Chapter 5
Isolation of Endothelial Progenitor Cells (EPCs)

Aaron Liew and Timothy O'Brien

5.1 Introduction

Endothelial progenitor cells (EPCs) are non-endothelial cells that are capable of differentiating into cells with endothelial phenotypes. They secrete angiogenic factors and enhance in vitro and in vivo vascular regeneration [1]. We defined EPC based on the following criteria: (a) cells derived from a non-endothelial source; (b) adherence to matrix molecule (our protocol utilises fibronectin); (c) demonstrate acetylated Low Density Lipoprotein (acLDL) uptake; and (d) positive staining with lectin. While the precise definition of EPCs is controversial, this terminology is often used to denote a heterogenous population of cells, consisting of two distinct cell types: (a) early EPCs or circulating angiogenic cells (CACs) and (b) late EPCs or outgrowth endothelial cells (OECs). These two different subpopulations have distinct cell growth patterns and the ability to secrete angiogenic factors [2, 3]. The former are spindle-shaped cells with a peak growth in culture at 2–3 weeks and die after approximately 4 weeks in vitro [3]. Late EPCs usually appear following 2–3 weeks of in vitro culture. They are cobblestone-shaped and have an exponential growth rate at 4–8 weeks which can be maintained for up to 12 weeks in culture [2, 3].

EPCs can be isolated from various sources including bone marrow, peripheral blood, and umbilical cord blood [2, 4, 5]. They can also be isolated by using either a single or a combination of haematopoetic (CD34) and endothelial (KDR) markers by using either FACS or a magnetic bead sorter [6]. The majority of early EPCs and late EPCs arise from a CD14 positive and CD14 negative subpopulation of peripheral blood mononuclear cells, respectively [2, 7]. The discussion regarding the surface markers of EPC is beyond the scope of this article. The late EPCs secrete a

A. Liew • T. O'Brien (✉)
Regenerative Medicine Institute (REMEDI), National Centre for Biomedical Engineering Science (NCBES), National University Ireland Galway (NUIG), Galway, Ireland
e-mail: Timothy.obrien@nuigalway.ie

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smaller array of cytokines as compared with the early EPCs [3, 8]. Even though late EPCs are more capable of in vitro tube formation than early EPCs, the data regarding their in vivo functional effects are conflicting [2, 3, 7].

EPC number and function inversely correlate with the number of cardiovascular risk factors and have been utilised as a surrogate marker for endothelial dysfunction [9–12]. Preclinical data showed that EPC based therapy has a therapeutic potential for the treatment of a variety of vascular disease states including ischaemia, restenosis and pulmonary hypertension [13–15]. Early human trials showed that EPC-based therapy is safe and feasible [16, 17].

Herein, we describe the protocol for isolation and characterisation of EPCs (CACs and OECs) from peripheral mononuclear cells. This protocol can be used for (a) direct quantification of EPC number as a surrogate marker of cardiovascular risk factor; (b) in vitro assessment of EPC biology; or (c) in vivo assessment of EPC biology by administration into animal model.

EPCs can also be isolated by two other methods which involved: (a) plating the mononuclear cells on a fibronectin-coated plate for 48 h and replating the non-adherent cells onto a fresh fibronectin-coated plate (These cells are termed colony forming unit-endothelial cells or CFU-ECs) [10]; and (b) plating the mononuclear cells onto a collagen-coated plate instead of fibronectin-coated plate (These cells are termed endothelial colony forming cells or ECFCs) [18].

5.2 Methodology

Our in-house protocol outlined below describe the protocol for isolation and characterisation of EPCs (CACs and OECs) derived from peripheral mononuclear cells. All research involving human subjects should be performed following the approval of relevant institutional ethical committee and blood samples should be obtained following informed consent. All experiments involving live animals must only be performed in accordance with the Guide for the Care and Use of Laboratory Animals or relevant regulations. Standard precautions for safe handling of blood products should be followed. All procedures described below are performed under strict aseptic technique in a Class II biohazard flow hood.

