

Isolation, Purification and Culture of Human Micro- and Macrovascular Endothelial Cells

NANCY LAURENS and VICTOR W.M. VAN HINSBERGH

■ Introduction

Endothelial cells, lining the inside of all blood vessels, are involved in many physiological processes, such as haemostasis, vasoregulation, and angiogenesis, but also play an important role in pathophysiological processes, such as inflammation, vascular leakage and tumor development. Therefore, the study of endothelial cells both *in vitro* and *in vivo*, gives investigators the ability to understand and to get more insight into these processes. It was in the early 1970s when the first endothelial cell culture was established (Jaffe et al. 1973; Gimbrone et al. 1974). Since then, the development of molecular and cellular techniques has improved and these tools are used to study extra- and intracellular responses of endothelial cells *in vitro* as well as *in vivo*.

Human microvascular endothelial cells (hMVECs) can be isolated from different human tissues, such as foreskin (Davison et al. 1980; Voyta et al. 1984), adult dermis (Davison et al. 1983), lung (Carley et al. 1992) glomerulus (van Setten et al. 1997), endometrium (Koolwijk et al. 2001), and brain (Gerhart et al. 1988). The umbilical cord can be used to isolate endothelial cells from the vein as described by Jaffe et al. (1973) and Gimbrone et al. (1974), but the arteries in the cord are also useful for isolating endothelial cells (van Hinsbergh et al. 1990). Endothelial cells from adult human aorta and vein can be obtained by a procedure comparable to the isolation of human umbilical vein endothelial cells (van Hinsbergh et al. 1987). This chapter describes the isolation of micro- and macrovascular endothelial cells and shows that a successful isolation can lead to a considerable number of endothelial cells derived from only one foreskin or umbilical cord.

The cells thus isolated can be used to study the properties of endothelial cells in a detailed biochemical way, without the interference of tissues and other cells. This has brought us in the last decade to a greater understanding of processes like vasoregulation, angiogenesis, barrier function, and leuko-

cyte endothelium interaction. However, one has to realize that during isolation and culture, the differentiated properties of the endothelial cells may be lost. Co-cultures between endothelial cells, astrocytes, pericytes, or smooth muscle cells may partly overcome this loss. Although in vivo experiments are needed to test the in vitro results, the in vitro systems will always be an important tool for getting more insight into the molecular properties and metabolic regulation of endothelial cells.

Human microvascular endothelial cells

■ Materials

Cells

- Human foreskin microvascular endothelial cells (hMVECs)

Reagents

- Cord buffer (4 mM KCl, 140 mM NaCl, 10 mM Hepes, 11 mM D-Glucose, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (p/s), pH at 7.4 and sterilized by filtration)
- M199 medium (Biowhittaker, www.biowhittaker.be) + 100 IU/ml penicillin and 0.1 mg/ml streptomycin (M199/p/s) (Biowhittaker)
- 0.4 % collagenase type II (GibcoBRL, lot number 1120228; www.gibco-brl.com) dissolved in M199/p/s and sterilized by filtration. Before use, an incubation period of 30 min at 37 °C is recommended to destroy contaminating proteolytic activity in some purified collagenase batches that may damage the isolated cells.
- M199/p/s supplemented with 10 % Human Serum, heat-inactivated, pH 7.4 (HS_i, heat-inactivation means an incubation period of 30 min at 56 °C, i.e., the serum has a temperature of 56 °C at the start of the 30 min period)
- M199/p/s supplemented with 20 % HS_i, 10 % Newborn Calf Serum heat-inactivated (NBCS_i, inactivation for 30 min at 56 °C with serum that already has a temperature of 56 °C), 5 U/ml heparin, 10 ng/ml bFGF, pH 7.4 (culture medium I)
- M199/p/s supplemented with 20 % HS_i, 10 % NBCS_i, 5 U/ml heparin, 5 ng/ml bFGF, and 0.0375 mg/ml ECGF, pH 7.4 (culture medium II)
- M199/p/s supplemented with 10 % HS_i, 10 % NBCS_i, 5 U/ml heparin, 0.075 mg/ml ECGF pH 7.4 (culture medium III)
- Human fibronectin (BD Biosciences, www.bdbiosciences.com) 2.5 µg/ml dissolved in M199/p/s and sterilized by filtration
- Solution A (137 mM NaCl, 5.4 mM KCl, 4.3 mM NaHCO₃, 5 mM D-glucose, and 0.002 % (w/v) phenol red, pH 7.4)

- 0.05 % (w/v) Trypsin/EDTA solution (137 mM NaCl, 5.4 mM KCl, 4.3 mM NaHCO₃, 5 mM D-glucose, and 0.67 mM EDTA, pH 7.4)
- M199/p/s supplemented with 0.1 % (w/v) pyrogen-free human serum albumin, pH 7.4 (HSA) (Sanquin, www.sanquin.nl)
- Mouse monoclonal antibody CD31 (2 µg/ml in M199/p/s supplemented with 0.1 % (w/v) HSA filtered by 0.22 µm filter)
- Dynal goat anti-mouse Ig coated beads (1 × 10⁷ beads/ml in M199/p/s supplemented with 0.1 % (w/v) HSA)
- 1 % gelatin (Merck, www.merck.com) in water (Milli-Q)

