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Isolation of Murine Brain and Lung Microvascular Endothelial Cells

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[Abstract] This protocol describes how to isolate murine endothelial cells from newborn mice brain and 3-month-old mice lung by modifying the original protocols (Sobczak *et al.*, 2010; Ruck *et al.*, 2014). We have used the protocol to analyze mRNA expression level in brain endothelial cells (Sawaguchi *et al.*, 2017). Isolated lung endothelial cells were expanded *in vitro* for various downstream experiments such as gene expression analysis and cell-based signaling assay.
Keywords: Endothelial cell, Mouse, Isolation, RT-PCR

[Background] This protocol describes experimental procedures for isolation of murine endothelial cells from mouse brain and lung. We used newborn mice for isolation of brain endothelial cells using anti-CD31 antibody. With this protocol, 2 μg of total RNA which is suitable for gene expression analysis can be isolated from brain endothelial cells, although pericytes and astrocytes cannot be totally eliminated. We also used 3-month-old mice lung to isolate lung endothelial cells using anti-CD31 and anti-CD102 antibodies. Isolated lung endothelial cells were expanded *in vitro* in 6 cm dish and used for various downstream experiments such as gene expression analysis and cell-based signaling assay.

Materials and Reagents

1. Microtube (INA•OPTIKA, BIO-BIK, catalog number: ST-0150F)
2. 50 ml conical centrifuge tubes (Greiner Bio One International, catalog number: 227245)
3. Syringe filter with a 0.22 μm pore size membrane (Pall, catalog number: 4192)
4. CellBIND surface culture dish (6 cm, Corning, catalog number: 3295)
5. Sterile blotting paper
6. Glass Pasteur pipette (25 ml, 10 ml) (IWAKI, catalog numbers: 73-0239, 73-0241)
7. 70 μm cell strainer (Corning, Falcon®, catalog number: 352350)
8. Mice (postnatal day 14)
9. Biotin-labeled anti-CD31 antibody (clone 390; BioLegend, catalog number: 102404)
10. Anti-CD102 antibody (clone 3C4; BioLegend, catalog number: 105604)
11. Bovine Serum Albumin (BSA, Equitec-Bio, catalog number: BAC62)
12. Collagenase type 2 (Worthington, catalog number: LS004174)
13. Collagenase/dispase (Roche Diagnostics, catalog number: 10 269 638 001)
14. DNase I (Boehringer Mannheim, catalog number: 104159)

15. Dynabeads Biotin Binder (Veritas Technologies, catalog number: 11047)
16. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: 172012-500ML)
17. Dulbecco's modified Eagle medium (NISSUI PHARMACEUTICAL, catalog number: 05915)
18. HuMedia-EG2 (Kurabou, catalog number: KE-2150S)
    a. hEGF
    b. hFGF-b
    c. Hydrocortisone hemisuccinate
    d. Heparin
    e. Gentamycin
    f. Amphotericin B
    g. FBS
19. Penicillin-streptomycin (Thermo Fisher Scientific, catalog number: 15140122)
20. 0.05% Trypsin/EDTA (Lonza, catalog number: CC-5012)
21. Na_2HPO_4 (Wako Pure Chemical Industries, catalog number: 196-02835)
22. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662-25G)
23. NaCl (Wako Pure Chemical Industries, catalog number: 191-01665)
24. KCl (Wako Pure Chemical Industries, catalog number: 163-03545)
25. Complete culture media (see Recipes)
26. Phosphate-buffered saline (PBS) (see Recipes)
27. 0.1% BSA in PBS (see Recipes)

Equipment

1. Forceps (Fine Science Tools, model: Dumont #5)
2. Incubator (SANYO, model: MCO-175)
3. Tube rotator (Taiyo, catalog number: RT-50)
4. Orbital mixer (Tokyo Rikakikai, EYELA, catalog number: CM-1000)
5. Centrifuge (KUBOTA, model: 8900)
6. Magnetic tube stand [*e.g.,* 6-Tube magnetic separation rack (New England Biolabs, catalog number: S1506S)]
7. Aspirator (handmade)

Procedure

A. Preparation of anti-CD31/CD102 antibody-conjugated magnetic beads (Dynabeads)
   1. Transfer 200 µl of resuspended Dynabeds Biotin Binder into a 1.5 ml Eppendorf tube and wash the beads with 0.1% BSA/PBS.
   2. Collect the beads using a magnetic tube stand.
   3. Remove the supernatant by aspiration.

4. Resuspend the beads in 1 ml of sterile 0.1% BSA/PBS.
5. Wash the beads three more times, for a total of four washes with 0.1% BSA/PBS.
6. Resuspend the beads in 500 µl of 0.1% BSA/PBS.
7. Add 10 µl anti-CD31 antibody or anti-CD102 antibody to the tube.
8. Incubate the samples under gently shaking on an orbital mixer at 4 °C (in the cold room) overnight, or for 2 h on a rotator at room temperature.
9. Wash the beads with sterile 0.1% BSA/PBS as described in Steps A5.
10. Resuspend the beads in 200 µl 0.1% BSA/PBS.
    Note: The anti-CD31 antibody-conjugated magnetic beads can be stored at 4 °C for up to 1 month.

