Neural Ganglioside GD2 Identifies a Subpopulation of Mesenchymal Stem Cells in Umbilical Cord

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Key Words

Umbilical cord • Mesenchymal stem cells • Neural Ganglioside GD2 • Purification • Differentiation

Abstract

In contrast to hematopoietic stem cells, there is still a lack of definitive cell markers for specific isolation and identification of mesenchymal stem cells (MSCs). Thus a homogenous population of MSCs is only obtained after several passages, when multilineage potential or other distinctive features of very early progenitors may be already somewhat compromised. Recently a novel surface marker the neural ganglioside GD2 has been reported to distinguish MSCs from all other cells within marrow. Here, we found that MSCs derived from umbilical cord (UC-MSCs) also expressed this marker at early-passages. More importantly, UC-MSCs were the only cells within umbilical cord expressing this marker. Compared to unsorted cells, GD2⁺-sorted cells not only possessed much higher clonogenicity and proliferation capacity but also had significantly stronger multi-differentiation potentials. Flow cytometric analysis revealed that GD2⁺-sorted cells showed increased expression of SSEA-4, Oct-4, Sox-

2 and Nanog, the typical markers expressed in embryonic stem cells, in comparison to unsorted or GD2-negative MSCs. Take together, our data demonstrate that the cells selected by GD2 are a subpopulation of MSCs with feature of primitive precursor cells and provide evidence that GD2 can be a cell surface marker suitable for the isolation and purification of UC-MSCs in early-passage culture.

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Introduction

Since Friedenstein et al. [1] first isolated the fibroblast-like cells from the nonhematopoietic compartment of bone marrow, a lot of reports have described the functional and phenotypical characteristics of these cells, which have the capacity for self-renewal and differentiation into a broad spectrum of lineages [2-4]. They are called mesenchymal stem cells or mesenchymal stromal cells (MSCs) [5, 6]. Bone marrow has been considered as the main source of mesenchymal stem cells until later studies demonstrate that MSCs exist in the stroma of other tissues. Recently, several groups

have succeeded in isolating MSCs from umbilical cord and have shown that UC-MSCs appear to be an attractive tool in the context of tissue engineering and cell-based therapy [7-10]. Because umbilical cord is a postnatal organ discarded after birth, the collection of cells does not require an invasive and painful procedure nor involves ethical issues. Moreover, UC-MSCs have higher proliferation rate and self-renewal capacity in comparison to adult bone marrow MSCs, which may attribute to the primary tissue origin of umbilical cord [11, 12].

However, MSCs are presently isolated simply depending on their ability to adhere to tissue culture plastic and identified by a panel of cell surface antigens. The caveat of such a method is heterogeneity of the resulting adherent cells [13, 14]. Because a homogenous population of MSCs is obtained only after several weeks in culture, when some distinctive features of very early progenitors may be already somewhat compromised, it become very difficult to isolate and identify MSCs in their primitive state. Several groups have done the best effort to find the specific cell markers for selectively isolating early progenitors from more mature cells in cultures of MSCs [15-18]. Notably, a novel surface marker neural ganglioside GD2 has been shown to be expressed on BM-MSCs, both freshly isolated from bone marrow and expanded in culture [19]. More importantly, MSCs are the only cells within normal marrow that express this marker. These results demonstrated that the immunomagnetic sorting of BM cells labeled with GD2 antibodies allowed the selection of phenotypically and functionally homogeneous cells, which are capable of expansion, self-renewal, and differentiation into osteoblasts, adipocytes, and chondroblasts. Upon this observation, we speculate GD2 may also serve to be a valuable marker for UC-MSCs since the biological similarity between BM-MSCs and UC-MSCs [7, 8].

Here, we first demonstrated the expression of GD2 in cultured UC-MSCs and then revealed specific spatial distribution of GD2-positive MSCs in umbilical cord. On the basis of these observations, we established an efficient method to isolate a homogenous cell population from early-passage cultures of UC-MSCs by GD2 immunosorting and further analyzed the phenotypic and functional characteristics of these sorted cells.

