Original Article

Prospective isolation and identification of human mesenchymal stem cells by flow cytometry

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Adult bone marrow contains endothelial cells, adipocytes, stromal cells, and blood cells, such as erythrocytes, lymphocytes, platelets. Stromal cells are a population of multipotent cells referred to as mesenchymal stem cells (MSCs), which not only support hematopoiesis, but also differentiate into multiple lineages, including fat, bone and cartilage. Because of this multipotency, MSCs are an attractive candidate for clinical application to promote repair or regeneration of damaged tissues of mesenchymal origin. However, the characteristics of bone marrow MSCs are still unclear, because of the lack of suitable markers for their prospective isolation. Here, we report the potential usefulness of CD271 (low-affinity nerve growth factor receptor: LNGFR) and CD133 (prominin1: AC133), as markers of the MSC population, for the prospective isolation of highly purified MSCs. This isolation method may provide hope for improving the starting population of stem cells for transplantation in diseases like spinal cord injury, cartilage repair and myocardial infarction.

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Introduction

In adults, one of the important sources of stem cells is the bone marrow (BM) compartment, which contains both hematopoietic stem cells (HSCs) and a nonhematopoietic stem cell population. HSCs are already in clinical use for cell therapy. The nonhematopoietic stem cell population, identified as mesenchymal stem cells (MSCs), is believed to not only support hematopoiesis, but also differentiate along various mesodermal lineages to generate osteocytes, chondrocytes and adipocytes.

MSCs were first identified more than 30 years ago by Friedenstein, who isolated these cells by simply exploiting their ability to adhere to plastic tissue culture dishes[1)]. MSCs referred to by different terminology as colony forming unit fibroblast (CFU-F)[2)], marrow stromal fibroblasts (MSF)[3)] and mesenchymal progenitor cells (MPC)[4)]. There are two major properties of MSC. One of the properties is that MSCs are long-term self-

renewal, which is an ability to form CFU-F and to sustain proliferation *in vitro*. Another is that they have multi-lineage differentiation potency that is a potential to give rise to multiple mesenchymal cell lineages. Despite the large number of studies that have focused on the biology of MSCs in the past decade[5-7], the method for their isolation has changed little from that used for their initial identification. In the case for cultured MSCs, very little information exists about the features of the tissue precursor cells that can give rise to the plastic adherent cells. HSCs, which are well-characterized counterparts of MSC, have developed effective methods for their prospective isolation[8,9]. On the other hand, there are still many unknowns about MSCs, because no suitable markers have been identified yet for the purification of the MSCs. Without prospective isolation and purification, it is very difficult to ascribe any attributes with certainty to the MSCs themselves. Other sources of MSCs have also been identified, such as fat tissue[10], cord blood[11] and peripheral blood[12], although these remain controversial.

To date, only a few markers have been developed and demonstrated to be suitable for the isolation of MSCs from primary tissues. Markers that meet the established criteria for their positive selection include STRO-1[13], CD73 (ecto-5'-nucleotidase: SH3, SH4)[14], CD105 (endoglin: SH2)[15], and CD271[16], whereas CD45 and CD235a (glycophorin A: GPA) are used for the negative selection of these cells. Here, we demonstrate that cell populations that are CD271+ and CD133+ represent an adult MSC population, and suggest that positive selection by using these markers may be a suitable method for prospective isolation of MSCs from whole human BM aspirates.

Materials and Methods

1)Preparation of Human BM Cells

The human trabecular bones were dissected out, and single cell suspensions were prepared as follows. The human trabecular bones were crushed with a pestle, and the marrow cells were suspended in HBSS+ (calcium- and magnesium- free Hank's Balanced Salt Solution supplemented with 2% FBS, 10 mM HEPES, and 1% Penicillin/Streptomycin (P/S)) (Gibco). The bone fragments were collected and incubated for 1 h at 37°C in 0.2% collagenase (Wako) / Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10 mM HEPES and 1% P/S. The suspension was filtered through a cell strainer (Falcon), and collected by centrifugation at 1200 rpm for 7 min at 4°C. The pellet was soused for 5-10 sec in 1 ml water (Sigma) to cause the red blood cells to burst, after which 1 ml of 2 x PBS (Wako) containing 4% FBS was added to it. The cells were resuspended in HBSS+ and the suspension was filtered through a cell strainer to remove debris. All experimental procedures were performed with the approval of the KEIO UNIVERSITY SCHOOL OF MEDICINE AN ETHICAL COMMITTEE (No, 18-26).

