

# **Original Article**

# Optimized method for culturing outgrowth endothelial progenitor cells

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Outgrowth endothelial progenitor cells (OECs) are expected to be a valuable source of blood vessels for regenerative medicine. Their properties and functions have been intensively studied, but the feasibility of reliable acquisition remains unclear. The aim of this study was to determine an optimized method for obtaining OECs from human umbilical cord blood (hUCB). We isolated mononuclear cells from hUCB and determined the OEC colony formation rate (OEC-CFR) under various conditions on the basis of the following 4 points: 1, cell density; 2, pre-selection of CD45<sup>(-)</sup> cells; 3, culture medium; and 4, influence of cryopreserva-tion. The OEC-CFR from CD45<sup>(-)</sup> cells was 0.250 colony/5  $\times$  10<sup>7</sup> , cells and was dependent on the initial cell density, while the OEC-CFR from total mononuclear cells was 0.347 colony/5  $\times 10^7$  cells. This result suggested that pre-selection of CD45<sup>(-)</sup> cells was not necessary to obtain OECs. Supplementation of the culture medium with microvascular endothelial growth medium (EGM-2-MV) caused an increase in the OEC-CFR. Furthermore, we obtained at least 1 colony from each sample of total mononuclear cells after cryopreservation, although the OEC-CFR was lower than that from fresh cells. Flow cytometric analysis and immunocytochemistry showed that isolated OECs expressed CD31, CD34, CD73, CD146, CD144 and CD309. By confocal microscopy, we confirmed the formation of tubes by OECs on Matrigel. In this study, we proposed an optimized method for the isolation of OECs. This method could be useful for the clinical application of OECs.

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### Introduction

Since the discovery of circulating endothelial progenitor cells  $(EPCs)^{1}$ , they have been used as a source for regenerative medicine, a biomarker for predicting cardiovascular disease and a vector for cancer therapy<sup>2</sup>). Generally, there are believed to be 2 types of EPCs *in vitro*, early EPCs and late EPCs, also known as outgrowth endothelial progenitor cells  $(OECs)^{3, 4}$ . Early EPCs are thought to provide angiogenic factors, whereas OECs bear typical endothelial characteristics *in vitro* and more directly contribute to neovascularization by providing new endothelial cells<sup>5</sup>. Therefore, isolation of OECs is useful for the creation of new vessels for transplantation.

Thus far, various studies have been attempted to identify the fraction in which OECs appear. Lin et al. reported a method for the purification of OECs using P1H12-conjugated beads with magnetic microbeads<sup>6)</sup>. Another group showed that OECs are originated from CD45<sup>(-)</sup> and CD34<sup>(+)</sup> fractions<sup>7)</sup>. In each study, methods for obtaining OECs have been modified to enrich the fraction containing OECs<sup>8)</sup>. Duda et al. proposed a protocol for the use of OECs as surrogate markers in patients with disease such as ischemic heart disease and cancer<sup>9)</sup>. The purpose of these study were to analyze the percentage of OECs in the blood or to develop a method to concentrate the fraction containing OECs. Definition of OEC using surface markers has not been decided yet and no detailed deta are available on the in vitro collection rate for the reported methods. For the clinical use of OECs, obtaining the required numbers of functional cells is important to develop new vessels for transplantations.

Thus, to use OECs in clinical settings, it is essential to establish a reliable method to obtain adequate numbers of OECs with certainty in an easy and cost-effective manner. Whether sorting of total mononuclear cells (MNCs) is necessary to obtain OECs is not clear. Although removal of cells other than OECs might be beneficial, there is a possibility that OECs might be lost while purifying MNCs. Therefore, the decision regarding detection method to be uses is preferentially made on the basis of factors such as convenience, cost-effectiveness and accuracy. We made a comparative analysis of the culture conditions used to obtain OECs for clinical application.

Cryopreservation is the accepted method for the

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long-term storage of hematopoietic stem cells for transplantation<sup>10, 11)</sup>. However, whether OECs can be derived from frozen umbilical cord blood MNCs is unclear. In this study, we also determined the amount of OECs that could be obtained by harvesting MNCs that were subjected freeze-thaw cycles.

