Gene Expression Profile of Adherent Cells Derived From Human Peripheral Blood: Evidence of Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) provide a novel option in cellular therapy and tissue engineering. Recent studies indicated that it is possible to obtain MSCs from peripheral blood by attachment ability to plastic surface. To evaluate adherent cells derived from peripheral blood, their expression profile and surface markers were investigated. The results of RT-PCR indicated that these cells expressed MECP2, SLITRK2, SLITRK4 as well as the neuronal markers Nestin, β III tubulin, NFM, MAP2 and NSE. In the present study, a panel of surface markers were analysed by flow cytometry. These cells display a fibroblast like morphology as well as known sources of MSCs. The results obtained from immune-staining represented that these cells were negative for CD14, CD45, CD15 and CD24, whereas they were positive for CD29, CD44, CD105, CD73, CD166 and CD184. Our data indicated that the peripheral blood could be considered as a source of MSCs with high yield.

Keywords: Mesenchymal stem cells; Neuronal markers; Expression profile; Immune-phenotyping.

Introduction

Peripheral blood (PB) contains a heterogeneous population of mononuclear cells including mesenchymal stem cells (MSCs) and hematopoetic stem cells (HSCs). In some studies, the peripheral blood has been introduced as a known source of MSCs [1-2]. Although mesenchymal stem cells are a rare population in peripheral blood, they could readily be isolated from other mononuclear cell residents in the blood by attachment ability to plastic surface. MSCs were one of the best-known stem cells that were characterized by

phenotype, surface marker expression and differentiation potential [3].

Mesenchymal stem cells exhibited the fibroblast like morphology and expressed specific pattern of surface markers. The studies indicated that these cells could differentiate into different cell types including adipocytes, myocytes, astrocytes and neurons [4-8]. These cells provided the great promise for treatment of wide range of diseases. The application of these cells for treatment of stroke, heart failure, Duchenne muscular dystrophy and liver failure have been reported [9-12].

Identification of a practical, cheap and feasible method for isolating MSCs is required in clinical studies. With this aim in mind, it was tried to develop an applicable method for isolation of MSCs from peripheral blood. In this study, the peripheral blood mononuclear cells (PBMCs) were cultured and determined their molecular genetic make-up by expression analysis of CD markers specific for MSCs as well as neuronal markers in adherent cells derived from peripheral blood.

Materials and Methods

The isolation and culture of PB-MSCs

Briefly, 10 mL of peripheral blood was diluted two times with PBS and then layered 1:2 over lymphodex. Then, it was centrifuged at 450g for 30 min at 20°C. The buffy coat containing peripheral blood mononuclear cells (PBMC) were washed with DMEM/F12 medium and centrifuged at 450g for 10 min at 20°C. After that, cells were washed with medium before culture by centrifugation at 450g for 5 min at 20°C.

PBMSCs were plated into untreated tissue culture flask in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 2mM L-glutamate and 100 units/ml Penicillin/Streptomycin (Pen/Strep) and incubated in a humidified atmosphere at 37°C with 5% CO2 .After 48-72 h, the medium containing non-adherent cells were removed. Cells reached 70-80% confluence after 6 days of culture. The morphology of cells was observed on an inverted microscope.

Flow cytometry analysis

The adherent cells derived from peripheral blood were subjected to flow cytometry analysis. These cells were detached with trypsin by the 6th day of culture and then, were labeled with the following anti-human antibodies: anti-CD15-FITC (Fluorescein isothiocyanate), anti-CD166-PE (Phycoerythrin), anti-CD24-FITC, anti-CD29-PE, anti-CD184-PE, anti-CD105-PE, anti-CD14-PE, anti-CD45-FITC, anti-CD73-PE and anti-CD44-FITC.The IgG1-FITC and IgG1-PE were used to

determine the level of nonspecific binding (Negative Control). Flow cytometry analysis was performed on Partec CyFlow Space cytometer using FloMax software.

