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CD271 Enrichment Does Not Help Isolating Mesenchymal Stromal Cells from G-CSF-Mobilized Peripheral Blood[1]

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Abstract—Reports on the isolation of mesenchymal stromal cells (MSCs) from granulocyte colony stimulating factor mobilized peripheral blood (G-CSF-mobilized PB) using regular culturing techniques are controversial. Enrichment techniques such as CD133 isolation have increased the success rates. CD271 is a well-known marker for enrichment of MSCs from bone marrow (BM). In the present study, we aimed to find out whether CD271 enrichment can help isolation of MSCs from G-CSF-mobiiized PB. Five G-CSF-mobilized PB samples were collected from the remnant parts of the bags used for BM transplantation. Five BM samples were used as the control. Mononuclear cells (MNCs) from both resources were collected and underwent magnetic sorting for CD271-positive cells. The isolated cells were cultured, undergoing flowcytometry and differentiation assays to determine if they fulfill MSCs characteristics. CD271-positive portion of G-CSF-mobilized PB did not yield any cell outgrowth but the BM counterpart could successfully form MSC colonies. Although the percentage of $CD271^+$ cells showed no difference between BM-MNCs and G-CSF-mobilized PB-MNCs, hematopoietic markers such as CD45, CD34 and CD133 composed a higher percentage of CD271-positive cells in the G-CSF-tnobiiized PB group. Results obtained indicated that CD271 enrichment does not help isolation of MSCs from G-CSF-mobilized PB. In this source, almost all of the $CD271^+$ cells are from hematopoietic origin and the frequency of MSCs is so low that possibly during the process of cell isolation most of them are lost and the isolation fails.

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Keyword: CD271, mesenchymal stromal cell, mesenchymal stem cell, granulocyte colony-stimulating factor, mobilized peripheral blood

INTRODUCTION

Multipotent mesenchymal stromal cell (MSCs), previously named mesenchymal stem cells, are multipotent cells with easy in vitro expansion characteristics and a capacity to differentiate into different cell types, like adipocytes, osteocytes, chondrocytes, hepatocytes and neural cells [1]. These cells can be obtained from different sources like adipose tissue [2], umbilical cord [3] and dental pulp [4], while they are mainly isolated from bone marrow (BM). The expansion ability and differentiation capacity of these cells were shown to be variable. There is also a few data regarding the isolation of MSCs from peripheral blood (PB-MSCs) but in trace amounts [5]. Our knowledge regarding the PB-MSCs is very limited. It is not obvious what their origin is and where they go. Furthermore, the very low amounts of MSCs in peripheral blood are a major obstacle [6].

Normally, there is just a trace of hematopoietic stem cells (HSCs) in the peripheral blood, and if HSCs could be demonstrated in high numbers within PB, this could be considered as an abnormality, though these cells can also migrate to PB by some interventions. One of the methods commonly used for the mobilization of HSCs from BM to peripheral blood is by use of granulocyte colony stimulating factor (G-CSF). It is not known whether administration of G-CSF can similarly lead to mobilization of MSCs or not. There are some studies that confirm migration of MSCs from BM to the peripheral circulation by administration of G-CSF [5], but there is a controversy in this issue. Villaron et al. [7] found that in the

Abbreviations: BM, bone marrow; G-CSF-mobilized PB, granulocyte colony-stimulating factor mobilized peripheral blood; MNC, mononuclear cell; MSC, mesenchymal stromal cell.

[1] The article is published in the original.

cases of BM transplantation receiving G-CSF transplants, post-transplantation MSCs in the patient's PB were from the recipient's source, while some other authors [8, 9] showed opposite results and claimed that some multipotent cells similar to BM-MSCs have been found post-transplantation in the PB which were from the donor. Lund et al. [5] showed that G-CSF-mobilized PB contains low amounts of fibroblast-like cells that could form colonies similar to BM-MSCs (termed "colony forming unit fibroblast, CFU-F"). However, in other studies the isolation of MSCs from G-CSF-mobilized PB barely succeeded. This poor yield may be a result of mismatches in methods used for MSC isolation, purification and culture [6]. Kassis et al. [10] observed that in the samples of mobilized PB of adult healthy human donors treated with G-CSF, spindle-like cells could be isolated by fibrin microbeads (FMB); these cells could further express MSC surface markers and also be differentiated to osteoblasts, chondrocytes and adipocytes while the number of plastic adherent cells was negligible in all the samples. Also in another study, other researchers used a positive selection method to isolate MSCs from G-CSF mobilized peripheral blood but this time with CD 133 [11].

