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Investigation of the Effective Action Distance Between Hematopoietic Stem/Progenitor Cells and Human Adipose-Derived Stem Cells During Their In Vitro Co-culture

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Abstract The in vitro suitable action distance between umbilical cord blood-derived hematopoietic stem/progenitor cells and its feeder cell, human adipose-derived stem cells, during their co-culture, was investigated through a novel transwell co-culture protocol, in which the distance between the two culture chambers where each cell type is growing can be adjusted from 10 to 450 μm. The total cell number was determined with a hemacytometer, and the cell morphology was observed under an inverted microscope each day. After 7 days of co-culture, the fold-expansion, surface antigen expression of $CD34^+$ and CFU-GM assay of the hematopoietic mononuclear cells (MNCs) were analyzed. The results showed that there was an optimal communication distance at around 350 μm between both types of stem cells during their in vitro co-culture. By using this distance, the UCB-MNCs and $CD34^+$ cells were expanded by 15.1±0.2 and 5.0±0.1-fold, respectively. It can therefore be concluded that the optimal action distance between stem cells and their supportive cells, when cultured together for 7 days, is of around 350 μm.

Keywords Adipose-derived stem cells · Hematopoietic stem/progenitor cells · Transwell · Co-culture · Expansion

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Introduction

The in vitro cultures of hematopoietic stem cells/progenitor cells (HS/PCs) can be divided into two kinds according to whether they are supported by stormal cells or not. There are current clinical practices that have accepted expanded HSCs with stroma as acceptable [1], and in fact, many studies have implicated the benefits of MSCs as a stroma support in facilitating both in vitro and in vivo hematopoiesis [2–5]. As the knowledge of the in vivo hematopoiesis process increases and the in vitro expansion study of HS/PCs develops, it is discovered that the co-culture protocol of stromal cells and HS/PCs obviously surpasses the non-stromal cell culture protocol [6, 7]. During the co-culture process, the contact style of two different cells can be divided into direct contact, cell villi indirect contact and a certain distance's non-contact [8, 9].

Amongst them, in direct contact co-culture system, it is very difficult to separate HS/PCs from stromal cells completely, thus, this direct contact co-culture protocol faces difficulties of purifying the cells and the security problems of immune rejection [10]. In 1999, Kawada et al. [11] proposed a method called "adjusted transwell" for the first time, which could both co-culture stromal cells with HS/PCs and prevent them from polluting the HS/PCs, leading a close indirect contact of the two cells. After that, Tagagi [12, 13] studied the effect of pore membrane's aperture on hematopoietic progenitor cell. The result showed that when the aperture was smaller than 0.4 μm, the stromal cells would not be able to transfer to the supine surface. This indirect contact co-culture, only having paracrine and no juxtacrine, results in the best culture outcome. In uncontact co-culture also only paracrine works, yet the effect is not as well, which demonstrates that the distance between the cells should not be too far. But in present transwell protocol, the distance of the co-cultured two cells is fixed and cannot be further changed.

Therefore, in order to explore the proper co-culture distance in vitro of the HS/PCs and its feeder cells, human adipose-derived stem cells (ADSCs), this study co-cultures them within a novel-adjusted transwell co-culture system which can alter the interaction distance of the two cells via alter the sidewall height of pore plate from 10 to 450 μm.

Materials and Methods

Cells

Umbilical cord blood (UCB) was obtained from the umbilical vein after full-term vaginal delivery with the informed consents of the parents. UCB was collected in bags containing heparin and processed within 24 h. After separation with the Ficoll-Hypaque (1.077 g/mL) method, the low-density mononuclear cells (MNCs) were washed in IMEM supplemented with 50 IU penicillin/mL and 50 μg streptomycin/mL. The cells were cultured overnight for co-culture use.

The ADSCs were separated from subcutaneous normal adipose issues of surgery patients (aged from 16 to 60) via our improved method [14] using co-digestion with 0.25% Trypsin (Sigma) and 0.1% collagenase (Sigma). After that, the below liquid containing mononuclear cells was diverted into a centrifuge tube, and DMEM (high-glucose) containing 10% FBS (Gibco) was added into this tube to terminate the digestion process. Following this, the left adipose tissues were digested for two to approximately three times again according to the above method. Then, the collected cells were resuspended and cultured in an incubator with 37 °C, 5% CO_2. The culture media were changed every 2 days. Cells over 11th passage were used for experiment.