Unlike adult peripheral blood, the human umbilical cord blood (hUCB) is enriched with OECs that can be collected without requiring invasive procedures. Umbilical cord blood-derived OECs that have vast proliferative potential represent a novel source of cells for regenerative medicine applications. These cells have a stable endothelial phenotype and can undergo at least 100 population doublings<sup>12)</sup>. Therefore, hUCB is attractive source for the isolation of OECs with clinical value. In this study, we describe a method to obtain OECs from hUCB.

### Materials and methods

#### Human samples

Human UCB was obtained from 18 healthy term newborns. Informed consent was obtained from all donors. The samples were handled according to the tenets of the Declaration of Helsinki, and the study protocol was approved by the university review board (No. 435). Human UCB samples were collected by gravity flow in a citrate phosphate dextrose (CPD) solution bag (C-200; Terumo, Tokyo, Japan). The samples were stored at 4°C and used within 24 hours. Some hUCB samples were divided into several aliquots to confirm the influence of culture conditions using the same donor's blood.

#### **Buffy coat cell preparation**

Human UCB (50–220 mL) was diluted 1:1 with phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and overlaid on Lymphoprep tubes (Axis-Shield, Oslo, Norway). Cells were centrifuged at  $280 \times g$  for 30 min at 18–20 °C. The resulting MNCs were collected and washed twice with PBS. In some experiments, the population of CD45-negative cells was isolated using magnetic activated cell sorting (MACS; Miltenyi Biotec, Paris, France).

#### Cell culture

Isolated cells were resuspended in (1) endothelial cell basal growth medium-2 (EBM-2, B3156; Lonza, Basel, Switzerland) with microvascular endothelial



growth medium (EGM-2-MV) supplements (Lonza, CC-3202), (2) EBM-2 with endothelial growth medium (EGM-2) supplements (Lonza, CC-4176), or (3) STK2 (DS Pharma Biomedical, Osaka, Japan). The cells were cultured in fibronectin-coated 6-well palates (BD Bioscience, MA, USA) at a density of 1  $\times 10^7$  cells, 5  $\times 10^7$  cells, or 1  $\times 10^8$  cells per well. The medium was changed either every day or every alternative day for 6 days, and then after 7 days of culture, the medium was changed 3 times a week. The characteristic OEC colonies were observed under a phase-contrast microscope (IMT-2; Olympus Optical, Tokyo, Japan), and OECs were cloned by colony isolation. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. OEC colony numbers were counted 4 weeks after MNC seeding. To characterize the cells, we used OECs up to 8th passage with a split ratio of 1:2.

#### **Cell cryopreservation**

Some isolated primary total MNCs were suspended in Bambanker (CS-02-001, Nippon Genetics, Tokyo, Japan) and cryopreserved for 1 month.

#### Flow cytometric analysis

OECs were detached using 0.05% trypsin-EDTA solution and washed with PBS. After cells were fixed with 4% paraformaldehyde at 4°C and washed with PBS, 50,000–200,000 cells were incubated with primary antibody for 15 min in the dark at room temperature. The antibodies used in this experiment were as follows, CD31-PE, CD34-PE, CD73–PE, CD146–PE and their corresponding isotype controls from BD Pharmingen (CA, USA). After the cells were washed with PBS containing 2% fetal calf serum, flow cytometric analysis was performed by using fluorescence activated cell sorter (FACS) Aria (Becton-Dickenson, NJ, USA).

#### Immunocytochemistry

OECs were seeded onto a 8-well chamber microscope slide (ibidi, München, Germany) coated with fibronectin (Sigma, MO, USA) and fixed with 4% paraformaldehyde (PFA) for 15–20 minutes. Immunocytochemistry was performed using primary antibodies against human vascular endothelial growth factor receptor 2 (VEGFR-2), (sc-6251; Santa Cruz Biotechnology, CA, USA; diluted 1:400) followed by incubation with Alexa 488-conjugated anti-mouse IgG (A-11001; Invitrogen, OR, USA; diluted 1:400) and against VE-cadherin (210-232-C100; Alexis, NY, USA; diluted 1:800) followed by incubation with Alexa 488-conjugated anti-rabbit IgG (A-11008; Invitrogen; diluted 1:400). Non-immune mouse IgG1 (sc-3877; Santa Cruz; diluted 1:100) and non-immune rabbit IgG (sc-2027; Santa Cruz Biotechnology; diluted 1:100) were used as controls. Immunoreactivities were detected using a laser confocal microscope (LSM 510, Zeiss, Oberkochen, Germany). Non-immune mouse and rabbit IgG were used as primary antibodies for negative controls.