Quirici and colleagues [12] showed that the antibody against the low-affinity nerve growth factor receptor (LNGFR also named as CD271 belongs to the low-affinity neurotrophin receptor and the tumor necrosis factor receptor superfamily) can stain BM mesenchymal stem cells. Isolated $CD271^+$ MSCs from BM had a higher proliferative capacity in comparison to MSCs isolated by plastic adherence. Now, CD27I is a well-known marker for the enrichment of MSCs from BM [13], In the present study, there was an attempt to isolate MSCs from G-CSF mobilized peripheral blood by selecting CD271 positive cells.

EXPERIMENTAL

Collecting G-CSF mobilized peripheral blood samples. The blood mobilization process consisted of 5 days of 10 mg/kg G-CSF-injections with Neupogen® (Amgen Inc., USA). Following the G-CSF treatment, the mononuclear cells (MNCs) were separated from the polymorphonuclear cells and collected using a cell separator (COBE Spectra Apheresis System operated with version 6.1 PBSC Software, Gambro BCT, Lakewood, USA).

The 5 samples for our experiments were obtained from minute residual waste materials remaining after such PB-based stem cell transplantation procedure. The use of this source of material was approved by Shiraz University of Medical Sciences' Ethics Committee due to the use of waste samples from human sources for research purposes, with no tracking of the donor's identity and no DNA examinations or manipulations.

Preparation of G-CSF-mobilized PB MNCs for isolation of MSCs. The G-CSF-mobilized PB samples were washed and centrifuged for 10 min at $300 \times g$. Cell debris was removed and 10^7 of the cells were resuspended within fresh medium and cultured directly or used for flow cytometry. Another 10^7 of the cells were subjected to CD271 isolation for culturingor flow cytometry analysis.

Collection of bone marrow units. Five samples with a volume of 2–3 mL of BM, anticoagulated with heparin, were obtained from patients undergoing diagnostic BM aspiration after taking informed written consent. Cultures from samples with normal results were included in this study.

Isolation of MNCs from BM. The BM was loaded on LymphoSep (1.077 g/mL, Biosera, Ringmer, UK) and centrifuged at $450 \times g$ for 25 min. Then MNCs were isolated from the buffy coat layer. Half of the isolated MNCs underwent CD271 isolation for culturing or flow cytometry, and the other half were cultured directly.

Magnetic cell sorting of MNCs based on CD271. The obtained MNCs from both resources primarily underwent dead cell elimination using Dead cell removal kit (Miltenyi Biotec GmbH, Germany) to decrease the chance of non-specific cell bindings of antibodies. Magnetic cell sorting was designed in order to separate the $CD271^+$ cells. MNCs were firstly labeled with the allophycocyanin (APC)–conjugated CD271 antibody (Miltenyi Biotec GmbH, Germany) by adding 10 μL of antibody to 100 μL of ceil suspension and incubating in a dark cold place for 10 min. Then, the target cells were labeled with anti-APC microbeads (Miltenyi Biotec) using 20 μL, of the antibody to 80 μL of cell suspension and incubated in a dark cold place for 30 min. The cell suspension was loaded onto a MACS column separator. The magnetically labeled $CD271^+$ cells were retained on the column while the unlabeled cells ran through. The isolated cells were used for the next steps.

Culturing of the isolated cells. MNCs, CD271 positive and negative cells from BM or G-CSF-mobilized-PB were plated in uncoated culture flasks in α-MEM supplemented with 10% FBS, 4 mM GlutaMAX, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco/Invitrogen, USA) and cultured for 72 h at 37°C in 5% CO_2 and 90% humidity. Unattached cells and debris were then removed and fresh medium was added to the adherent cells. Culture media was changed twice weekly until the flask became confluent or no cell growth was noted for 14 days. The cells were cultured to 80% confluency before being released with trypsin–EDTA (Gibco/Invitrogen) and subcultured. The cells were passaged three times and then underwent further experiments including differentiation assay and flow cytometry analysis.