Adjustment and Determination of the Action Distance Between Two Kinds of Cells

The distances between transwell film and the bottom of 6-pore plate, ranged from 10 to 450 μm, measuring by a high-precision vernier caliper, were changed via grinding the sidewall of the pore plate carefully adopting 100, 150, 280, and 600 mesh sandpapers, respectively.

Co-culture of MNCS and ADSCs

The ADSCs were seeded into a pretreated 6-well plate at 2×10^5 cells per milliliter, the transwell chamber was inserted into the pore plate when majority of cells were confluent. After this, the MNCs were seeded into the chamber at 1×10^6 cells per milliliter. The experiment was composed of 12 groups, the UCB-MNCs cultured alone in the first group were regarded as control group, and the second group was direct co-culture group, i.e., co-culture two kinds of cells directly. In respect to other ten groups, we designed indirect contact co-culture with different cellular action distance ranging from 10 to 450 μm. During 7 days of culture, the cell number was counted each day to detect the expansion of MNCs. The expansion fold of the MNCs, $CD34^+$ cells and CFU-GM under different culture conditions were also compared and analyzed after 7 days of culture.

Flow Cytometry Analysis of $CD34^+$ Cells

For flow cytometry, approximately 10^6 freshly separated cells or expanded hematopoietic cells were washed once in PBS and afterwards resuspended in 100 μL flow cytometry buffer (0.1% sodium azide in PBS) and stained with 10 μL anti-CD34-PE or anti-CD34-FITC (BD Pharmingen) and incubated at 4 °C in the dark for 20 min. Isotype and positive controls of both antibodies were also prepared. The cells were then washed twice with PBS and finally resuspended in 1 mL flow cytometry buffer. Finally, the samples were analyzed with a FACSCalibur. At least 20,000 events were acquired per sample and analyzed using CellQuest Pro.

Colony-Formation Capability Assay

IMDM containing 0.9% methyl cellulose, 10% horse serum, 10% FBS, and 5×10^{-5} mol/L 2-mercaptoethanol were used as culture medium of CFU-GM colony. Besides, this medium also contained cytokines including 8 ng/mL SCF, 3.735 ng/mL FL, 3.735 ng/mL TPO, 2.56 ng/mL IL-3, 1.665 ng/mL G-CSF, and 1.065 ng/mL GM-CSF. After 7 days of culture in a humidified environment at 37 °C and 5% CO_2, the colonies consisting of 50 or more cells were counted under an inverted microscope.

Multilineage Differentiation Potential of Expanded ADSCs

After co-culture with MNCs, ADSCs were induced to differentiate into osteoblasts, chondrocytes, and adipocytes to detect whether they still remained multilineage differentiation capacity. ADSCs were seeded into a 24-well plate at 1×10^5 cells per milliliter and cultured in IMDM until they reached 80% of confluence. Then, osteogenic differentiation was induced by incubating them with IMDM consisting of 10% FBS, 10 mM β-glycerophosphate, 50 μM vitamin C, and 0.1 μM dexamethasone, and detected by ALP staining after 1 and 2 weeks of induction and von Kossa staining after 3 weeks of

induction, respectively. Chondrogenic differentiation was induced by IMDM consisting of 0.1 μM dexamethasone, 50 μg/mL vitamin C, 100 μg/mL sodium pyruvate, 10 ng/mL transform growth factor (TFG-β3), 500 ng/mL bone morphogenetic protein (BMP-6), and 50 mg/mL ITS$^+$, and detected by toluidine blue staining after 1, 2 and 3 weeks of induction, respectively. Adipogenic differentiation was induced by IMDM including 10% FBS, 0.1 mM 3-isobutyl-1-methyl xanthine, 1 μM dexamethasone, 0.2 μM indomethacin, and 10 μM insulin, and assayed with oil red staining after 1, 2 and 3 weeks of induction, respectively [15, 16].