Materials and Methods

Cell isolation and culture

Umbilical cords (n=3) were obtained from local maternity hospitals after normal deliveries. Tissue collection for research

was approved by the institutional review board of the Chinese Academy of Medical Science and Peking Union Medical College. UC-MSCs were isolated from the whole umbilical cords according to the protocols described previously [20]. Human MSCs isolated from fetal bone marrow (FBM-MSCs) and adult bone marrow (ABM-MSCs) were chosen as controls and used for evaluation of the experimental results. Blood and fetal tissue collection for research purposes was approved by the institutional review board of the Chinese Academy of Medical Science and Peking Union Medical College. Pregnant women gave written consent for the clinical procedure and for the use of fetal blood or tissue for research purposes. Bone marrow samples were obtained from 3 fetuses and FBM-MSCs were harvested as described previously [21]. ABM-MSCs were isolated by bone marrow aspirates from the iliac crest of three normal healthy volunteers after informed consent and were prepared as described previously [22]. UC-MSCs, FBM-MSCs and ABM-MSCs were plated at a density of 1×10^6 cells/cm² in non-coated T-25 or T-75 cell culture flasks (Beckon Dickinson). Growth medium consisted of DMEM/F12 medium (Gibco) and 10% fetal bovine serum (FBS; Hyclone) or human AB serum (AB-HS; Hyclone), supplemented with 10 ng/mL vascular endothelial growth factor (VEGF; Sigma), 10 ng/mL epidermal growth factor (EGF; Sigma), 100 U penicillin/streptomycin (Sigma) and 2 mM L-glutamine (Gibco). Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C. After 3 days of culture, the medium was replaced and non-adherent cells were removed. The medium was then changed twice weekly thereafter. Once 60%-80% confluence had been reached, adherent cells were replated at a density of 1×10^4 /cm² in growth medium for expansion.

Foreskin fibroblasts were isolated from the foreskin obtained from circumcision and were passaged in RPMI 1640 (Gibco) with 20% FBS (Hyclone). Human umbilical cord vein endothelial cells (HUVEC) were isolated and cultured according to the protocol described by Jaffe et al [23]. Umbilical cord blood mononuclear cells (MNC) were prepared by gradient centrifugation. A melanoma cell line was cultured in DMEM (Gibco) with 20% FBS (Hyclone), serving as positive control.

Cell separation

Human UC-MSCs (P2) were incubated with murine monoclonal antibody against human GD2 (BD Biosciences) at a concentration of 1-2 µg per 10^6 cells. Cell suspensions were gently agitated for 30 min at 4°C. Then cells were incubated with beads coated with goat anti-mouse IgG microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Cell suspensions were separated by using a magnetic separation column (Miltenyi Biotec). The uncoated cells (GD2⁻ cells) were removed by several washes with PBS/BSA. The retained cells were termed the GD2⁺ cells.

Flow cytometry

Antibodies were obtained from BD Biosciences unless stated otherwise. UC-MSCs (n=3, P2), FBM-MSCs (n=3, p2) and ABM-MSCs (n=3, p2) were stained with murine monoclonal antibody against GD2 and FITC-conjugated goat anti-mouse secondary antibody. Staining without the primary anti-GD2

antibody served as a negative control. Using the same method, the GD2 expression of unsorted, GD2⁺ and GD2⁻ cells was monitored from p2 to p10. Three fractions were stained with PE-conjugated antibodies against SSEA-4, Oct-4, Sox-2 and FITC-conjugated antibodies against Nanog (eBioscience). Mouse isotypic antibodies served as the control. Cells were stained in single label and then analyzed by flow cytometry with a FACScan (Becton Dickinson).

Immunocytochemistry and immunofluorescence

ABM-MSCs, UC-MSCs, HUVEC, MNC, fibroblasts and melanoma cells were cultured on the sterile chamber slides. After 2 days, cells were fixed in 4% paraformaldehyde (Sigma). After non-specific binding, the cells were incubated with primary antibodies against GD2. The reaction was visualized with a biotinylated goat anti-mouse secondary antibody with ABC substrate (Zymed). All slides were counterstained with hematoxylin for 2 minutes.

For detection of the distribution of GD2-positive MSCs in cord section, immunohistochemistry and immunofluorescence were performed using murine monoclonal antibody against GD2. In immunocytochemistry, the reaction was visualized with a biotinylated goat anti-mouse secondary antibody with ABC substrate. In immunofluorescence, the reaction was followed by incubation with FITC-conjugated goat anti-mouse secondary antibody (KPL). Finally, the sections were counterstained with hematoxylin or DAPI (Biochemika) respectively.