5.3 Materials (and Company Name)

5.3.1 Collection of Blood (60 mls)

5.3.1.1. Eight to ten 8 ml Venous blood collection tubes (vacutainer) coated with K3 EDTA 15 % solution (BD Biosciences)
5.3.1.2. Standard phlebotomy set

5.3.2 Isolation of Mononuclear Cells

5.3.2.1. Fourteen 50 ml conical tubes
5.3.2.2. One 10 ml Pasteur pipette
5.3.2.3. Hanks' Balanced Salt solution (SIGMA)
5.3.2.4. Bovine Serum Albumin (SIGMA)
5.3.2.5. EDTA pH8 (SIGMA)
5.3.2.6. Endothelial Growth Medium 2 (EGM-2) with Bullet Kit (Lonza)
5.3.2.7. Ficoll-Paque PLUS (GE Healthcare Life Sciences)
5.3.2.8. Red Cell Lysis Buffer (SIGMA)
5.3.2.9. Phosphate Buffered Saline (PBS) (Gibco)

5.3.3 Counting and Plating of Mononuclear Cells

5.3.3.1. Haemocytometer
5.3.3.2. Trypan blue (SIGMA)
5.3.3.3. The BioCoat™ fibronectin-coated six well plates (BD Bioscience)

5.3.4 Cell Culture

5.3.4.1. Endothelial Growth Media 2 (EGM-2) with Bullet kits (Lonza)
5.3.4.2. Incubator with 5 % CO_2 at 37 °C

5.3.5 acLDL Uptake and Lectin Staining

5.3.5.1. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled Ac-LDL (DiI-Ac-LDL) (Molecular Probes)
5.3.5.2. Phosphate Buffered Saline (PBS) (Gibco)
5.3.5.3. 3 % Paraformaldehyde (PFA)
5.3.5.4. FITC-labeled Ulex europaeus agglutinin-I lectin (SIGMA)

5.4 Basic Protocol

5.4.1 Isolation of EPCs

5.4.1.1. Prepare 3 separate 50 mls tubes containing 20 mls of Hank's Complete Solution each (Please see Sect. 5.7 for the preparation of Hank's Complete Solution)

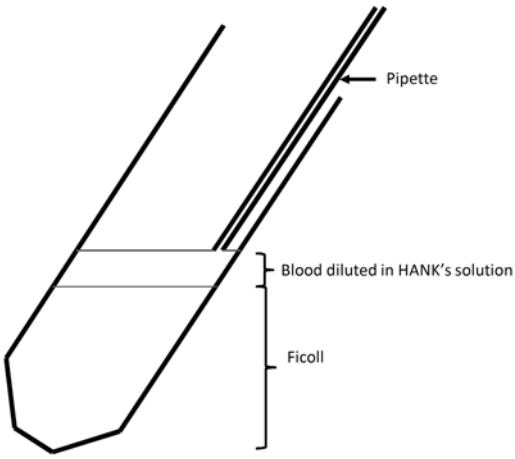

Fig. 5.1 The diluted blood can be gently layered on the Ficoll layer by the 'tilting' method

5.4.1.2. Prepare 8 separate 50 mls tubes containing 21 mls of Ficoll each
5.4.1.3. Prepare 2 separate 50 mls tubes containing 25 mls of PBS each
5.4.1.4. Add 20 mls of blood into each 50 mls tube containing 20 mls of Hank's solution prepared in step 5.4.1.1.
5.4.1.5. Gently layer 15 mls of the diluted blood from step 5.4.1.4. into each 50 mls tube containing 21 mls of Ficoll prepared in step 5.4.1.2. using the 'tilting' method as shown in Fig. 5.1 (Care should be taken to avoid mixing the diluted blood with Ficoll)
5.4.1.6. Centrifuge for 30 min at 1,800 rpm WITH ZERO DECELERATION (without the break following a deceleration phase)
5.4.1.7. Remove cloudy buffy coat containing mononuclear cells with Pasteur pipette and transfer it equally into two separate 50 mls tubes (approximately 5–7 mls of cloudy buffy coat containing mononuclear cells from each 4 tubes into a new tube) containing 25 mls of PBS (approximately 1:1 dilution) prepared in step 5.4.1.3. (Care should be taken to avoid collecting serum or Ficoll) as shown in Fig. 5.2
5.4.1.8. Centrifuge for 10 min at 1,600 rpm without zero deceleration (with the break following a deceleration phase)
5.4.1.9. Aspirate and discard the supernatant
5.4.1.10. Add 5 mls of Red Blood Cell Lysis Buffer to the pellet
5.4.1.11. Gently resuspend the pellet with the Red Blood Cell Lysis Buffer
5.4.1.12. Centrifuge for 10 min at 1,600 rpm without zero deceleration (with the break following a deceleration phase)
5.4.1.13. Repeat step 5.4.1.9 if residual RBC remains (If the pellet still red in colour)
5.4.1.14. Aspirate and discard the supernatant
5.4.1.15. Add 25 mls of PBS in to each 50 mls tube
5.4.1.16. Gently resuspend pellet with PBS
5.4.1.17. Transfer the resuspended pellets with PBS from two 50 mls tubes into a new 50 mls tube