Flow cytometry. To analyze the cell surface marker panel of MNCs from BM and G-CSF-mobilized PB, at least 10^6 cells underwent flow cytometry regarding

rare cell analysis standards. Dead ceils were excluded during analysis using propidium iodide (Miltenyi Biotec). The cells were incubated 10–30 min in a dark environment with the following antibodies against human cell markers: CD90–APC, CD271–phycoerythrin (PE), CD271–fluorescein isothiocyanate (FITC), CD271–APC, MSCA1 (mesenchymal stem cell antigen 1)–APC, CD34–FITC, CD133–PE, CD56–PE, CD45–peridinin chlorophyll protein (PerCP) (all from Miltenyi Biotec) and CD146-FITC (BD Biosciences, USA).

To analyze the cell surface marker panel of the adherent cells within cultures, the cells from the 3rd passage were harvested by 0.25% trypsin–EDTA. Trypsin was neutralized by FBS-containing media, and the isolated cells were washed twice with PBS. Then the cells were incubated 10–30 min in a dark with the following anti-human antibodies: CD90–FITC, CD105–PE (AbD Serotec, UK), CD14–PE, CD34–FITC, CD166–PE, CD45–FITC, CD44–PE, CD73–PE (BD Biosciences).

Mouse IgG1–PE, IgG1–APC, IgG2a–FlTC (AbD Serotec), IgG2a–PerCP, IgG2b–FITC and IgG2a–PE (Miltenyi Biotec) were used as the isotype controls. Cells were analyzed on a BD FACS-Calibur instrument. Data were analyzed using WinMDI Software.

Osteogenic differentiation. For osteogenic differentiation, MSCs from the 3rd passage were harvested by trypsin–EDTA 0.25%. The ceils were cultured in NH-osteoDiff Medium (Miltenyi Biotec) at a density of 3×10^4 cells/mL in 2-chamber culture slides (BD Biosciences) for 3 weeks according to the manufacturer's guides and medium exchange was performed twice a week. To confirm differentiation, after the appropriate morphological changes the cells were analyzed by alizarin red staining. Briefly, to perform the staining, the cells were washed once with PBS, fixed in methanol for 10 min and stained with the solution of 0.1 M alizarin red (Sigma, USA) in 25% ammonia water for 24 h, after that the cells were washed once with distilled water.

Adipogenic differentiation. For adipogenic differentiation, the cells from the 3rd passage were harvested as mentioned above and cultured in MesenCult medium supplemented with 10% adipogenic stimulatory supplements (both from Stem Cell Technologies, Canada) at a density of 1.5×10^4 cells per milliliter in 2-chamber culture slides according to the manufacturer's guides. The cells were cultured for 3 weeks, and half of the medium was exchanged only when the color of the media changed to yellow. As the cells showed appropriate morphological changes, they were analyzed with Oil-red O staining. Briefly, to perform the staining, the cells were fixed in 4% formalin containing 1% of calcium chloride for an hour. Afterwards, the cells were stained with Oil-red O solution for 10–15 min, and then counterstained with 70% ethanol for a minute and washed with distilled water.

Statistical analysis. SPSS Software version 19.0 for windows (IBM, USA) was used for statistical analysis. Data were analyzed using Kruskall–Wallis and Fisher's exact test. p value < 0.05 was considered statistically significant.

RESULTS

Culture Characteristics

The primary seeded G-CSF-mobilized PB MNCs showed non-uniform rounded appearance with variable sizes (Fig. 1a) but as time passed many cells died, and, interestingly, a few cells were noticed with oval/round or elongated appearance and visible borders, some showing cytoplasmic extensions and occasionally multiple nuclei (Fig. 1b). These cells are similar to osteoclast-like cells contaminating MSC cultures from the umbilical cord blood [14]. In contrast, BM-MNCs cultured cells formed typical fusiform fibroblast-like cells in all 5 samples, apart from a few rounded cells (Fig. 1c). These fibroblast-like cells formed homogenous colonies that became confluent within 15–21 days (Fig. 1d).