#### Cell patterning and tube formation on Matrigel

In a previous report, we described the tube formation assay using our unique printing technique <sup>13</sup>. Glass substrates on which a line pattern is printed were placed on cell culture dishes. In all,  $2.5 \times 10^5$ cells were seeded per substrate ( $25 \times 15$  mm) and incubated for 20-22 h at 37°C. During incubation, cells in hydrophobic areas migrated to hydrophilic areas and formed lines. The cells in a line pattern on the substrate were turned over onto Matrigel (354230; BD Bioscience, MA, USA) and incubated with the culture medium for 24 h. When the substrate was removed, the cells had transferred from the substrate to the Matrigel. The structure consisting of OECs was pre-loaded with calcein-AM for 30 min on Matrigel and observed by a phase-contrast microscopy. Three-dimensional capillary structures were analyzed using a laser confocal microscope.

#### Statistical analysis

All data are presented as mean $\pm$ SE. Intergroup comparisons were performed using Student *t* test and one-factor analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant.

# Results

#### Effect of cell density on OEC-CFR

On the basis of the report by Timmermans et al., we first used the CD45-negative fraction of MNCs to obtain OECs<sup>7)</sup>. To analyze the effect of cell density, hUCB from a single donor was divided into 3 aliquots. The colony numbers derived from each sample are summarized in Table 1. We examined OEC colony formation at 3 different densities; (1)  $1 \times 10^7$  cells, (2)  $5 \times 10^7$  cells and (3)  $1 \times 10^8$  cells per one well of 6-well plates. A positive correlation between OEC-CFR and cell densities was observed, as shown in Fig. 1 (R<sup>2</sup>=0.9553). We used EBM-2 with EGM-2 supplements in this assay.





# Figure 1. Relationship between OEC-CFR and inoculated cell density

Human UCB from a single donor was divided into 3 samples. Total MNCs were pre-selected using CD45 magnetic beads and the CD45-negative population was inoculated in 6-well fibronectin-coated plates. OEC colony numbers were counted 4 weeks after MNC seeding. OEC-CFR is displayed on a scatter diagram. OEC-CFR showed a positive correlation ( $R^2 = 0.9553$ ) with cell density.



#### Figure 2. Differences in colony number from each cell fraction

The CD45<sup>(-)</sup> or total MNCs were inoculated on fibronectin-coated culture plates at a density of  $5 \times 10^7$  cells/well. EBM2 with EGM-2 supplements was used in this assay. OEC colony numbers were counted 4 weeks after MNC seeding. OEC-CFR is displayed on the graph. No significant difference in OEC-CFR was observed between CD45-negative and total MNCs. Mean ± SE: CD45(-); 0.250 ± 0.250, total MNCs; 0.347 ± 0.200 (p = 0.8095)

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#### Influence of culture medium

We investigated whether there was any difference in OEC-CFR when we used the 3 different kinds of media: (1) EBM-2 with EGM-2 supplements, (2) EBM-2 with EGM-2-MV supplements, and (3) STK2. We inoculated total MNCs at  $5 \times 10^7$  cells per well in this assay. When we used STK2, no colonies appeared as is shown in Table 1, and we found that this medium was inadequate for OEC aquisition. Next, we examined the effect of EGM-2-MV supplements on OEC-CFR. The addition of EGM-2-MV supplements resulted in a significant increase in OEC-CFR. The results showed a significant difference between the 2 media. (Fig. 3, p = 0.0018)



Figure 3. Comparison of OEC-CFR in different media

Total MNCs were inoculated at  $5 \times 10^7$  cells/well on fibronectin-coated culture plates. OEC colony numbers were counted 4 weeks after MNC seeding. EBM-2 with EGM-2-MV supplements, EGM-2 with EGM-2 supplements, and STK2 were used in this assay. When we used STK2, no colonies appeared. A significant difference was observed in OEC-CFR between EBM-2 + EGM-2 and EBM-2 + EGM-2-MV. Results presented are the mean  $\pm$ SE (p = 0.0018).