CFU-F assay

Equal number (2×10^3) of unsorted, GD2⁺ or GD2⁻ cells were seeded in growth medium in T-25 flasks. Medium was replaced every three days. After 14 days in culture, the flasks were washed twice, fixed with 100% methanol, and stained with 3% Crystal violet. Cell clusters consisting of at least 50 fibroblasts were scored as a CFU-F colony. The CFU equals the total number of colonies divided by the initial number of cells multiplied by 100 as a percentage.

Proliferation assays

Unsorted, GD⁺ or GD2⁻ cells were plated in a six-well plate at the density of 2.5×10^4 cells/well. Three replicas for each cell population were performed at P2, P6 and P10. At 72 hours post-seeding, three fractions were harvested and a total cell count was determined. The proliferation rate was calculated by dividing the total cell number at 72 hours of culture by the initial plating number.

In addition, cell proliferation was measured using Cell Proliferation ELISA, BrdU (Roche Molecular Biochemicals). Unsorted, GD⁺ or GD2⁻ cells were seeded in triplicate in 96-well plates at the density of 5×10^3 cells/well. Three replicas for each cell population were performed at P2, P6 and P10. At 72 hours post-seeding, cell proliferation was measured using the cell proliferation BrdU ELISA kit according to manufacturer's instructions. Briefly, BrdU was added during the last 4 h of culture. After fixation, a peroxidase conjugated anti-BrdU antibody was added and incubated for 60 min after washing, a peroxidase substrate was added and absorbance was measured

in an ELISA plate reader.

Multi-lineage differentiation

A) Osteogenic and adipogenic differentiation. In the same conditions, unsorted, GD⁺ and GD2⁻ cells were induced to osteoblasts and adipocytes as previous described [20]. All the reagents for induction were purchased from Sigma. The adipocytes were stained with Oil Red O and the osteoblasts with von Kossa according to published protocols. The unsorted cells were plated in expansion medium as the control. The osteogenic specific gene, alkaline phosphatase (ALP); bone sialoprotein (BSP) and osteocalcin (OC), as well as the adipogenic specific gene, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor2 (PPAR α) were further detected by reverse transcriptase polymerase chain reaction (RT-PCR) with specific primers (unshown data).

B) Neurogenic differentiation. To induce neurogenic differentiation a multistep protocol was used [24]. Cells kept in regular growth medium were regarded as the control. IMDM was purchased from Gibco. bFGF, SHH and BDNF were purchased from R&D (Minneapolis, MN, USA). All the other reagents were purchased from Sigma. Neurogenesis was assessed by immunofluorescence on day 10 of induction. Cells were fixed in 4% paraformaldehyde (Sigma) and permeabilized in 0.1% Triton X-100 (Sigma). After non-specific binding, the cells were incubated with primary antibodies against Nestin, neuronspecific enolase (NSE) and fibrillary acidic protein (GFAP). Then cells were incubated with FITC-labeled appropriate secondary antibodies, followed by counterstaining with DAPI. All the antibodies were purchased from Chemicon (Temecula, CA, USA). Control experiments were performed with cells cultured in regular growth medium. The neurogenic specific gene, Nestin, Musashi, NSE and GFAP were further detected by RT-PCR with appropriate primers (unshown data).

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (QRT-PCR) analysis

Total RNA was isolated using Trizol (Invitrogen) as recommended by the manufacturer. First-strand cDNA was produced using Superscript II reverse transcriptase (Invitrogen) with Oligo dT and then used for RT-PCR analysis with specific primers (unshown data). For real-time PCR, primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). The following primer sets were used: SSEA-4 primers 5'-TGG ACG GGC ACA ACT TCA TC-3' and 5'-GGG CAG GTT CTT GGC ACT CT-3'(119bp); Oct-4 primers 5'-CAG GAG TCC CA GGA CAT GAA-3' and 5'-GTG GTC TGG CTG AAC ACC TT-3'(151bp); Sox-2 primers 5'-CAT GCA CCG CTA CGA CGT GAG-3' and 5'-TGG GAG GAA GAG GTA ACCA CAG G-3'(192bp); Nanog primers 5'-GCC GAA GAA TAG CAAT GGG TGT G-3' and 5'-CCA GGA CTG GAT GTT CTG GGT C-3' (175bp). Subsequently, we analyzed the mRNA levels of these genes in unsorted and GD2⁺ cells. Real-time PCR analysis was carried out using 2x SYBR Green PCR Master Mix (Applied Biosystems). PCR reactions were carried out in an ABI7500 sequence detection system (Applied Biosystems, CA) as follows: 10 minutes at 95°C and 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 34 seconds.