2)Antibody Staining and Flow Cytometry

The human BM cells were resuspended in ice cold HBSS+ at 2.5-5 x 10^7 cells/ml, then stained for 30 min on ice with the following mAb: FITC-conjugated CD45 (Dako), and FITC-conjugated GPA (Dako), PE-conjugated CD133 (Miltenyi Biotec), and APC-conjugated CD271 (Miltenyi Biotec). After antibody staining, the cells were washed with an excess amount of HBSS+ and resuspended at 1 x 10^7 cells/ml in HBSS+ containing 2μg/ml propidium iodide (Sigma Chemical Co.). Cell sorting and analysis was conducted using Moflo (Beckman Coulter) and a FACSCalibur (Becton Dickinson) instrument and the data were analyzed using Flowjo software (TOMY DIGITAL BIOLOGY CO., LTD.).

3)Culture of MSCs

Cells were allowed to adhere overnight to the culture dishes, and the nonadherent cells were washed out by changing the medium. Thereafter, medium change was carried out twice weekly. The expansion medium consisted of DMEM and 20% FBS (Hyclone) supplemented with 20 ng/mL bFGF (peprotech) and 1% P/S.

4)Colony Forming Unit Fibroblast (CFU-F) Assay

The CFU-F assay was performed by plating 5 x 10^3 FACS-selected cells into a 35-mm dish in the presence of 20% FBS / DMEM containing 20ng/ml bFGF, 10 mM HEPES and 1% P/S. After 14 days of culture, the adherent cells were washed twice with HBSS+. Adherent colonies containing more than 50 cells were counted as a colony.

5)*In vitro* Differentiation

Osteogenic differentiation:

To induce osteocyte differentiation, the adherent cells were cultured in an 8-well chamber (Iwaki) containing Osteogenic Induction Medium (LONZA), with medium change every 3 to 4 days. After 14 days, we confirmed the differentiation of the cells into osteocytes by alkaline phosphatase enzymatic staining.

Adipogenic differentiation:

To induce adipocyte differentiation, the adherent cells were cultured in an 8-well chamber containing Adipogenic Induction Medium (LONZA), with medium change every 3 to 4 days. After 14 days, we confirmed the differentiation of the cells into lipid-laden adipocytes by Oil red O staining.

Chondrogenic differentiation:

To induce chondrocyte differentiation, the adherent cells were

cultured in a polystyrene tube containing Chondrogenic Induction Medium (LONZA), in the presence of 10 ng/ml TGF-β3, with medium change every 3 to 4 days. After 21 days, we confirmed the differentiation of the cells into chondrocytes by toluidine blue staining.

Neurogenic differentiation:

Fifth- to seventh- passage cells, seeded at a density of 1000 cells/cm^2, were treated with Sphere medium for 14 days. The Sphere medium consists of DMEM/F12 (1:1) (Gibco) supplemented with 20 ng/ml bFGF, 20 ng/ml EGF, 5 μg/ml Heparin, 10ng/ml LIF (Chemicon), and 20ng/ml B27 (Gibco). The spheres were plated on to 8-well chamber slides coated with poly-L-ornithine / fibronectin and cultured for 10 days in the following differentiation medium: DMEM/F12 supplemented with 1% FBS and B27, not containing any growth factors. For immunocytochemistry, the cells were fixed in 4% PFA and stained with the following primary antibodies: anti-βIII tubulin (mouse IgG2b, Sigma)[17], anti-GFAP (Glial Fibrillary Acidic Protein) (rabbit IgG, Dako) and anti-αSMA (Smooth Muscle Actin) (mouse IgG2a, Sigma). The following were used as the secondary antibodies: anti-mouse IgG2b (Alexa Fluor 488, Invitrogen), anti-rabbit IgG (Alexa Fluor 568, Invitrogen) and anti-mouse IgG2a (Alexa Fluor 350, Invitrogen).