# OEC colonies derived from cryopreserved total MNCs

We evaluated whether OEC colonies could be derived from cryopreserved total MNCs under the defined culture conditions (cell density,  $5 \times 10^7$  cells/well; medium, EBM-2 with EGM-2-MV sup-



plements). As shown in Table 1, OEC colonies appeared in all samples; however, the colony number

was less than that from non-cryopreserved total MNCs.

1						nies / well
1	1	CD45	1×10 <sup>7</sup>	no	EBM2+EGM2	0.00
2	2	CD45	1×10 <sup>7</sup>	no	EBM2+EGM2	0.33
3	2	CD45	5×10 <sup>7</sup>	no	EBM2+EGM2	0.50
4	2	CD45	1×10 <sup>8</sup>	no	EBM2+EGM2	1.00
5	3	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	0.33
6	4	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	0.50
7	5	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	1.25
8	6	CD45	5×10 <sup>7</sup>	no	EBM2+EGM2	0.00
9	6	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	0.00
10	8	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2MV	3.33
11	8	Total MNCs	5×10 <sup>7</sup>	no	STK2	0.00
12	9	Total MNCs	$5 \times 10^{7}$	no	EBM2+EGM2MV	3.33
13	9	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	0.00
14	10	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2	0.00
15	10	Total MNCs	$5 \times 10^{7}$	no	STK2	0.00
16	11	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2MV	2.80
17	12	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2MV	0.83
18	13	Total MNCs	5×10 <sup>7</sup>	no	EBM2+EGM2MV	2.00
19	14	Total MNCs	5×10 <sup>7</sup>	yes	EBM2+EGM2MV	0.60
20	15	Total MNCs	5×10 <sup>7</sup>	yes	EBM2+EGM2MV	0.25
21	16	Total MNCs	5×10 <sup>7</sup>	yes	EBM2+EGM2MV	1.17
22	17	Total MNCs	5×10 <sup>7</sup>	yes	EBM2+EGM2MV	1.00
23	18	Total MNCs	5×10 <sup>7</sup>	yes	EBM2+EGM2MV	0.09

#### Table 1. OEC-CFR data of each sample

MNCs were isolated from hUCB of 18 healthy term newborns. Some hUCB samples were divided into several aliquots to confirm the influence of culture conditions using the same donor's blood. OEC colony numbers were counted 4 weeks after MNC seeding.

#### **Characterization of OEC**

Immunohistochemical analysis was performed to confirm whether the expanded cells still expressed endothelial cell markers. Each OECs obtained by our method expressed VE-cadherin (CD309, Fig. 4b) and VEGFR-2 (CD144, Fig. 4c). Therefore, these cells were defined as OECs as previously described<sup>12,</sup> <sup>14, 15)</sup>. All the characteristics were identical to all OECs, independent of the cell culture fraction (MNC versus CD45-negative cells) and the culture conditions described above. FACS analysis showed that these OECs expressed CD31, CD34, CD73, and CD14 (Fig. 4d). These results indicated that the cells

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expressed endothelial and endothelial progenitor cell markers. Furthermore, confocal laser microscopic analysis revealed that these OECs formed capillary-like tubular structures on Matrigel using the technique based on offset printing<sup>16</sup>.

# Confocal laser scanning microscopy of tubular structures formed by OECs

During OECs on the substrate were turned over

onto Matrigel and incubated in EBM-2 with EGM-2-MV supplements for 24 hours, the cells were transferred from the substrate to the Matrigel and changed their cellular morphology to form capillary-like tubular structures (Fig. 5a). These structures consisting of OECs were observed using confocal laser-scanning microscopy. Cross-sections showed the assembly of cells with a central cavity (Fig. 5b).



cobblestone appearance. Scale bar = 50  $\mu$ m.

(b, c) Immunohistochemistry of VE-cadherin and VEGFR-2 in OECs. Scale bars = 20  $\mu$ m. (d) Flow cytometric analysis of CD31, CD34, CD73, and CD146 expression in OECs. Isotype controls are overlaid as lines on each histogram.