### *Reverse transcription polymerase chain reaction (RT-PCR) amplification*

Total RNA was extracted using Total RNA purification kit (Jena Bioscience, Germany) on the 14th dayof culture. Five micrograms of total RNA isolated from PB-MSCs was subjected to DNase I treatment (Fermentas, Thermo Scientific, Waltham, MA, USA) to remove any DNA contamination. The electrophoresis on a 1% agarose gel was used to confirm complete removal of DNA. Then, the first-strand cDNA synthesis was performed using random hexamer primers and dART reverse transcriptase (EURx Ltd, Gdansk, Poland). The RT-PCR was carried out in reactions of 25μl total volume containing 4μl cDNA product, 12.5 μl RealQ plus master mix Green (Ampliqon, Denmark) and 0.08μM of each primers. The RT-PCR reaction with no cDNA was used as the negative control. The PCR amplification conditions consisted of initial denaturation for 10 min at 95°C, 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C primer sequences were represented in Table 1. RT-PCR reaction with primers of HSP90AB1 wasconducted as positive control. PCR products were confirmed by electrophoresis on 3% agarose gel (staining with GelRed).

Results

After plating peripheral blood mononuclear cells (PBMCs), colonies of cells began to appear in the 12.5 cm^2 flasks after 72 hours of culture [Figure 1]. On days 5-6, the cells grew to 80%-90% confluence. These cells displayed a spindle-shaped morphology under inverted microscopy. Figure 1 represented the fibroblast-like appearance of adherent cells derived from peripheral blood on day 6.

When culture of these cells continued for 14 days, the morphology of MSCs derived from peripheral blood changed from spindle-shaped to flat appearence. The expression of specific neural genes including Nestin, β III tubulin, NFM, MAP2, NSE, SLITRK2, SLITRK4 and MECP2 were analyzed by RT-PCR on the 14th dayof culture. The results indicated that all genes under investigation were expressed in PB-MSCs [Figure 2 and Supplementary 1]. The size of RT-PCR products were presented in Table 1.

These cells were trypsinized on day 6 of culture and the expression of CD markers was evaluated by flow cytometry. These cells were CD166+, CD29+, CD184+,
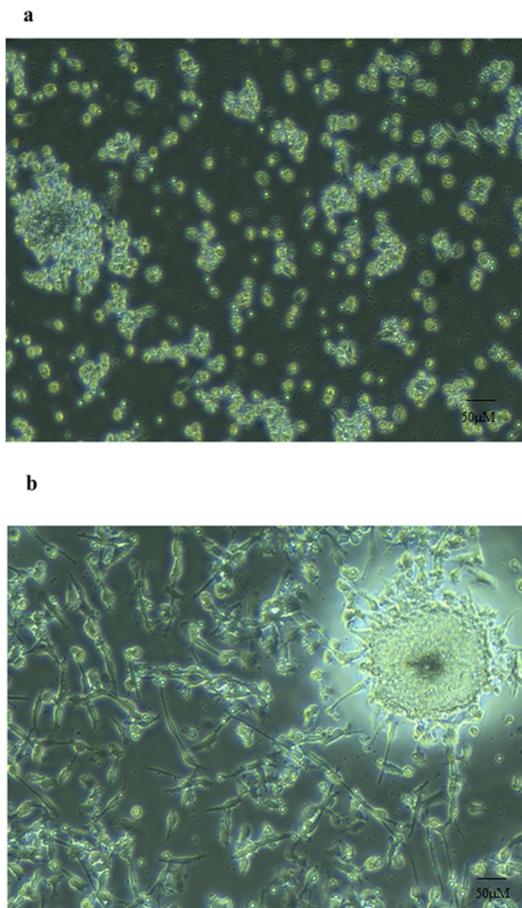
Figure 1. Graphs indicating the morphology of adherent cells derived from peripheral blood after (a) 3 days and (b) 6 days of culture.

CD105+, CD73+ and CD44+ [Figure 3, Table 2]. These cells were also negative for hematopoietic marker CD45 as well as monocyte marker CD14.

Discussion

More than 290 clinical trials using MSCs transplantation were reported in NIH database (http://clinicaltrials.gov/). The results obtained from different studies demonstrated that MSCs could be useful in treatment of autoimmune disorders [13-17]. Tse et al., have reported that cardiac function could improve in patients with myocardial infarction following transplantation of MSCs derived from bone marrow [18]. However, the regeneration of cartilage via MSC transplantation was not successful in patients with rheumatoid arthritis and osteoarthritis [19].