All CD271 negative cell cultures from both G-CSF-mobilized PB and BM led to no cell outgrowth after 1 month (Figs. 1e–1h). G-CSF-mobilized PB $CD271^+$ isolated cells after the primary seeding showed non-uniform rounded appearance with variable sizes (Fig. 1i). After 4 weeks, most of the cells died in all samples and no colony formation was noticed (Fig. 1j) BM derived CD271 positive cells formed adherent spindle-shaped 2 days after primary cultivation (Fig. 1k). The cells expanded rapidly during 5–6 days, showing homogenous colonies that confluented the flask within 15 days (Fig. 1l).

Generally, no MSCs were grown from cultures of all 5 samples in the three mentioned G-CSF-mobilized PB categories. In contrast, all 5 samples of both BM derived CD271 positive cells and BM-MNCs formed typical MSC cultures ($p = 0.014$). BM derived CD271 negative could not form any CFU-F (Table 1).

Differentiation Assay

Adipogenic differentiation from cultures showing CFU-F cells was documented by both morphological and staining criteria. One week after the seeding in the adipogenic medium, the cells showed small isolated vacuoles that increased in number and size with time and all were stained by Oil-red O staining.

The earliest evidence of MSC differentiation to osteoblasts was matrix deposition around the cells in the second week after seeding. Full differentiation to osteoblasts took 3 weeks. Mineralization was documented by alizarin red staining.

