

Characteristics of *Ex Vivo* Expansion of Endothelial Progenitor Cells*

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Summary: The characteristics for the *ex vivo* expansion of the endothelial progenitor cells (EPCs) were explored. CD34⁺ cells were selected from umbilical cord blood mononuclear cells (MNC) by MiniMACS system, expanded under the same conditions as those for total MNC, cocultivation of CD34⁺ and CD34⁻ from the same donor for EPCs. In addition, the effects of vessel endothelial growth factor (VEGF) and passage on cell differentiation, expansion kinetics and apoptosis were examined. EPCs were determined and quantified by immunocytochemistry and flow cytometry. The results showed that both coculture of CD34⁺ and CD34⁻ and total MNC led to a significant increase in the expansion of CD34⁺ cells as compared with CD34 enrichment ($P < 0.05$). There was a tendency toward decreased apoptosis in cultures when early passage was performed immediately after cord like structures appeared. VEGF had no significant effect on apoptosis ($P > 0.05$). These differentiated EPCs were positive for CD34⁺, von Willebrand factor (vWF), KDR, CD31 staining and phagocytized acetylated low-density lipoprotein (LDL). CD34⁺ cells accounted for (68.2±6.3) % of attaching (AT) cells at day 7 of culture. It was suggested the most efficient method to *ex vivo* expansion of EPCs was coculture of CD34⁺ and CD34⁻ or total MNC. Early passage makes cell apoptosis rate decrease. VEGF had no significant effect on *ex vivo* expansion of EPCs.

Key words: endothelial progenitor cell; expansion; CD34⁺; apoptosis

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Recently, endothelial progenitor cells (EPCs) were isolated from peripheral blood and proved to participate in neovascular formation^[1-3]. This will be useful in regenerating therapeutic vasculogenesis, provide suitable coating for vascular grafts or deliver lethal toxins to tumor vascular beds. But EPCs in peripheral blood is very limited in number, its introduction into transplantation protocols has been hampered by the limited number of progenitors contained in blood harvested. EPCs were expanded *ex vivo* for clinical use^[2, 4].

In this experiment, we investigated the influences of CD34 enrichment, coculture of CD34⁺ and CD34⁻, early passage, and VEGF on cell differentiation and expansion kinetics, and discussed the characteristics of *ex vivo* expansion of EPCs.

1 MATERIALS AND METHODS

1.1 Cell Separation

Human cord blood anticoagulated with heparin was collected and diluted with phosphate-buffered saline (PBS) Dulbeccos without magnesium and calcium (pH=7.2). The blood diluted was layered gently onto Ficoll (1.077 g/mL), and mononuclear cells (MNCs) from the interface band were collected after density gradient centrifugation at 800 r/min for 30 min and washed three times with PBS.

1.2 Isolation of MNCCD34⁺

MNCs were enriched by using the magnetic activated cell-sorting system according to the manufacturer's instructions to enrich CD34⁺. Briefly, 1×10^8 cells/mL was suspended in 300 μ L degaussed PBE (PBS with 0.5 % bovine serum albumin and 2 mmol/L EDTA) at 4 °C. The cells were first incubated with 50 μ L rat against human CD34 Ab coupled with microbeads at 4 °C, washed two times with aforementioned buffer and centrifuged at 100 r/min for 5 min to discard unconjugated Ab, which was followed by suspension of the cells with 1 mL PBE. The bead-positive cells (MNCCD34⁺) were enriched on positive-selection columns set in magnetic field.

1.3 Expansion Cultures

The medium used for cell culture was medium-199 supplemented with 20 % FBS, antibiotics (penicillin 100 U/mL, streptomycin 100 μ g/mL), VEGF 10 ng/mL, FGF-b 1 ng/mL, IGF-2 1 ng/mL (Peprotec, USA), 10^{-4} mol/L ascorbic acid (Sigma, USA), 1.6 mmol/L L-glutamine. A part of CD34⁺ enrichment cells labeled with DiI-Ac-LDL were cocultured with their unlabeled counterpart at a cell count ratio of 1:99, (MNCCD34⁺/MNCCD34⁻, 1×10^5 cells/mL). At the same time MNCCD34⁺ (1×10^4 cells/mL), MNC (1×10^5 cells/mL) were cultured on human fibronectin-coated plastic plates. The plate was incubated at 37 °C in a humidified environment in 5 % CO₂. At day 4 of culture, medium was replaced, and then, cells were fed every 4 - 7 days. The numbers of spindle-shaped and attaching (AT) cells and cell clusters were counted under a phase-contrast microscope at day 7 of culture. Five randomly selected microscopic fields were evaluated, and mean numbers of AT

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cells and cell cultures were calculated for each cord blood.