Statistic Analysis

All the experiments should be repeated for three times. The statistics were showed as mean±SD to determine the level of significance with *t* test, analyzed by Origin7.0 software.

Results

Expansion of Mononuclear Cells

The co-cultures of two kinds of cells with ten different distances were investigated in this research. The expansion fold of MNCs with time was showed in Fig. 1a and Table 1; meanwhile, the relationship between the expansion fold and cellular distance was showed in Fig. 1b. Table 1 demonstrated that the distance between two different stem cells had a significant effect on the expansion of mononuclear cells. The expansion fold of MNCs increased with the increase of cell distances initially, and then it reached to 15.1±0.2-fold when the cell distance was around 350 μm, which achieved the top value. After that, it decreased with the increasing action distance.

Expansion of CD34$^+$ Cells

The relationship between expansion fold of CD34$^+$ cells and cellular action distance was showed in Fig. 2a. It demonstrated that the expansion fold of CD34$^+$ cells fluctuated obviously when the cell distance increased. There was a highest expansion, 5.0±0.1-fold,

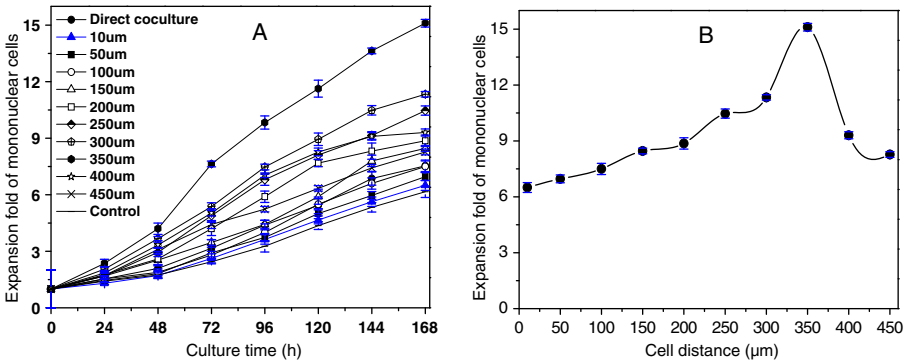

Fig. 1 Expansion of mononuclear cells co-cultured with ADSCs with time and different cellular distance. **a** Effect of culture time on the expansion of mononuclear cells at different distances. **b** Effect of distance between co-cultured cells on mononuclear cells' expansion, on the seventh day

Table 1 Expansion fold of nucleated and $CD34^+$ cells

Culture fashion	Day 0		Day 7		
	MNCs expansion fold	$CD34^+$ content (%)	MNCs expansion fold	$CD34^+$ content (%)	$CD34^+$ expansion fold
Control group	1	1.2	6.2±0.3	0.6	3.1±0.2
Direct co-culture	1	1.2	7.5±0.3	0.5	3.1±0.1
10 μm	1	1.2	6.5±0.3	0.5	2.7±0.1
50 μm	1	1.2	7.0±0.2	0.5	2.9±0.1
100 μm	1	1.2	7.5±0.3	0.6	3.8±0.2
150 μm	1	1.2	8.5±1.5	0.4	2.8±0.3
200 μm	1	1.2	8.9±0.3	0.5	3.7±0.1
250 μm	1	1.2	10.5±0.3	0.4	3.5±0.1
300 μm	1	1.2	11.3±0.1	0.4	3.8±0.3
350 μm	1	1.2	15.1±0.2	0.4	5.0±0.1
400 μm	1	1.2	9.3±0.2	0.4	3.1±0.1
450 μm	1	1.2	8.3±0.2	0.4	2.8±0.1

when the cell distance reached to 350 μm. After that, it decreased with the increasing action distance.

GFU-GM Colony Culture

The relationship between GFU-GM expansion fold and cellular distance was showed in Fig. 2b. When the co-culture distance was 350 μm, the expansion of mononuclear GFU-GM colony reached to 8.5±0.5-fold, while the expansion at other cell distances was not so well. It indicated that the HS/PCs, co-cultured with ADSCs at a cell distance of 350 μm, were superior in terms of remaining its original state and multiplication capacity than that at other cellular distances.