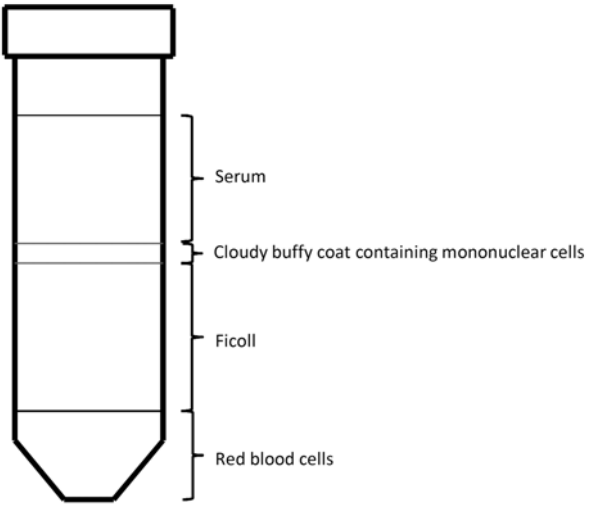

Fig. 5.2 Following Ficoll density centrifugation step, the cloudy buffy coat containing mononuclear cells can be seen between the serum and Ficoll layers

5.4.1.18. Centrifuge for 10 min at 1,600 rpm with no zero deceleration (with the break following a deceleration phase)
5.4.1.19. Aspirate and discard the supernatant
5.4.1.20. Gently resuspend pellet with 50 mls of PBS
5.4.1.21. Centrifuge for 10 min at 1,600 rpm (with no zero deceleration)
5.4.1.22. Repeat steps 5.4.1.19–5.4.1.21
5.4.1.23. Aspirate and discard the supernatant
5.4.1.24. Gently resuspend the pellet in EBM-2 media supplemented with growth factors
5.4.1.25. Count the mononuclear cells with haemocytometer with trypan blue exclusion
5.4.1.26. Gently resuspend the mononuclear cells at a concentration of 10 million mononuclear cells per 2 mls of EBM-2 media supplemented with growth factors
5.4.1.27. Gently transfer the resuspended mononuclear cells into each well of the 6-well plate
5.4.1.28. Incubate for 4 days at 37°C in 5 % CO_2 incubator
5.4.1.29. Gently add 2 mls of EBM-2 media supplemented with growth factors 2 days after plating the mononuclear cells
5.4.1.30. Change media every second day from Day 4 onwards
5.4.1.31. The number of adherent cells can be assessed from Day 4 onward (step 5.4.2.1.)
5.4.1.32. To isolate late EPCs or OECs, continue the culture for up to 3 weeks from step 5.4.1.30. for the appearance of colonies of late EPCs or OECs, which may start to appear from week 1 onwards (However, this is only seen in 5–10% of cultures)

5.4.2 acLDL Uptake and Lectin Staining

5.4.2.1. Prepare DiI-acLDL (5ug/ml) in 1 ml of media
5.4.2.2. Gently add 1 ml of media containing DiI-acLDL (5ug/ml) to the adherent cells, following the media change after step 5.4.1.31.
5.4.2.3. Incubate for 3 h at 37°C
5.4.2.4. Aspirate and discard the media containing DiI-acLDL (5ug/ml)
5.4.2.5. Gently wash the adherent cells with 5 mls of PBS
5.4.2.6. Repeat step 5.4.2.5.
5.4.2.7. Fix the adherent cells with 3 % PFA for 10 min
5.4.2.8. Prepare lectin-FITC (10 ug/ml) in 1 ml of media
5.4.2.9. Gently add 1 ml of media containing lectin-FITC (10ug/ml)
5.4.2.10. Incubate in a dark room at room temperature for 1 h
5.4.2.11. Gently wash the adherent cells with PBS
5.4.2.12. Repeat step 5.4.2.11. twice
5.4.2.13. Visualise adherent cells under fluorescent microscope (Adherent cells which are dual positive for acLDL and lectin are considered EPCs)