B. Isolation of murine brain microvascular endothelial cells
1. Ten mice at P14 are sacrificed, and brains are isolated. Remove the Cerebellum and brainstem with forceps. Detach the meninges by rolling the brains on sterile blotting paper.
2. Transfer meninges-free brains to a 50 ml Falcon tube filled with 13.5 ml of DMEM (Figure 1A).
3. Mince the brains first with a 25 ml pipette, then with a 10 ml pipette until the medium becomes milky (Figure 1B).
4. Digest the tissue homogenates by adding 0.6 ml of 10 mg/ml collagenase type 2 in DMEM and 0.2 ml of 1 mg/ml DNase I in PBS for 1 h at 37 °C using an orbital mixer at 180 rpm.
5. After digestion (Figure 1C), add 10 ml of DMEM and centrifuge the tissue suspension at 1,000 *x g* for 10 min at 4 °C (Figure 1D).
6. Remove the supernatant by aspiration (Figure 1E).
7. Resuspend the pellet using a 25 ml pipette in 25 ml of 20% (w/v) BSA in DMEM approximately 25 times and centrifuge at 1,000 *x g* for 20 min at 4 °C (Figure 1F).
8. After centrifuging (Figure 1G), remove the upper myelin layer with glass Pasteur pipette (Figure 1H).

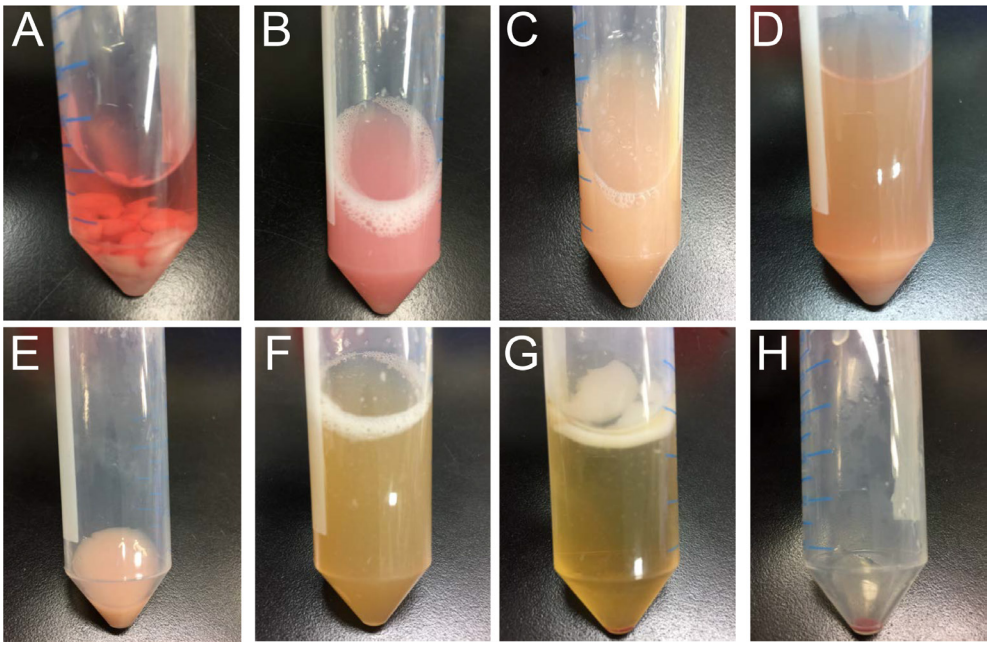

Figure 1. Isolation of murine brain microvascular endothelial cells. See text for details.

9. Resuspend the pellet in 9 ml of DMEM and supplement with 1 ml of 10 mg/ml collagenase type 2 and 0.1 ml of 1 mg/ml DNase.
10. After digestion for 1 h at 37 °C, add 15 ml of 20% FBS in DMEM containing penicillin/streptomycin to stop digestion.
11. After centrifugation at 1,000 *x g* for 5 min, resuspend the pellet with 3 ml of 0.1% BSA in PBS.
12. Mix the cells with 22.5 µl of Dynabeads Biotin Binder pre-coated with 5 µg of biotin-labeled anti-CD31 antibody. Incubate the mixture using a tube rotator at RT for 15 min.
13. Collect the beads with bound endothelial cells using a magnetic tube stand.
14. Wash the beads with PBS.

C. Isolation of murine lung microvascular endothelial cells

1. Mice at 3 months old are sacrificed.
2. Mince the lungs and digest it with 1 mg/ml of collagenase/dispase in DMEM for 45 min at 37 °C.
3. Pass the cells suspension through a 70 µm cell strainer, collect the cells by centrifugation.
4. Resuspend the cells in 1 ml 0.1% BSA/PBS.
5. Isolate endothelial cells using Dynabeads Biotin Binder precoated with biotin-labeled anti-CD31 antibody.
6. Resuspend the Beads in 10 ml HuMedia-EG2 containing 2% FCS and 10 ng/ml hEGF, 5 ng/ml hFGF-b, 1.34 µg/ml hydrocortisone hemisuccinate, 10 µg/ml heparin, 50 µg/ml gentamycin, 50 ng/ml Amphotericin B, and then, plate onto CellBIND Surface Culture Dishes.
7. After reaching 70-80% confluent, detach the cells are by using 0.05% Trypsin/EDTA and collect by centrifugation.
8. Resuspend the cells in 1 ml 0.1% BSA/PBS.

9. Purify the endothelial cells using Dynabeads Biotin Binder precoated with biotin-labeled anti-CD102 antibody.
10. Resuspend the beads in 2 ml complete HuMedia-EG2 media and plate onto CellBIND surface culture dishes.

Recipes

1. Complete culture media
   DMEM containing 10% FBS and penicillin-streptomycin
2. Phosphate-buffered saline (PBS)
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   137 mM NaCl
   2.7 mM KCl
3. 0.1% BSA in PBS
   0.1% BSA in PBS is prepared by dissolving 50 mg BSA in 50 ml PBS
   The solution is sterilized by filtering through a 0.22 μm syringe filter (can be stored at 4 °C)

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