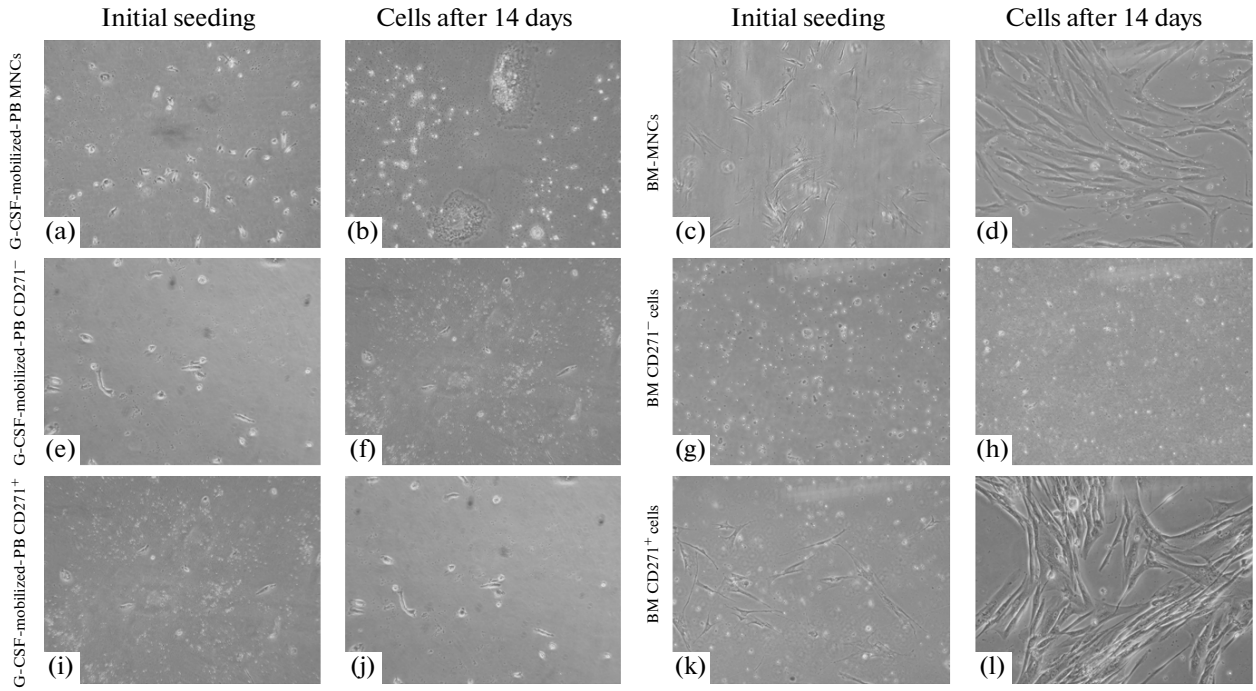

Fig. 1. Characteristics of the cells cultured. (a) The primary seeded G-CSF-mobilized PB MNCs had non-uniform rounded appearance with variable sizes; (b) after one month many cells died but interestingly a few cells were noticed with oval/round or elongated appearance and visible borders, some with cytoplasmic extensions and occasionally multiple nuclei; (c) BM cultured MNCs formed typical fusiform fibroblast-like cells; (d) the cells formed homogenous colonies that became confluent within 15–21 days. (e) $CD271^-$ cell cultures from both G-CSF-mobilized PB; (f) no cell outgrowth after 1 month was noticed in this group. (g) In the cultures from BM derived $CD271^-$ cells no fibroblast-like cell was present after the initial seeding; (h) and not even after one month; (i) After the primary seeding G-CSF-mobilized PB $CD271^+$ isolated cells had round appearance but differed in forms and sizes; (j) after 4 weeks most of the cells died; (k) BM-derived $CD271^+$ cells 2 days after primary cultivation formed adherent spindle-shaped cells; (l) the cells expanded rapidly in 5–6 days and formed homogenous colonies confluenting the flask within 15 days.

Flow Cytometry Analysis

Data from flow cytometric analysis of MNCs from both G-CSF-mobilized PB and BM before and after CD271 isolation are displayed in table 2. Flow cytometry analysis of the fibroblast-like cells harvested from BM-derived cultures showed that the cells were positive for mesenchymal markers such as CD73 ($98.68 \pm 1.24\%$), CD105 ($97.66 \pm 1.93\%$), CD44 (100%), CD166 ($99.88 \pm 0.1\%$), CD90 ($89.28 \pm 10.31\%$) and negative for hematopoietic antigens such as CD45 ($1 \pm 0.87\%$), CD34 ($0.91 \pm 0.62\%$) and CD14 ($0.47 \pm 0.21\%$, Fig. 2).

DISCUSSION

In this study, magnetic cell sorting and culture techniques were used to isolate mesenchymal stem cells from G-CSF-mobilized PB and characterize them using flow cytometry and differentiation assays, taking the bone marrow MSCs as the controls.

The presence of MSCs in BM and other sources like the placenta and umbilical cord blood is confirmed [14, 15], but highly successful isolation of MSCs from PB is still a controversy [6]. Despite reports of successful isolation of MSCs from PB [16], in two studies even one colony outgrowth of MSCs could not be reached from healthy donors [17, 6] and

Table 1. Isolation of mesenchymal stromal cells from different origins

Origin	Successful/total isolation number, %
G-CSF-mobilized PB MNCs	0/5 (0%)
BM MNCs	5/5 (100%)
G-CSF-mobilized PB $CD271^+$ cells	0/5 (0%)
BM $CD271^+$ cells	5/5 (100%)
G-CSF-mobilized PB $CD271^-$ cells	0/5 (0%)
BM $CD271^-$ cells	0/5 (0%)