Results

1)Clonogenic cells reside in the Lineage- (Lin-) GPA- CD271+ CD133+ population

Human cells derived from trabecular bones have been used for many years in the study of bone cell biology[18]. Recently, several studies reported[19,20] that human trabecular bone is a good source of MSCs with the characteristics of self-renewal and multilineage differentiation potential. To isolate MSCs from trabecular bones, we digested human trabecular bone with collagenase to release cells. Then, collagenase-released cells were stained with Lineage markers (CD3, CD14, CD16, CD19, CD20, CD56), GPA, CD271, and CD133 antibodies and analyzed by flow cytometry. The hematopoietic cells were removed by Lineage markers and GPA negative gate. To analyze the clonogenic potential of the rare Lin- GPA- CD271+ CD133+ (R2) and other populations (R1, R3 and R4), the CFU-F capacity of the sorted cells from each region was determined: Lin- GPA- CD271+ CD133- cells (R1): 1.90 $\pm$ 0.1% (n=3), Lin- GPA- CD271+ CD133+ (R2): 0.75 $\pm$ 0.13% (n=3), Lin- GPA- CD271- CD133- (R3): 77.2 $\pm$ 0.28% (n=3) and Lin- GPA- CD271- CD133+ cells (R4): 20.0 $\pm$ 0.3% (n=3) (Fig.1A). As shown in Figure 1B, the R1-4 fractions were isolated by FACS and each population assayed for its CFU-F content. Selection of the R2 fraction resulted in a 720-fold enrichment of CFU-F (17.4 $\pm$ 3.6 colonies per 5,000 sorted cells: n=4) relative to their incidence in the unfractionated cells (1 colony per 2 x 10^5 cells). A few CFU-F (2.95 $\pm$ 1.2 colonies per 5,000 sorted cells: n=4) were also found in the R1 fraction. The majority of CFU-F was recovered in the minor R2 subpopulation and correspondingly depleted or absent from the R1, R3 and R4 fractions.

2)Proliferation and differentiation of Lin- GPA- CD271+ CD133+ cells in single colony culture

The cells derived from Lin- GPA- CD271+ CD133+ population indicated spindle-shape morphology (Fig.2B). Growth kinetic analyses revealed that the cells in single colony could be grown for more than 7 weeks (Fig.2A), and that their doubling time was 50.6 hours (Clone A: 44.5h, Clone B: 51.5h, Clone C: 55.9h). We examined surface epitopes of Clone A at fifth-passage. Clone A was positive for CD73, CD105, and CD133; showed negative levels of CD45, GPA, and CD271 (Data not shown). To investigate the differentiation potential of Lin- GPA- CD271+ CD133+ derived cells, Clone A was cultured under conditions appropriate for inducing differentiation for each lineage. Clone A formed clusters of Oil red O positive lipid-laden adipocytes when exposed to adipogenic media (Fig.2C). Similarly, Clone A was assayed for their chondrogenic potential using the well established aggregate culture system in the presence of TGFβ3. In the chondrogenic media, the cells differentiated into chondrocytes, as shown by toluidine blue staining (Fig.2D). When induced to differentiate under osteogenic conditions, the spindle shape of Clone A was flattened and broadened with increasing time of induction. Differentiation into osteocytes was confirmed by alkaline phosphatase staining (Fig.2E).

3)Differentiation potential of Lin- GPA- CD271+ CD133+ cells into neuron-like and smooth muscle-like cells

Several *in vitro* studies have shown that stem cells could be isolated from BM and that these stem cells were capable of transdifferentiation towards neural-like cells[21]. Also, the formation of spheres from neural crest derived tissue has been reported[22]. To evaluate the differentiation potential of Lin- GPA- CD271+ CD133+ cells, Clone A were collected and cultured for 14 days in Sphere medium containing bFGF, EGF, Heparin, LIF, and B27 (Fig.3A). To assay differentiation, spheres were plated on poly-L-ornithine / fibronectin-coated chamber slides and allowed to differentiate without growth factors for 10 days. The differentiated cells were identified by triple immunostaining: neurons with βIII tubulin, glial cells with GFAP, and myofibroblasts with αSMA. Lin- GPA- CD271+ CD133+ spheres demonstrated