The MNCs co-cultured with ADSCs using this novel-developed transwell system could achieve the highest expansion at a distance of 350 μm. This is mainly because under this

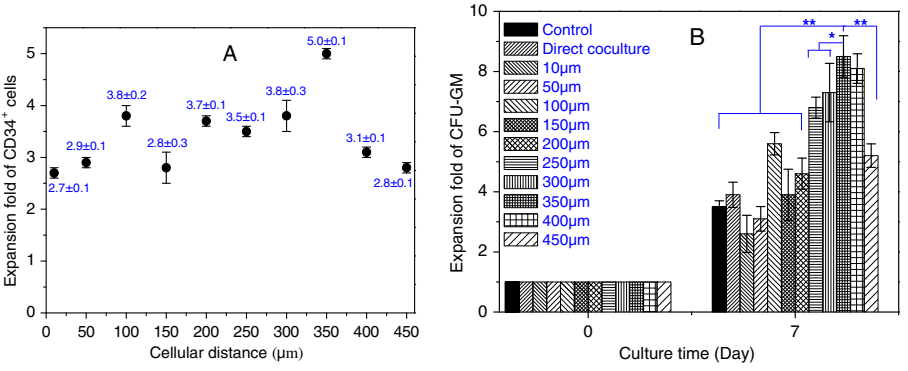

Fig. 2 Effects of distances between two co-cultured cells on the expansion folds of $CD34^+$ cells and CFU-GM. **a** Expansion folds of $CD34^+$ cells at different distances between cells on the seventh day. **b** Expansion folds of CFU-GM at different distances between cells

distance, two kinds of stem cells both carried out cellular signal transmission including paracrine and juxtacrine, in which paracrine played a more important role. At this distance, slight nutrient for the MNCs was absorbed by ADSCs, and thus, the remaining nutrient was still enough for cell consumption of MNCs. At other cell distances, either the ADSCs fought for the nutrient with mononuclear cells, or the signal transmission between cells was not so ideal, paracrine reduced while juxtacrine abounded, which therefore resulted in a worse expansion.

Surface Antigen of Co-culture Cells

ADSC is a group of cells without unique phenotype; it expresses various kinds of surface markers, and up to now, there is no unique surface marker that could identify it alone. At present, markers of $CD29^+$, $CD34^-$, $CD44^+$, $CD45^-$, and $CD166^+$ were usually adopted to identify ADSCs. The surface markers of expanded ADSC analyzed using flow cytometry was shown in Fig. 3. The expressions of CD29 (member of integrin family), CD44 (receptor of fibronectin and hyaluronate), and CD166 were positive, while the expressions of CD34 (hematopoietic stem/progenitor cells and endothelial cells were positive) and CD45 (surface antigen of hematopoietic cells) were negative. It, therefore,