5.5 Results

Both the early EPCs and late EPCs are adherent cells of non-endothelial origin, which demonstrated dual positivity for acLDL and lectin. The phenotypes for early EPCs and late EPCs are spindle-shaped and cobblestone appearance, respectively, as demonstrated in Fig. 5.3.

5.6 Troubleshooting

5.6.1. Perform phlebotomy at the same time in the morning
5.6.2. Mix blood with EDTA within the vacutainer tube by gently shaking the tube immediately following phlebotomy
5.6.3. Process the blood within an hour following phlebotomy
5.6.4. Gently layer the diluted blood on the Ficoll as shown in Fig. 5.1
5.6.5. Ensure that the deceleration break is off so that a distinct buffy coat of mononuclear cells is obtained
5.6.6. Care should be taken to avoid leaving the mononuclear cells in red cell lysis buffer for too long
5.6.7. Care should be taken to avoid agitating the cells too much when washing them
5.6.8. Gently layer the mononuclear cells on fibronectin-coated plate to prevent bubble formation

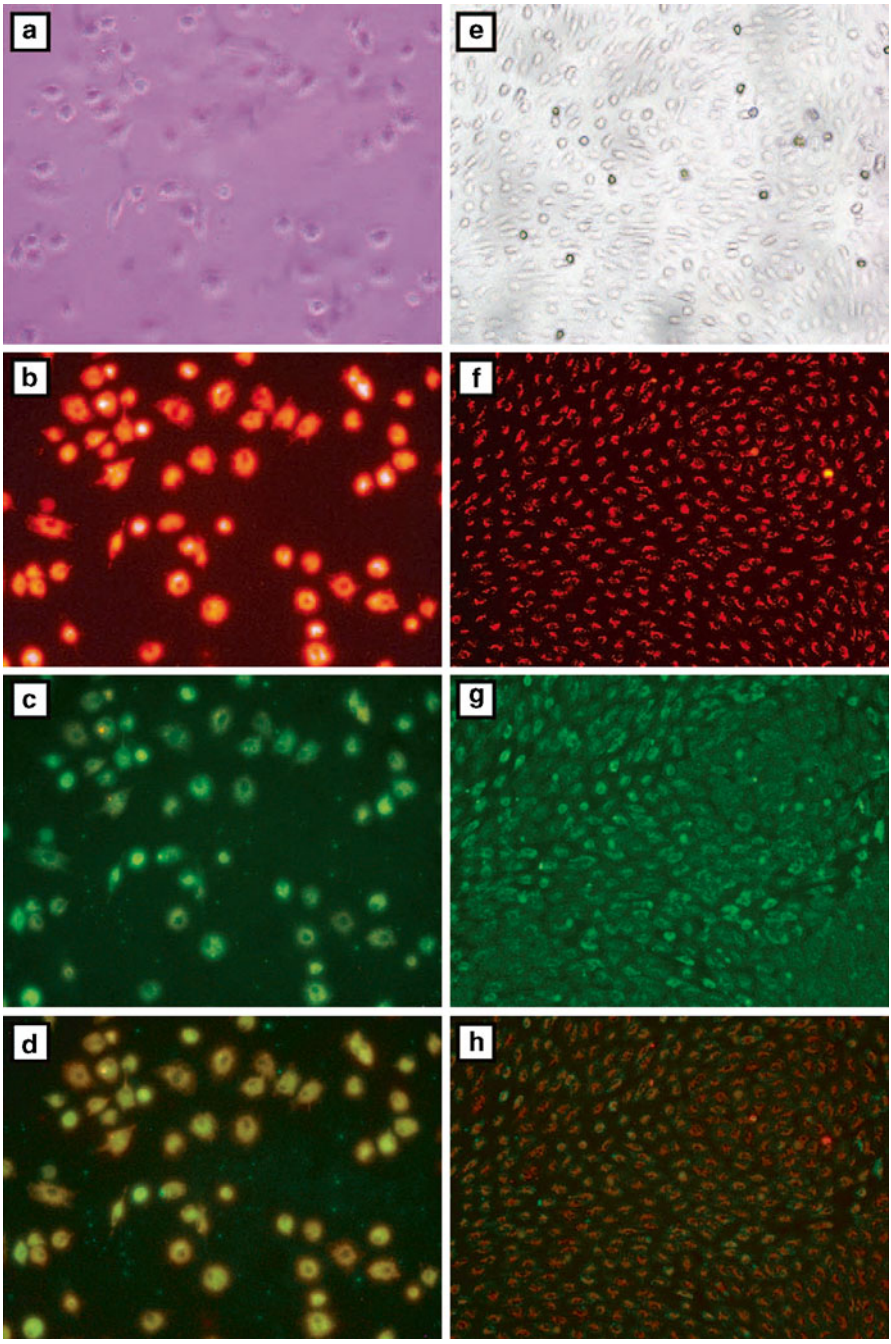

Fig. 5.3 (**a**) Early endothelial progenitor cells (EPCs) under light microscopy; (**b**) early EPCs stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled Ac-LDL acetylated low-density lipoprotein (diI-acLDL) (*red*); (**c**) early EPCs stained with fluoroscein isothiocyanate (FITC)-lectin (*green*); (**d**) early EPCs stained with both diI-acLDL and FITC-lectin; (**e**) late EPCs under light microscopy; (**f**) late EPCs stained with diI-acLDL (*red*); (**g**) late EPCs stained with FITC-lectin (*green*); (**h**) late EPCs stained with both diI-acLDL and FITC-lectin (Adapted from Liew et al. [19] with permission from Wiley Materials)

5.6.9. Care should be taken to avoid leaving the EBM-2 media in the water bath for too long to prevent degradation of the growth factors within the media
5.6.10. Try to use fresh EBM-2 media if possible (less than 3 weeks old)
5.6.11. Avoid repeated thawing of the EBM-2 media (Use aliquot instead)
5.6.12. Try to recruit a homogenous group of subjects for EPC assessment since there are many confounding factors including cardiovascular risk factors which can affect the EPC number and function [6] (The common confounding factors include current smoking history, significant difference in age among subjects and medications including statin, oral contraceptive pills and ACE inhibitors)