Fig. 1. GD2 expression on cultured UC-MSCs and BM-MSCs. (A) Immunocytochemical staining of cultured ABM-MSCs and UC-MSCs for GD2, in addition to MNC, HUVEC and fibroblast. Staining of melanoma cells served as a positive control, scale bar=100 μ m. (B) RT-PCR for GD2 synthase. RT-PCR of total RNA isolated from ABM-MSCs and UC-MSCs demonstrating the expression of GD2 synthase (230bp). No products expanded from total RNA of MNC, HUVEC and fibroblast. PCR products of GD2 synthase in melanoma cells served as a positive control. β -actin served as a control for the quality and quantity of RNA (527bp). (C) Comparison of GD2 expression of FBM-MSCs, ABM-MSCs and UC-MSCs in 10% FBS and human AB serum. Data represented mean $\pm$ SD (n=3). * p <0.05, ** p <0.01. (D) Detection of GD2 expression during culture. Abbreviations: FBM, fetal bone marrow; ABM, adult bone marrow; UC, umbilical cord; FBS, fetal bovine serum; AB-HS, human AB serum; MNC, umbilical cord blood mononuclear cells; HUVEC, human umbilical cord vein endothelial cell; P, passage.

Relative quantities of the gene of interest were determined by the comparative threshold cycle (delta CT) method and normalized to β -actin quantities.

Statistical analysis

Data are presented as arithmetic mean $\pm$ SD. Standard analyses were carried out using the Student t-test. Significant differences were considered those with a P value of <0.05.

Results

GD2 expression on cultured UC-MSCs

Using the protocols described previously, we succeed in isolating fibroblastic-like cells from three umbilical cord, fetal bone marrow and adult bone marrow samples respectively. These cell populations displayed the characteristics of MSCs as defined by the International Society for Cellular Therapy (ISCT) [6]. The immunocytochemical staining revealed that cultured ABM-MSCs and UC-MSCs expressed GD2, which were consistent with the following results. However MNC, HUVEC and fibroblasts did not express GD2. Melanoma cells served as positive control. (Fig. 1A). These results were further supported by RT-PCR analysis which showed ABM-MSCs and UC-MSCs also expressed the mRNA for GD2 synthase, a key enzyme for GD2

biosynthesis, while MNC, HUVEC and fibroblasts lacked GD2 synthase expression. 230 bp PCR products of GD2 synthase in melanoma cells served as a positive control (Fig. 1B). Furthermore flow cytometric analysis demonstrated that cultured UC-MSCs expressed GD2 either in FBS or in AB-HS. However, we observed that the expression of GD2 were significantly higher when UC-MSCs were cultured in AB-HS rather than in FBS. Moreover, flow cytometric analysis of FBM-MSCs and ABM-MSCs showed the same results. Either FBM-MSCs or ABM-MSCs cultured in AB-HS showed a significantly higher level of GD2 compared to FBS (Fig. 1C p <0.01 and p <0.05 respectively). Importantly, the flow cytometric analysis revealed that unsorted and GD2⁺ cells continued to express GD2 though 10 passages, which showed a gradual decrease with the passage. However, GD2⁻ cells never expressed GD2 until P10 (Fig. 1D).

Distribution of GD2-positive MSCs in umbilical cord

Umbilical cord is briefly classified into three distinctive zones; from outer to inner, i) subamnion; ii) intervacular regions (Wharton's jelly, IVCs); iii) perivascular regions (subdivided into peri-vein, peri-artery, vein wall and artery wall, PVCs) (Fig. 2A) [25].

Fig. 2. Distribution of GD2-positive MSCs in umbilical cord. (A) The structure of umbilical cord. Umbilical cord was divided into three distinctive zones. From out to inner, i) subamnion; ii) intervacular regions; iii) perivascular regions. (B) GD2- positive MSCs reside in umbilical cord. GD2-positive MSCs was demonstrated to locate in the entire cord by immunohistochemistry (upper panels) and immunofluorescence (lower panels). In immunohistochemistry GD2-positive MSCs (brown) were indicated by the arrow, nuclei were counterstained with hematoxylin (blue). The areas within the white rectangles were further magnified to show details of GD2-positive MSCs (green) in immunofluorescence, nuclei were counterstained with DAPI (blue). Scale bar=100µm.