The original source of MSCs is usually bone marrow (BM). However, there are some limitations in their application in regenerative medicine. For example, the isolation of MSCs from BM is an invasive procedure. Therefore, the peripheral blood could be considered as a suitable alternative source of MSCs. Mesenchymal stem cells were characterized according to the surface marker pattern, morphology, ability to adhere to a plastic surface. The aim of this study was to identity the MSCs derived from peripheral blood. In other published protocols, the RBC lysis buffer was used to isolate PBMSCs [20-21]. In the present study, this buffer was applied after isolation of PBMCs by Ficoll to remove the remaining red blood cells. This process decreased the adherence ability of MSCs to plastic surface. Although, some other authors isolated the MSCs from
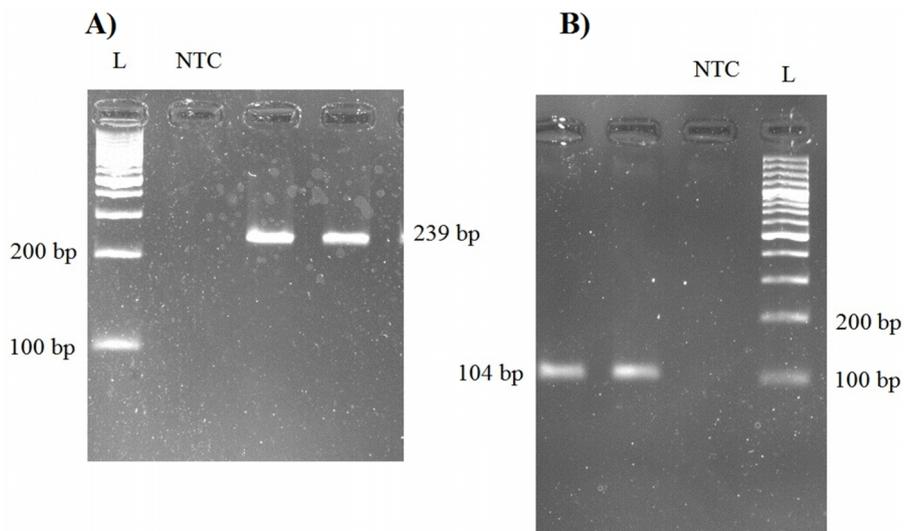
Figure 2. The results obtained from RT-PCR on RNA extracted from PB-MSCs. The NSE (a) and NFM (b) were expressed in PB-MSCs. The approximate size of the RT-PCR products was determined by a 100bp DNA Ladder. NTC= Non Template Control

Table 1. Primer sequences used in the RT-PCR amplification of adherent cells derived from peripheral blood.

Target	Forward Primer	Reverse Primer	Size (bp)
Nestin	5-ATCGCTCAGGTCCTGGAA-3	5-AAGCTGAGGGAAGTCTTGGA-3	146
NSE	5-GGAGAACAGTGAAGCCTTGG-3	5-GGTCAAATGGGTCCTCAATG-3	239
β-tubulin III	5-CTCAGGGGCCTTTGGACATC-3	5-CAGGCAGTCGCAGTTTTCAC-3	160
NFM	5-GTCAAGATGGCTCTGGATATAGAAATC-3	5-GTCAAGATGGCTCTGGATATAGAAAT-3	104
MAP2	5-CATGGGTCACAGGGCACCTATTC-3	5-GGTGGAGAAGGAGGCAGATTAGCTG-3	233
SLITRK2	5- TGCAGTCATTCAGGAAGGTG-3	5- GCTCTGCAGTCCATCAAACA-3	112
SLITRK4	5- TCAGCCCTGATTTCTTCGACA-3	5- CTCACAGTTGACATAGAGCACAT-3	105
MECP2	5- TGAGATGCCTGGTGAGCATTACAG-3	5- TCCACCTTCCATACCACTCCCA-3	101

peripheral blood by Ficoll, they needed almost 5-6 days to adhere MSCs derived from peripheral blood to plastic surface [22] In this study, the adherent cells were isolated from other cells of PBMCs after 2-3 days.

Furthermore, these cells had the ability to reach confluence 6 days after beginning of culture.

