| 0.05% Trypsin-EDTA (1X), Phenol Red | Invitrogen | 25300054 |
| Sterile Cloning Cylinders | Fisher Scientific | 07-907-10 |
| Phosphate Buffered Saline | Invitrogen | 14190250 |
| Trypan Blue Stain | Invitrogen | 15250-061 |
| Glacial Acetic Acid | Fisher Scientific | A38-500 |
| Sample Needles & Collection sets | BD Bioscienes | 367344 |
| Vacutainer Holder | BD Biosciences | 364815 |
| Methanol | JT Baker | 9070-01 |
| Normal Donkey Serum | Millipore | S30-100mL |
| Bis-benzamide | Invitrogen | H3569 |
| Mouse anti-human PECAM (Clone P2B1) | Santa Cruz Biotechnology | sc-20071 |
| Mouse anti-human CD146 (Clone P1H12) | Santa Cruz Biotechnology | sc-18837 |
| Mouse anti-human CD34 (Clone QBEnd/10) | Santa Cruz Biotechnology | sc-52312 |
| Goat anti-human VE-Cadherin | Santa Cruz Biotechnology | sc-6458 |
| Alexa Fluor 488 Donkey anti-Mouse IgG | Invitrogen | A-21202 |
| Alexa Fluor 555 Donkey anti-Goat IgG | Invitrogen | A-21432 |
| Mouse IgG1-APC | BD Biosciences | 550854 |
| Mouse IgG1-FITC | BD Biosciences | 349041 |
| Mouse IgG1-PE | BD Biosciences | 555787 |
| Mouse anti human CD34-APC | BD Biosciences | 340441 |
| Mosue anti-human CD133-PE | Miltenyi Biotec | 130-080-801 |
| Mouse anti-human CD31 FITC | eBioscience | 11-0319-42 |
| Mouse anti-human CD146 FITC | Santa Cruz Biotechnology | sc-18837 FITC |
| Mouse anti-human VEGFR2 APC | R&D Systems | FAB357A |
| Mouse anti-human CD45 FITC | BD Biosciences | 347463 |
| Mouse anti-human CD14 FITC | eBioscience | 8011-0149-120 |

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