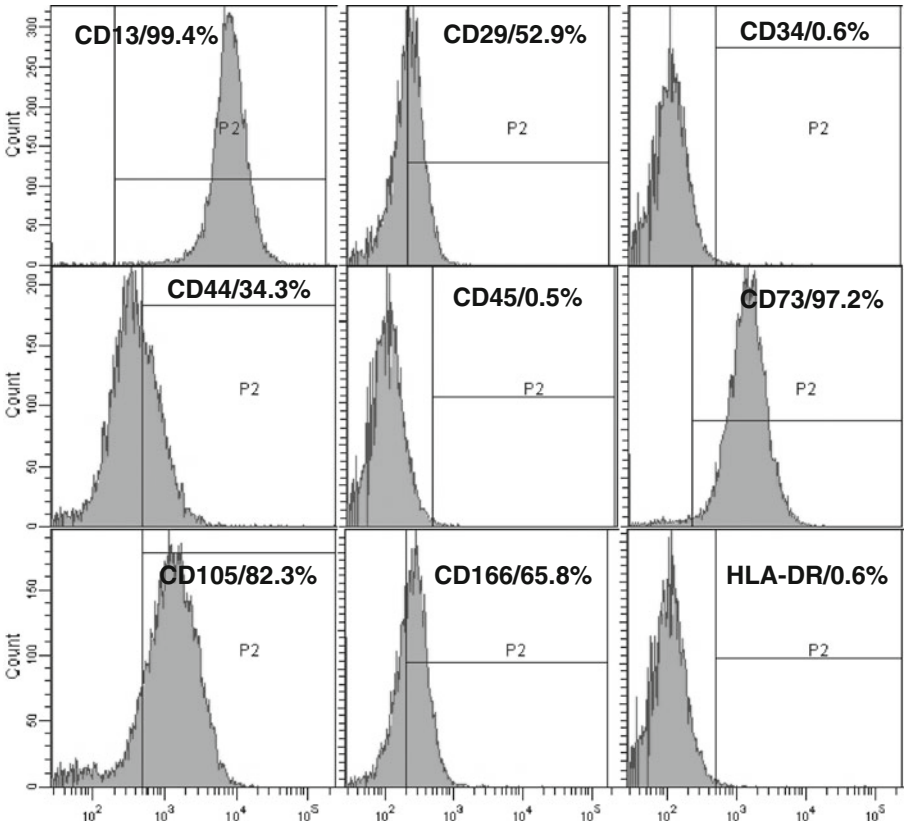

Fig. 3 Fluorescence-activated cell shorter analysis of CD13, CD29, CD34, CD44, CD45, CD73, CD105, CD166, and HLA-DR expression

demonstrated that the ADSCs were undifferentiated stem cells distinguished from hematopoietic cells.

Induced Differentiation Results of ADSCs

ADSC is a stem cell with multi-lineage differentiation potential, which can differentiate into osteoblasts, cartilage cells, adipose cells, nerve cells, and so on, under proper induced condition. In order to confirm the multi-lineage differentiation potential of co-cultured ADSCs, the harvested ADSCs were induced to differentiate into osteoblasts, chondrocytes, and adipocytes. Figure 4(OD) showed that osteogenic differentiation was confirmed by the increased alkaline phosphatase (ALP) expression by histochemical staining after 1 and 2 weeks of induction, respectively; the cytoplasm presented black. The ALP expression demonstrated that the osteoblasts induced from ADSCs achieved mature status, and it indicated the calcification. After 3 weeks of induction, ADSCs showed obvious calcium deposition and calcified nodules by von Kossa staining, which confirmed that the osteoblasts differentiated from ADSCs got preferably capability of osteogenesis. Figure 4(CD) showed that after 1, 2 and 3 weeks of chondrogenic induction, large amount of mucopolysaccharide-rich extracellular matrix appeared around ADSCs after toluidine blue staining, indicating the features of chondrocytes. This demonstrated that majority of