# Discussion

In previous studies, many markers were used to concentrate the OEC fraction such as CD34, CD133 and CD45<sup>8)</sup>. We first used the CD45-negative fraction in order to eliminate early EPC contamination in OECs. OECs can be easily distinguished from other cell types by morphology. After individual clones were obtained, expanded clones showed endothelial cell phenotype without contamination by other cells. Certainly, we often observed the growth of early EPCs in the same culture from total MNCs. However, we could differentiate early EPCs from OECs because unlike OECs, early EPCs appeared at day 3-7 of culture and disappeared after day 14; thus, early EPCs had poor proliferative and survival potency. Therefore, we could obtain OECs from total MNCs without contamination of early EPCs. Considering the loss of cells by sorting, it is not necessary to prepare the CD45-negative fraction before culturing. Actually, we found that MNC sorting is not required because there is no significant difference in the OEC-CFR between the CD45-negative cell culture and the total MNC culture. Moreover, OEC-CFR of MNCs tended to be higher than that of the CD45-negative fraction.

Next, we analyzed the effect of initial cell density on OEC-CFR. Hur et al. reported that they used  $1 \times 10^7$  cells/well of 6-well plates to obtain OECs<sup>3)</sup>. OEC colonies could be obtained from culture at a density of  $1 \times 10^7$  cells/well (CFR, 0.33 colonies/well), and OEC-CFR was positively correlated with cell density. Several types of media have been used for OEC culture. Shi et al. used M199 medium with 10% fetal bovine serum (FBS) containing VEGF (10 ng/mL), basic fibroblast growth factor (bFGF; 1 ng/mL), and insulin-like growth factor-1 (IGF-1; 2 ng/mL) in their first report on OEC culturing<sup>17)</sup>. Ingram et al.



used EBM-2 with EGM-2 supplements<sup>12)</sup>. We compared OEC-CFR of MNCs cultured in EBM-2 media containing different supplements, and found that OEC-CFR was higher when EBM-2 with EGM-2-MV supplements was used. EGM-2 supplements contain 2% FBS, human epidermal growth factor (hEGF), hydrocortisone, VEGF, human fibroblast growth factor (hFGF)-B, R3-IGF-1 (human recombinant analog of insulin-like growth factor-I with the substitution of Arg for Glu at position 3), ascorbic acid, heparin, and GA-1000 (gentamicin/amphotericin-B). The difference between the EGM-2 and EGM-2-MV supplements is that EGM-2 has a lower concentration of FBS and has soluble heparin added. It is possible that an excess of soluble heparin affects OEC-CFR, because soluble heparin inhibits endothelial cell proliferation through the inhibition of VEGF signaling<sup>18</sup>.



#### Figure 5. Tube formation by OECs

OECs were inoculated on line patterning glass substrates, and then turned onto Matrigel. When they were incubated for 24 hours, OECs were transferred to Matrigel and formed tubular structures. These structures were stained by calcein-AM and observed under (a) a phase-contrast and (b) a laser-scanning microscope. 3D images showed a lumen within the tubular structure. Scale bars =  $50 \mu m$ .

The first successful hUCB transplantation was performed in 1998<sup>19)</sup>. At present, it is possible to treat or support more than 85 different illnesses with hUCB transplantation<sup>20)</sup>. Human UCB is usually stored as frozen MNCs for hematopoietic stem cell transplantation. To determine whether cryopreserved MNCs could be used for endothelial cell or vascular transplantation is important to reveal another use for cryopreserved hUCB. In this study, we investigated whether OECs could be obtained from cryopreserved MNCs. Although the OEC-CFR of cryopreserved MNCs was lower than that of freshly obtained MNCs, OECs were isolated from all samples of hUCB. Our results showed that it is possible to obtain OECs from cryopreserved MNCs such as those that were stored for hematopoietic stem cell transplantation or other purposes. This result contributes

to clinical use of hUCB in regenerative medicine.

In this study, we compared the influence of cell sorting and medium on obtaining OECs. The best method is to use total MNCs and EBM-2 with EGM-2-MV supplements at more than  $5 \times 10^7$  cells per well of 6-well plates.

It is important to reliably obtain cells for use in clinical applications. In the present study, we focused on the methodology for obtaining OECs. Human UCB-derived OECs seem to be a promising source for cardiovascular tissue engineering because of their physiologic properties and easy accessibility. More detailed investigation should be performed on the use of OECs in clinical applications, but tube formation by OECs will help to further develop advanced tissue engineering constructs. Our results support the therapeutic potential of using human



OECs in regenerative medicine.

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