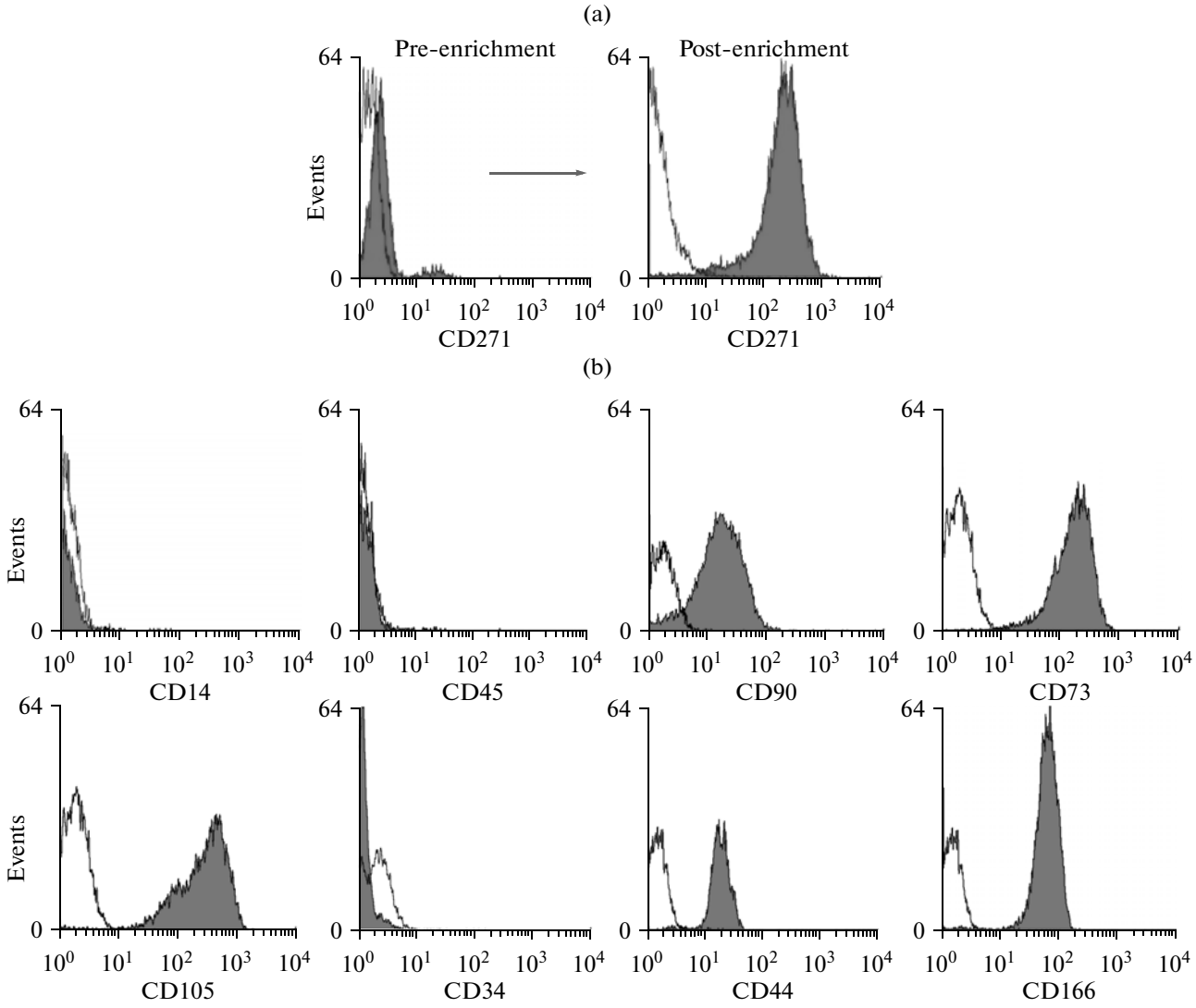

Fig. 2. Flow cytometry analysis of successfully isolated BM MSCs. (a) A content of $CD271^+$ cells in BM MNCs before and after enrichment; (b) Antigen pattern: of bone marrow-derived fibroblast-like colony-forming cells.

in another one only two out of ten samples led to successful isolation of MSCs [18]. In general, the yield of PB-MSCs is usually poor, and it is quite common to fail [19, 20]. Also maintenance of PB-MSCs seems to be difficult [6].

As some evidence suggesting mobilization of MSCs from bone marrow after treatment with G-CSF was found [7], G-CSF-mobilized PB was introduced as a new possible source for MSCs. Further studies showed the presence of fibroblastic-shaped cells in G-CSF-mobilized PB samples with the same surface markers as MSCs. Lund and colleagues separated cells positive for several markers like CD13, CD29, CD105 and CD166 and negative for CD14, CD34, CD45 and CD133. However, they found these cells at a very low incidence (0.0002%). Moreover, the mobilized peripheral blood MSCs showed a limited expansion potential and became senescent 20–25 days after isolation [5]. Fernández et al. [17] claimed isolation of MSCs from G-CSF-mobilized PB of breast cancer patients in 11 out of 14 patients; however, the isolation from normal donors totally failed. It was reported that some modifications in the enrichment techniques, such as consecutive CD6 depletion [21], using the fibrin microbead-based method [10] or CD133 selection [11], may give rise to isolation of MSCs from G-CSF-mobilized PB from healthy donors. These data suggest that immunoselection may enrich G-CSF-mobilized PB-MSCs [6]. One possible candidate for this purpose is CD271 which was shown to be a useful marker for isolation of MSCs from BM [11]. In our study, CD271 positive cells isolated from all BM samples could successfully form CFU-Fs with appropriate cell surface marker panel and differentiation potential, all fulfilling the criteria of The International society of cell therapy for MSC definition [22], Nevertheless,