5.7 Additional Notes

Preparation of Hank's Complete Solution can be prepared by adding 2.5 g of Bovine Serum Albumin (BSA) and 1 ml of 500 mM EDTA pH8 to 500 mls of Hank's Balanced Salt Solution. Filter and aliquot the Hank's Complete Solution and store in fridge at 3°C. Thaw in water bath at 37°C prior use in step 5.4.1.1.

5.8 Applications and Discussion

Direct assessment of EPC number can be used as a surrogate marker for endothelial dysfunction [1, 9–12]. Many confounding factors including cardiovascular risk factors can affect the EPC number and function [6]. The common confounding factors are current smoking history, significant difference in age among subjects and medications including statin, oral contraceptive pills and ACE inhibitors. It is important to recruit a homogenous group of subjects for EPC assessment, especially, if the proposed sample size is small. The timing for phlebotomy for EPC isolation needs to be standardised since EPC level exhibits diurnal variation [20]. We recommend performing phlebotomy between nine and ten o'clock in the morning and proceed to immediate EPC isolation within 1 h of the phlebotomy.

Preclinical data showed that EPC based therapy has a therapeutic potential for the treatment of a variety of vascular disease states including ischaemia, restenosis and pulmonary hypertension [13–15]. Early human trials showed that EPC based therapy is safe and feasible [16, 17]. These cells can be genetically modified to enhance their therapeutic potential [21]. Furthermore, we have shown that patients with diabetes mellitus have less EPCs and that their EPCs are dysfunctional, and pre-treatment of EPCs with osteopontin restore its function [1].

This protocol can be used for (a) direct quantification of EPC number as a surrogate marker of cardiovascular risk factor; (b) in vitro assessment of EPC biology; or (c) in vivo assessment of EPC biology by administration into animal model [1].

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