The cells used in this study were plastic-adherent. However, morphology is not sufficient to confirm that
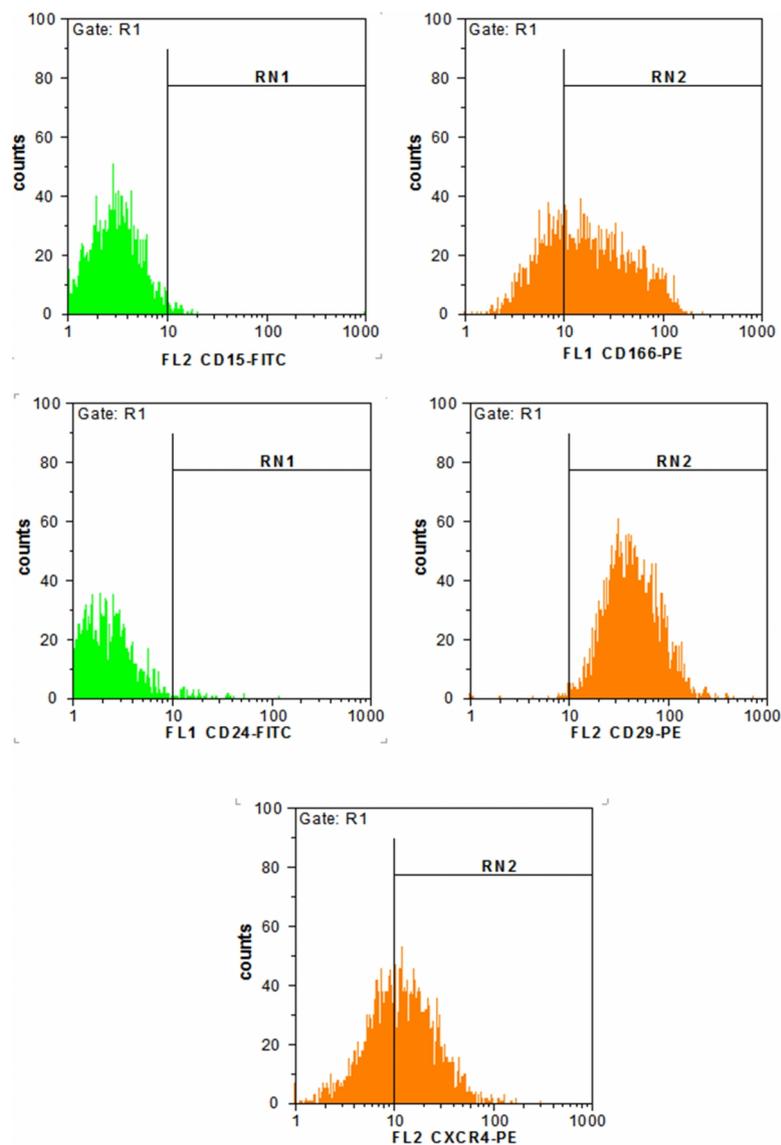
Figure 3. Immune phenotype of adherent cells derived from peripheral blood. The histograms indicated the negative expression of markers CD15 and CD24 as well as the positive expression of CD29, CD184 (CXCR4) and CD166.

Table 2. Summary of surface markers expression in adherent cells derived from peripheral blood

Surface marker	Percentage of positive cells
CD166	65.8%
CD15	1.9%
CD24	1.87%
CD29	99.17%
CD184	58.32%
CD105	74.99%
CD14	18.37%
CD45	2.43%
CD73	72.08%
CD44	97.43%

MSCs are found in mononuclear cells derived from peripheral blood. They should be characterized using specific CD markers. To demonstrate their identity as MSCs, expression of CD markers were determined by flow cytometry analysis. Our data revealed that human peripheral blood-derived MSCs (PB-MSCs) were highly positive for CD44 and CD29 expression (>90%). CD29 expression has been shown to involve in MSCs migration [23-24]. It has been also known that the expression of CD44 is important for proliferation and apoptosis of stem cells [25-26].

The adherent cells in the present study were also positive for CD166, CD184, CD105 and CD73. However, the percentage of positive cells for these markers was less than 80%. However, the previous studies indicated that MSCs freshly derived from adipose showed low levels of CD105 expression. The studies indicated that CD105 expression increased in culture of MSCs upon passaging [27-28]. Other studies have demonstrated that CD105 expression could play an important role in regenerative potential of MSCs [29].