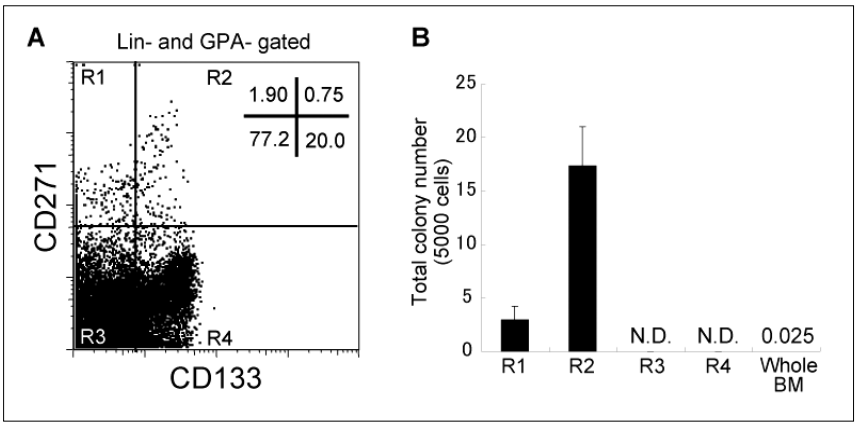

Fig1 CD271+ CD133+ population can be used to prospectively isolate human MSCs

(A) Representative flow cytometric profile of BM cells stained with CD133 and CD271, negatively gated with Lineage markers and GPA. Lineage markers= CD3, CD14, CD16, CD19, CD20, CD56. (B) Colony-forming efficiency is shown. Number of CFU-F at 14 days after plating 5000 cells. Whole BM= unfractionated cells, N.D.= Not Detected

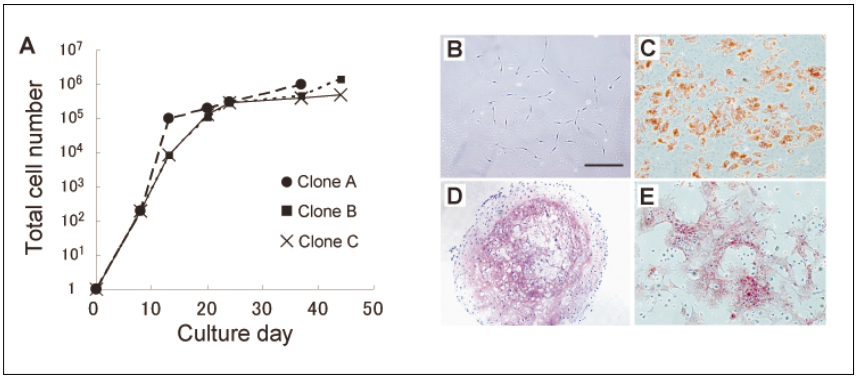

Fig2 Proliferative and differentiation potential of CD271+ CD133+ cells

(A) Growth curve of single colony derived from Lin- GPA- CD271+ CD133+ cells. (B) Clonal cells have spindle-shape morphology. (C) Multi-lineage differentiation of Clone A at fifth-passage for adipocytes demonstrated by Oil red O staining, (D) for chondrocytes shown by toluidine blue staining, and (E) for osteocytes demonstrated by alkaline phosphatase staining. Scale bars= 250 μm

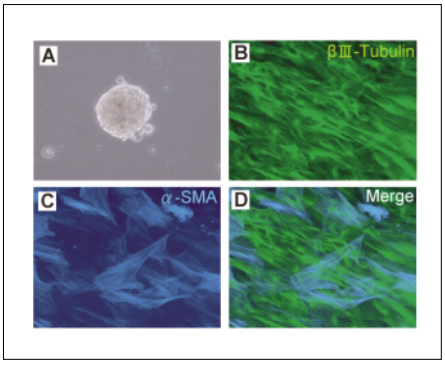

Fig3 Differentiation into neuron-like and smooth muscle-like cells

(A) Spheres formed from Lin- GPA- CD271+ CD133+ cells differentiated into (B) βIII tubulin+ neurons, and (C) αSMA+ myofibroblasts. (D) Merged image.

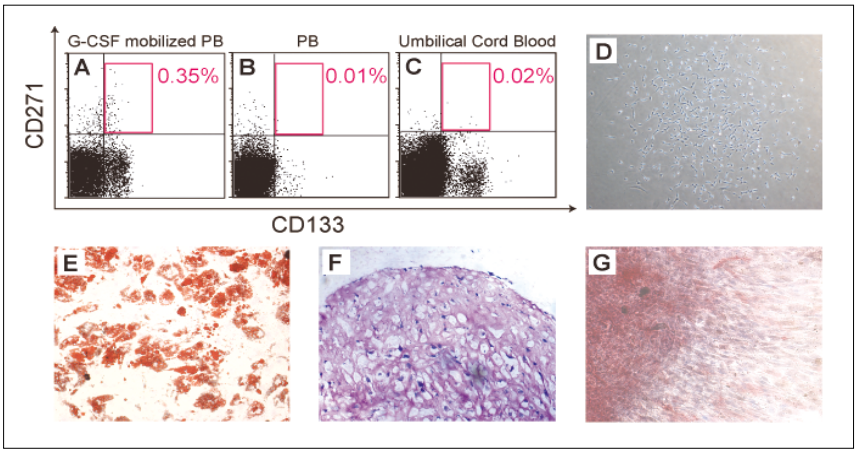

Fig4 CD271+ CD133+ cells in other tissues

(A) Flow cytometry analysis of Lin- GPA- CD271+ CD133+ Cells in G-CSF mobilized peripheral blood, (B) peripheral blood and (C) umbilical cord blood. (D) Multi-lineage differentiation of Lin- GPA- CD271+ CD133+ formed colony from G-CSF mobilized peripheral blood (E) for adipocytes, (F) for chondrocytes and (G) for osteocytes. PB = Peripheral blood

differentiation potential into neurons (Fig.3B) and myofibroblasts (Fig.3C), not into glial cells.