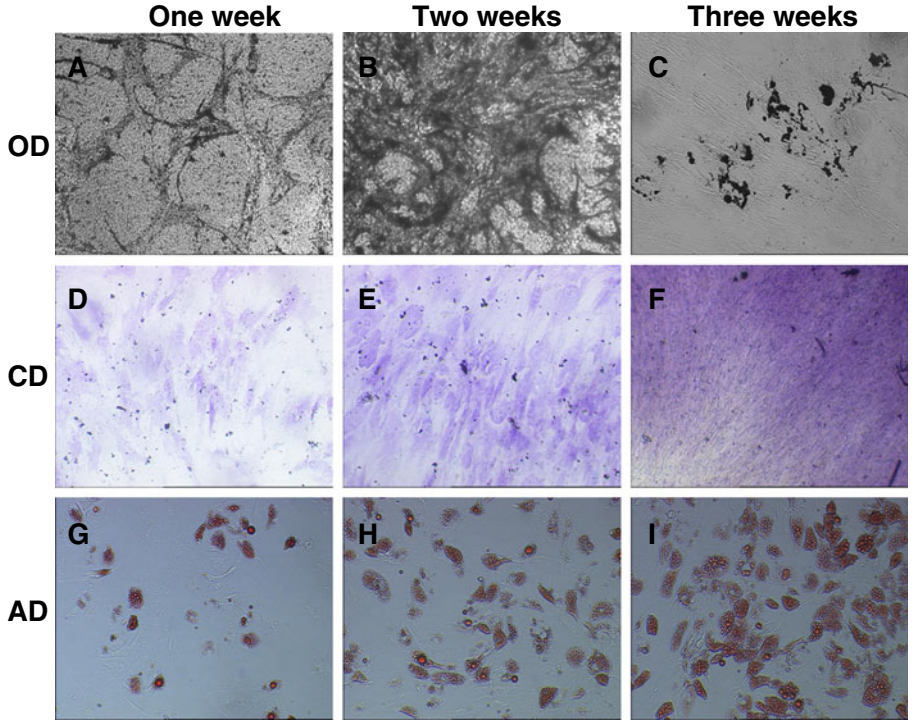

Fig. 4 Multi-differentiation potential assays of expanded human-derived ADSCs. *OD* osteogenic differentiation, *CD* chondrogenic differentiation, *AD* adipogenic differentiation. **a**, **b** ALP staining for osteogenic differentiation, **a** 1 week and **b** 2 weeks; **c** Von Kossa staining for osteogenic differentiation, 3 weeks; **d**–**f** Toluidine blue staining for chondrogenic differentiation, **d** 1 week, **e** 2 weeks, and **f** 3 weeks; **g**–**i** Oil red staining for adipogenic differentiation, **g** 1 week, **h** 2 weeks, and **i** 3 weeks; **a**–**i** ×100

ADSCs could be induced into chondrocytes after determined time of induction. Figure 4(AD) showed that after 1, 2 and 3 weeks of adipogenic induction, respectively, large quantity of red lipid droplets appeared inside the cells by oil red staining, which demonstrated that a large number of cells could be induced into adipocytes.

Taking the results from flow cytometry and multilineage differentiation assay into account, it demonstrated that ADSCs could not only support the in vitro expansion of UCB-MNCs but also remain its phenotype and stemness at a suitable co-culture cell distance within this novel co-culture system.

Discussion and Conclusion

Recent studies keep proving that HS/PCs plays a significant role [17, 18] in the clinical treatment of blood diseases (hemophthisis, etc.) in the hematopoietic reconstitution of patients with cancer (leukemia, etc.) after high-dose chemotherapy and radiation therapy, and in the gene treatment, immunotherapy, among which UCB-MNCs gradually become hotspots because of its advantages [19, 20]. However, the problem which hindered the wide application of UCB-MNCs is the limited resource of stem cells, which is far from meeting the clinical need. Thus, the in vitro expansion of UCB-MNCs has become a most effective method.

Although many studies [21, 22] try to add in various known growth factors to imitate the nourishing function of stromal cells, but people still do not understand the interaction mechanism between stromal cells and hematopoietic stem/progenitor cells, and many kinds of cell factors still have not been identified. However, as knowledge develops, the idea [23, 24] that simulating microenvironment in vivo could be the most suitable method has gradually been realized. In vivo microenvironment, stromal cells and hematopoietic cells coexist, and stromal cells play an important role in supporting the expansion of hematopoietic cells and regulating haematogenesis via secreting growth factors, extracellular matrix, and interacting with hematopoietic cells [25]. Thus, the co-culture of stromal cells and HS/PCs is obviously far more advantaged than that in the absence of stromal cells [6, 7].

Basing on realizing the co-culture of two kinds of cells, direct contact, indirect contact, and non-contact co-culture have also been investigated [9, 26–30]. Most studies adopt the methods in which stromal cells directly contact with hematopoietic cells, which can lead to a large scale of expansion of $CD34^+$ cells, but the direct contact co-culture is not beneficial to clinical application. As for the indirect contact and non-contact methods, which is better that has not been decided yet. For these methods, the most important difference of indirect contact and non-contact co-culture is that the different effective cell distance between stromal cells and MNCs, thus the influence of cell distance on cell expansion could be of much significance.

Conclusion

In summary, we adopted a modified transwell culture system to co-culture UCB-MNCs and human ADSCs under different cell distance for 7 days, and observed the in vitro expansion characteristics of UCB-MNCs. By contrast, under cell distance of 350 μm, the expansions of MNCs, $CD34^+$ cells, and CFU-GM colony were 15.1±0.2, 5.0±0.1, and 8.5±0.5-fold, respectively, which was obviously more superior to other cell distances. It was, therefore, concluded that the optimal cellular distance for co-culture of UCB-MNCs and human ADSCs was of around 350 μm.

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