Equipment

- CO₂ incubator (water-conditioned)
- Laminar flow hood (preferably down-flow)
- Inverted phase contrast microscope
- Laboratory centrifuge
- Thermostatic water bath
- Clean laboratory coat
- Sterile gloves
- Sterile endotoxin-free pipettes
- Sterile scissors
- Sterile rubber plug, 6–8 cm diameter
- Sterile needles
- Sterile scalpel
- Tweezers
- 50 ml sterile centrifuge tubes (e.g., Falcon)
- Tissue culture 6-well dishes (e.g., Falcon or Costar)
- Dynal Magnetic Concentrator
- 12 ml sterile tubes

■ Procedure

Isolation of microvascular endothelial cells

1. Collect one foreskin in 20 ml cord buffer and preserve it at 4 °C for 1 or 2 days before isolating the endothelial cells, in order to obtain a high yield.
2. Perform the isolation in a sterile environment, e.g., a down-flow laminar flow hood. Use sterile gloves and a laboratory coat to prevent contamination of the cultures, and to protect yourself.
3. Stretch the foreskin using needles onto a sterile rubber plug.
4. Put a few drops M199/p/s on the foreskin to prevent drying.

5. Use a scalpel to scrape the upper layer of the inside of the foreskin from the outside to the middle of the piece.
6. Put the collected layer in a 50 ml tube containing 5 ml of 0.4 % collagenase solution mixed with 5 ml M199/p/s and incubate 2 hours at 37 °C under continuous shaking. Add subsequently 5 % HS_i to protect the cells.
7. Pellet the cells by centrifugation for 5 min at 200 × g.
8. Resuspend the pellet in culture medium I and seed them into 2–4 fibronectin-coated 10 cm² culture dishes, depending on the size of the pellet.
9. Put the culture dishes in a (water-conditioned) incubator at 37 °C under a 5 % CO₂/95 % air atmosphere.
10. Add fresh culture medium I to the wells with the attached ('endothelial') cells.
11. Renew the medium after 2 days with culture medium I (1.5 ml/10 cm²). Visually inspect the cells by using an inverted phase contrast microscope.
12. After a few days, replace culture medium I by culture medium II and inspect the cell growth every two or three days.
13. When contaminating cells start to overgrow the endothelial cells, select the endothelial cells by treating them with CD31 IgG and an anti-IgG-coated dynabeads solution (see below).

Purification by α -CD31 dynabeads

1. Wash the cells with 1.0–1.5 ml Solution A per 10 cm² well and detach the cells with 0.5 ml trypsin per 10 cm² well. Add 5 volumes M199/p/s supplemented with 10 % HS_i to the well.
2. Collect the medium with cells and centrifugate for 5 min at 200 × g.
3. Wash the cells by resuspending the pellet in 10 ml M199/p/s supplemented with 0.1 % (w/v) HSA by resuspending the medium.
4. Centrifugate for 5 min at 200 × g.
5. Dissolve the pellet in 5 ml M199/p/s supplemented with 0.1 % (w/v) HSA and divide the medium over at least three 12-ml tubes (depending on the amount of cells).
6. Centrifugate for 5 min at 200 × g.
7. Dissolve the pellet in 200 μ l CD31 IgG solution.

8. Incubate the cells for 30 min at 4 °C under end over rotation.
9. Add 10 ml 0.1 % HSA to each tube and resuspend the medium.
10. Centrifugate for 5 min at 200 × g.
11. Dissolve the pellet in 5 ml M199/p/s supplemented with 0.1 % (w/v) HSA and resuspend the medium.
12. Add several μ l IgG-coated beads solution (for example, for 20 cm² confluent cells with approximately 50 % endothelial cells, add 40–60 μ l of 1×10^7 beads/ml) to each tube and incubate for 30 min at 4 °C under regular but gentle shaking.
13. Coat 10 cm² wells with 0.5 ml fibronectin and incubate the wells for at least 5 min at 37 °C. Remove the medium before the cells are plated.
14. Wash the cells 3–5 times with 5 ml M199/p/s supplemented with 0.1 % (w/v) HSA per tube by bringing the tube into the magnetic concentrator for 1 min per wash. Remove the supernatant and add again 5 ml M199/p/s supplemented with 0.1 % (w/v) HSA per tube.
15. After 3–5 washing rounds of purification the CD31-positive cells are resuspended in culture medium II and plated on fibronectin- or gelatin-coated wells. After several days, the cells can be cultured with culture medium III.

Human macrovascular endothelial cells

■ Materials

Cells

- Human umbilical vein macrovascular endothelial cells (HUVECs)

Reagents

- Cord buffer (4 mM KCl, 140 mM NaCl, 10 mM Hepes, 11 mM D-Glucose, 100 IU/ml penicillin, and 0.10 mg/ml streptomycin (p/s), pH at 7.4 and sterilized by filtration)
- M199 medium (Biowhittaker) + 100 IU/ml penicillin and 0.1 mg/ml streptomycin, pH 7.4 (Biowhittaker) (M199/p/s)
- 0.2 % collagenase type II (GibcoBRL, lotnumber 1120228) dissolved in M199/p/s and sterilized by filtration. Before use, an incubation period of minimal 30 min at 37 °C can be helpful to destroy contaminating proteolytic activity in some purified collagenase batches that may damage the isolated cells.

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