1.4 Influence of VEGF and Passage on Cell Proliferation

MNCs (1×10^5) were plated onto four flasks of 25 cm² respectively and divided into two groups. In one group, once linear cord like structure appeared, adherent cells were detached with 0.25 % trypsin-EDTA and re-plated at 1:4 dilution. After that, passage was performed following cells confluence. In another group, no passage was performed, subgroup with or without VEGF was set for each group.

1.5 Immunocytochemistry

Spindle-shaped and AT cells at day 7 of culture were subjected to immunocytochemistry to analyze the expression of Von Willebrand factor (vWF), KDR, CD34 and CD31. In brief, cells were grown on chamber slides, and fixed with 4 % paraformaldehyde for 10 min at 4 °C. Non-specific mAb binding was blocked by incubation with 10 % horse serum. Primary mAbs direct against human vWF, KDR, CD34, CD31 were then applied. These mAbs were all mouse IgG1. Negative control slides were incubated with appropriate dilution of MOPC-21, a non-immune mouse IgG1. After two washes with PBS, the mixture was incubated with biotinylated horse anti-mouse IgG1, and then subject to the treatment with avidin-biotin immunoperoxidase.

1.6 Flow Cytometry

Cord blood-derived AT cells at day 7 of culture ($n=6$) were subjected to flow cytometric analysis to examine the surface expression CD34. AT cells were iso-

lated from culture plates at day 7 of culture by incubation with PBS with 0.25 % trypsin (pH=7.4) for 15 min at 37 °C, $5 \times 10^6 - 1 \times 10^7$ cells were suspended in 1 mL M199 with 20 % FCS and incubated with 40 μL FITC-CD34 for 20 min at 4 °C, and then analyzed by flowcytometry. To examine the influence of cell density, passage and VEGF on apoptosis, propidium iodide (PI) and annexin V staining was performed in all MNC at day 9, followed by flow cytometry, PI/Annexin V⁺ signals served as indicators of apoptosis.

1.7 Cellular Uptake of Acetylated LDL

AT cells cultured on fibronectin were incubated in medium containing 15 μg/mL DiI-labeled Ac-LDL (DiI-Ac-LDL molecular probes) for 24 h at 37 °C, and cells were then examined under a fluorescence microscope.

1.8 Statistical Analysis

All values were presented as $\bar{x} \pm s$, all data were subjected to ANOVA followed by LSD. Probabilities less than 0.05 were considered to be statistically significant.

2 RESULTS

2.1 Enhancing Effect of the Expansion

After 7 days, AT cells were counted. It was found that AT cells and cell clusters developed from coculture of MNCCD34⁺ and MNCs MNCCD34⁻ were more than those from the culture with MNCCD34⁺ alone. The spindle-shaped AT cells formed tuber-like structure (fig. 1A, 1B).

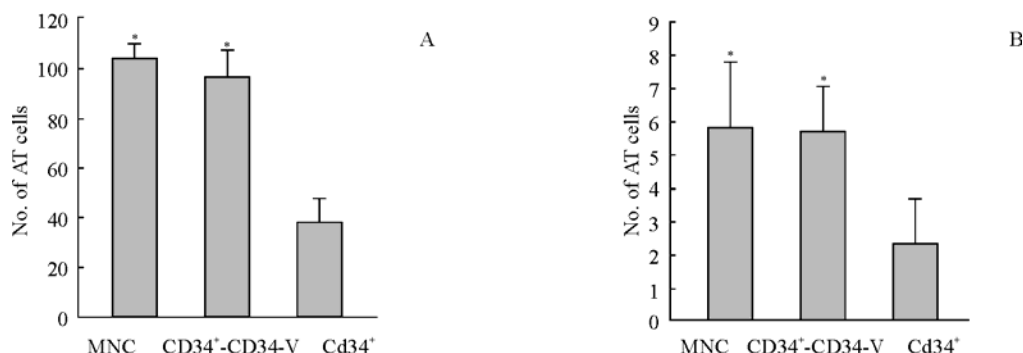


Fig. 1 Comparison between spindle-shaped AT cells and cell clusters

A and B show the numbers of AT cells and clusters from three different cell cultures at day 7 of culture. *

$P < 0.05$ vs Cd34⁺

2.2 Multiple Endothelial Phenotypes of AT Cells

AT cells had multiple endothelial phenotypes. AT cells were positively stained for vWF, CD31, CD34 and KDR. In addition, (65±7.2) % of the AT cells of culture took up DiI-ac-LDL after 7 days.

2.3 Flow Cytometry

Flow cytometry revealed that (68.2±6.3) % of AT cells were positive for CD 34 at day 7 of culture. When DiI-labeled MNCCD34⁺ was cocultured with MNCCD34⁻ at the ratio of 1:99, at day 7 of culture (60.3±7.7) % of the AT cells were from DiI-labeled MNCCD34⁺. Hence, AT cells mainly came from MNCCD34⁺.