Table 2. Expression of various cell surface markers on target populations*

	CD271	CD45	CD56	MSCA1	CD146	CD90	CD34	CD133
BM-MNCs	1.59 ± 0.46	79.82 ± 5.72	3.17 ± 0.58	0.22 ± 0.1	2.56 ± 0.45	0.29 ± 0.22	1.86 ± 0.2	1.46 ± 0.2
BM-CD271$^+$ cells	89.07 ± 1.2	53.81 ± 17.64	49.17 ± 2.7	1.65 ± 0.3	34.62 ± 0.5	10.51 ± 2.1	21.03 ± 1.3	15.08 ± 0.3
G-CSF-mobilized PB MNCs	1.5 ± 0.5	94.64 ± 1.02	12.49 ± 1.76	2.54 ± 0.4	1.12 ± 0.34	1.73 ± 0.2	5.16 ± 0.6	4.89 ± 0.2
G-CSF-mobilized CD271$^+$ cells cells	88.26 ± 1.25	97.11 ± 0.4	94.79 ± 4.56	2.37 ± 0.2	44.23 ± 2.3	39.98 ± 1.5	69.3 ± 2.5	60.85 ± 2.1

* Data of flow cytometry analysis are shown as the percentage of analyzed cells.

colony outgrowth was seen neither in the CD271-enriched portion nor in the non-enriched part of G-CSF-mobilized PB ($p = 0.014$). The results obtained are in agreement with previous data indicating that CD271 is a useful marker for isolation of MSCs from BM [12, 13]. However, it should be indicated that this fact cannot be extended to G-CSF-mobilized PB. The profile of CD271$^+$ cells among MNCs from BM and G-CSF-mobilized PB differs. Although the frequency of CD271$^+$ cells among MNCs of BM and G-CSF-mobilized PB did not differ ($p = 0.21$), the percentage of hematopoietic markers such as CD45, CD34 and CD133was higher in the G-CSF-mobilized PB. This shows that most of CD271$^+$ cells in the G-CSF-mobilized PB MNCs are from hematopoietic origin rather than MSCs.

Virtual absence of CD271-positive MSCs (and apparently any MSCs) in the peripheral blood denotes that mobilization of MSCs from BM is not critically influenced by stimulation of G-CSF and is mainly controlled by other mechanisms. Mobilization of MSCs is a key step in the process of MSC related tissue repair [23] that involves mobilization of MSCs from their normal places of residence, migration towards the pathological tissues and homing there. Factors released by tissues after damage or apoptosis occurs can recruit stem cells to the damaged site, leading to their proliferation, differentiation and eventually replacement of the damaged tissues [24, 25]. Evidence confirms that interactions of stromal cell-derived factor-lα (SDF-1α) and C-X-C chemokine receptor type 4 (CXCR4) mediate the trafficking of MSCs. Furthermore, inflammatory cytokines, transforming growth factor (TGF)-β1, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α can upregulate the production of matrix metalloproteinases (MMPs) in MSCs resulting in their migration through the extracellular matrix [26]. These data can explain the results obtained by Fernandez et al. [17]—they had isolated MSCs from G-CSF-mobilized PB of breast cancer patients, in 11 out of 14 cases, whereas the isolation from normal donors totally failed. It seems that mobilization of MSCs was not necessary induced by G-CSF. More probably, it happens due to the other chemokines which are specific for the pathological status of breast cancer patients but are absent in normal. It can be concluded that there is no convincing evidence regarding the association between PB MSCs and the leukopheresis technique or solid tumors [6], as there were conflicting reports on success [5, 17] and failure [19, 20, 27] in detecting MSCs in PB under these conditions. Furthermore, the results of our study clarify that CD271 enrichment cannot enhance the success rate of isolation of MSCs from G-CSF mobilized PB. In this source, almost all of the CD271$^+$ cells are of hematopoietic origin and the frequency of MSCs is so low that during the processes of cell isolation most of them are lost and the isolation fails.

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