CD166 showed to be expressed in adherent cells derived from peripheral blood (Table 2). CD166 involved in cell-to-cell interaction and has an important role in regulation of stem cell differentiation [30]. Although some studies showed the low level of CD166 expression on Wharton's jelly-MSCs (WJ-MSCs) [31], it was observed in this study that CD166 was expressed on more than 60% of PB-MSCs.

The expression of CD73 was observed on PB-MSCs as well as human bone marrow derived MSCs (BM-MSCs) in other studies [32-33]. However, it was revealed that CD73 was not expressed on rabbit MSCs [32]. More than 50% of PB-MSCs also showed a positive expression of CD184 (CXCR4). Different studies indicated that only a small proportion of MSCs derived from bone marrow showed the CD184 expression [34-38]. Furthermore, it has been demonstrated that CD184 expression could increase the therapeutic potential of MSCs following engraftment

[39-40].

The data obtained from flow cytometry analysis showed that PB-MSCs used in this study were CD45 negative. CD45 was known to be expressed on surface of leukocyte cells [41]. The lack of CD45 expression was previously observed on BM-MSCs [42-43]. However, it was demonstrated that the expression of CD45 can be inducted on MSCs under certain culture conditions [44].

In this study, less than 10% of the PB-MSCs stained positive for CD15, showing similar results with bone marrow derived MSCs [5, 7]. Flow cytometric analysis also revealed that PB-MSCs were negative for CD24. The previous studies showed a negative expression of CD24 in human nucleus pulposus derived mesenchymal stem cells (NP-MSCs) [45].

In the present study, low expression of CD14 was observed on PB-MSCs. The CD14 is considered as the lipopolysaccharide receptor (LPSR) [46]. Although the placenta-derived MSC (P-MSC) and bone marrow-derived MSCs (BM-MSCs) were negative for CD14, the CD14 expression was observed on adipose tissue-derived MSC (AT-MSC). The studies indicated that there is a CD14 cross-reactive epitope on MSCs [47-49].

Several other studies indicated that the MSCs had the ability to differentiate into neuronal cells [50-52]. In the present study, the researchers also investigated whether PB-MSCs can express the neuronal markers. The obtained results showed that the neuronal markers including Nestin, β III tubulin, NFM, MAP2 and NSE were expressed by PB-MSCs. This observation suggested that these cells are prone to neuronal differentiation. The previous studies indicated that the MSCs derived from bone marrow expressed Nestin [53]. It has been also demonstrated that the Nestin + MSCs had the tri-lineage differentiation potential [54]. In this study, the nestin expression was also observed in the PB-MSCs as well as bone marrow derived MSCs. As expected, the PB-MSCs were also positive for the early neuronal marker, β III tubulin, through RT-PCR analysis. Deng et al believed that the MSCs could spontaneously express the neuronal markers [55]. However, there is controversy about these observations. For example, Woodbury et al did not detect any expression of neuronal markers in the MSCs derived from bone marrow [56]. In contrast, some other studies have reported that the MSCs expressed the neuronal markers such as Nestin, GFAP, Vimentin, β-III tubulin and MAP2 [53, 57-58]. According to the data obtained from different studies, it has been suggested that spontaneously induction of neuronal marker expression may result from the isolation of MSCs from in vivo and

then their culture in vitro [59-60].

In the present study, the expression of MECP2 in the PB-MSCs was confirmed by RT-PCR. The study of MECP2 expression in MSCs derived from bone marrow has demonstrated that MECP2 implicated in proliferation senescence and neuronal differentiation of MSCs [61-62].

The expression of SLITRK gene family (SLITRK1-SLITRK6) has been observed in embryonic stem cells as well as in the central nervous system (CNS) [63-64]. The studies indicated that SLITRKs play different roles in regulating neuronal development [65-66]. The researchers found that the PB-MSCs were positive for the expression of the SLITK2 and SLITKR4 as well as other studied neuronal markers. The observation of SLITRK2 and SLITRK4 expression in PB-MSCs could provide more evidence from their differentiation ability into neuronal cells.

In summary, we report the profile of gene expression in MSCs from peripheral blood in culture. Although some studies reported that mesenchymal stem cells cannot be found in peripheral blood under normal physiological conditions [67], the obtained results of this study indicated that these cells can be successfully obtained from peripheral blood with high yield.

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