2.4 Apoptosis

At day 29, the percentage of apoptotic cells in the

passed cells with or without VEGF was (7.1±3.1) % and (6.5±3.2%), respectively ($P > 0.05$), against (13.1±4.9) % and (12.1±4.7) % ($P > 0.05$) in non-passaged cells with or without VEGF ($P < 0.05$, fig. 2). The passed cells assumed cobblestone shape.

3 DISCUSSION

A few cells with phenotypic characteristics of endothelial cells have been found in both the bone marrow and the peripheral blood^[5]. The functions of these cells were unknown, and it was speculated that new vessel formation in adult animals could occur only via angiogenesis^[6]. Recently, EPCs were isolated from peripheral blood in adult and they were found to be involved in the neovascularization in ischemic area, and interest in

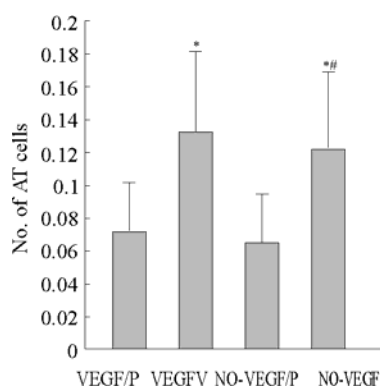


Fig. 2 Cell apoptosis rate under 4 different conditions at day 29 of culture

VEGF/P: VEGF and passage group, NO-VEGF/P: group without VEGF or passage, VEGF: VEGF group, NO-VEGF: group without VEGF)

* $P < 0.05$ vs VEGF/P, # $P < 0.05$ vs NO-VEGF/P

these cells has been rekindled by these findings^[6, 7]. We obtained AT cells from MNCs of human umbilical blood by using various methods and we found that most of these cells expressed KDR, CD31, vWF, CD34 antigens, and took up DiI-Ac-LDL. AT cells had the phenotypes of endothelial cells and functioned like ECs. Therefore, AT cells were believed to be mainly EPCs. Fluorescence-labeling suggests that AT cells predominantly came from MNCCD34⁺.

CD34 selection is an efficient way to debulk cord blood samples by removing non-clonogenic cells^[8, 9]. Those cells might otherwise deplete the culture system of nutritive factors thereby leading to changes in lactate and the pH of culture media. Theoretically, this notion is valid. In contrast with some previous findings^[8, 10], we found that CD34 enrichment was not the best method for *ex vivo* expansion of EPCs, and our results were consistent with those reported by Asahara *et al.*^[3]. When MNCCD34⁺ and MNCCD34⁻ and MNC were re-mixed and co-plated, much more cell clusters and AT cells developed as compared with cultures of MNCCD34⁺ alone. Co-incubation of MNCCD34⁺ and MNCCD34⁻, MNCs increased the proliferation rate more than 2 times that of MNCCD34⁺ cultured alone. Coculture of MNCCD34⁺ and MNCCD34⁻ cells also showed enhanced MNCCD34⁻ differentiation, including the formation of cellular networks and tuber-like structures on fibronectin-coated plates. The reasons may be as follows. First MNCCD34⁺ seems to require either cell-to-cell interaction with MNCCD34⁻ or exposure to soluble factors released from MNCCD34⁻ or both, to differentiate into AT cells *in vitro*. In addition, it was unclear whether EPCs derived from hematopoietic stem cells (HSC) or other groups of cells in mobilized bone marrow. Furthermore a portion of AT cells may be derived from MNCCD34⁻AC133⁺, enriched AC133⁺ progenitors, a small portion of which did not coexpress CD34⁺, but could generate adherent cells. Phenotypic analysis of the adherent cells revealed that the majority displayed endothelial features^[2]. And *in vitro* CD34⁻AC133⁺ multipotent adult progenitor from postnatal human bone marrow cultured with VEGF differentiate into CD34⁺, VE-cadherin, Flk1⁺ cells^[11]. Whether MAPC exists in

blood is unknown. Moreover, peripheral blood usually contains very few endothelial cells^[12].

No significant difference in proliferation efficiency and differentiation was found between coculture of MNCCD34⁺ and MNCCD34⁻ and culture of MNC. The results of coculture of MNCCD34⁺ and MNCCD34⁻ was similar to those of culture of MNC, hence there was no need to isolate MNCCD34⁺ from MNC for AT cells culture, which makes it simple and economic.

The rate of apoptosis is known to increase in CD34⁺ progenitor during *in vitro* culture and it may serve as a useful marker for the assessment of the quality of stem cell grafts. The increased rate of apoptosis may be associated with proliferation and cell division induced by the cytokines^[4]. The apoptosis rate of EPCs measured by annexin positivity was related to initial density of cultured cells. The culture with low cell density and early passage tend to decrease apoptosis when the cord-like structures appeared. Therefore, increased percentage of apoptotic cells may be due to contact inhibition between cells.

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