4)Lin- GPA- CD271+ CD133+ cells also exist in other tissues

We next proceeded to analyze the presence of Lin- GPA- CD271+ CD133+ cells in other tissues by flow cytometry. Fresh tissues were dissociated enzymatically, and cell suspensions were stained directly with labeled antibodies and run on FACSCalibur. The Lin- GPA- CD271+ CD133+ cells, represented in red, were used for the gating (Fig.4). A similar cell subset was detected in G-CSF stimulated peripheral blood, accounting for 0.347 ± 0.033% (n=3) (Fig.4A), the percentage of cells was much lower in peripheral blood (PB) (0.010 ± 0.010%: n=3) (Fig.4B), and umbilical cord blood (0.017 ± 0.016%: n=3) (Fig.4C). To determine multi-lineage differentiation potential, colony cells derived from Lin- GPA- CD271+ CD133+ cells in G-CSF stimulated peripheral blood were tested for the multiple lineages differentiation ability *in vitro*. The colonies were shown to differentiate appropriately to the adipogenic, chondrogenic, and osteogenic lineages (Fig.4D-G).

Discussion

A large body of evidence demonstrates that stromal tissue derived from the adult BM of mammalian species contains clonogenic progenitor cells, some of which are considered to be multipotent MSCs with the capacity to differentiate into a range of mesenchymal cell lineages, including adipose tissue, bone, cartilage, tendon, and ligament[5]. Despite considerable interest in the potential therapeutic applications of these cells, there is no well-defined protocol for the prospective isolation of human MSCs in order to properly study their biological characteristics prior to cell culture. Current methodologies, which rely upon the rapid adhesion of the stromal progenitor populations to plastic tissue culture dishes and their subsequent rapid proliferation *in vitro*, for the isolation of primitive MSCs are based upon those initially described by Friedenstein and colleagues[1,6]. Such protocols result in a heterogeneous starting population of adherent BM cells, of which only a minor proportion represents multipotent MSCs. Moreover, these protocols select the progeny of CFU-F and are not suitable for characterizing the clonogenic progenitors themselves.

In a recent report, Quirici have shown that CD271 is expressed on the cell surface of multipotent MSCs derived from the human BM[16]. In this study, we analyzed whether CD271 may also be a marker of naive MSCs. We determined the expression pattern of CD271 on freshly isolated BM cells and analyzed the CFU-F potential of the isolated CD271 cells. The cell surface expression of CD271 on naive BM was found only in a minor subset (2.65%) of cells. Co-expression analysis revealed that CD271 cells were also positive for CD133 (0.75%) (Fig.1A), but did not express CD45 or CD34 (data not shown). After the sorting of the CD271+ CD133+ subsets, we showed that the clonogenic potential resides almost exclusively in the CD271+ CD133+ fraction (Fig.1B). The CD271+ CD133+ cells sorted directly from BM were a highly enriched population of CFU-F, and contained approximately 720-fold the number of cells as compared with whole BM. These cells could be induced to further differentiate into adipocytes and osteocytes, indicating their multi-lineage differentiation capacity (Fig.2). In conclusion, CD271 and CD133 are suitable markers for the prospective isolation of MSCs from human BM.

Although there are an increasing number of reports describing the presence of MSCs in distinct tissue, only a few markers have been established to identify these cells in the respective organ. In this study, we demonstrated that CD271 and CD133 were expressed at high levels on collagenase-released cells and G-CSF stimulated peripheral blood, but only at near background levels in umbilical cord blood and peripheral blood (Fig.4). These molecules, CD271 and CD133, are expressed at high levels and with high selectivity on the somatic MSCs, making it an attractive marker for the prospective isolation of MSCs from human tissues.

In conclusion, the antigens recognized by the monoclonal antibody used in this study are novel candidate targets for the prospective isolation of MSCs. The selected cells may be used either directly after isolation or after *in vitro* expansion for transplantation in diseases like osteogenesis imperfecta, cartilage repair and myocardial infarction.

Acknowledgments

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