Fig. 3. Clonogenic and proliferative capacity of unsorted, GD2⁺ and GD2⁻ cells. (A) The clonogenic potential of unsorted and GD2-sorted cells was assessed by CFU-F assay. Note the different colony number and size of unsorted, GD2⁺ and GD2⁻-derived CFU-F, Scale bar=200µm. (B) The proliferation rate of unsorted, GD2⁺ and GD2⁻ cells was determined by cell count assay. Data represented mean $\pm$ SD (n=3). * p <0.001. (C) Cell proliferation was determined by BrdU assay. Data represented mean $\pm$ SD (n=3). ** p <0.01. Abbreviations: CFU-F, colony forming unit - fibroblast; UN, unsorted cells; GD2⁺, GD2⁺-sorted cells; GD2⁻, GD2⁻-sorted cells; P, passage.

Using immunohistochemistry and immunofluorescence in situ, we showed that GD2-positive MSCs distributed in the entire cord, including subamnion, intervacular and perivascular regions (Fig. 2B).

Clonogenicity and proliferation assays

The clonogenic potential of unsorted and GD2-sorted cells was assessed by colony-forming unit-fibroblast (CFU-F) assay. Our results showed that GD2⁺ cells

Fig. 4. FACS and QRT-PCR analysis of the expression of SSEA-4, Oct-4, Sox-2 and Nanog. (A) A representative FACS profile of unsorted, GD2⁺ and GD2⁻ cells analyzed for SSEA-4, Oct-4, Sox-2 and Nanog expression at p2. Each plot represents the indicated cell marker (red line) and the relevant isotype control (green line). Numbers on each individual plot refers to the percentage of positive cells. (B) The flow cytometric analysis of SSEA-4, Oct-4, Sox-2 and Nanog expression on unsorted, GD2⁺ and GD2⁻ cells. Data represented mean $\pm$ SD (n=3). ** $p < 0.01$. (C) Quantitative real-time PCR analysis of the mRNA levels of SSEA-4, Oct-4, Sox-2 and Nanog in unsorted and GD2⁺ cells. RNA was harvested separately from three samples and β -actin was used as an endogenous standard. Each of the cDNA was tested in duplicate. Diagram bars represent the gene expression levels normalized to β -actin values, assuming as one unit gene levels of unsorted cells. Data represented mean $\pm$ SD (n=3). * $p < 0.001$. Abbreviations: UN, unsorted cells; GD2⁺, GD2⁺-sorted cells; GD2⁻, GD2⁻-sorted cells.

displayed significantly more colony forming units-fibroblasts than unsorted cells in vitro. Moreover, the colony size in GD2⁺ cells was much larger than that observed in unsorted cells. However, GD2⁻ cells never showed any residual CFU-F activity. GD2⁺ cells possessed significantly higher level of CFU ($68.33 \pm 5.03\%$) than unsorted cells ($36.21 \pm 3.6\%$) (Fig. 3A; $p < 0.01$). In addition, GD2⁺ cells had much higher proliferation rate (27.33 ± 0.77 folds and 18.6 ± 0.63 folds) compared to unsorted cells (15 ± 0.45 folds and 9.2 ± 0.36 folds) at p2 and p6. Although the proliferation rate decreased gradually in culture, GD2⁺ cells (11.77 ± 0.52 folds) still possessed far more proliferative capability than do unsorted cells (5.9 ± 0.4 folds) at p10. However, GD2⁻ cells had a very low proliferation rate (2.9 ± 0.2 folds and 1.3 ± 0.2 folds) at p2 and p6, they hardly grew at p10 (Fig. 3B; $p < 0.001$). Similar to the results of cell count assay, BrdU incorporation assay showed that BrdU incorporation in GD2⁺ cells was significantly higher than that of unsorted and GD2⁻ cells at p2, p6 and p10 (Fig. 3C; $p < 0.01$).

FACS and quantitative RT-PCR (QRT-PCR) analysis

Following expansion of GD2⁺ and GD2⁻ cells, we investigated the expression of SSEA-4, Oct-4, Sox-2 and Nanog on each cell population (Fig. 4A). The flow cytometric analysis showed that these stem cell markers were significantly increased in GD2⁺ cells compared to unsorted cells. Notably, GD2⁻ cells did not express these markers (Fig. 4B; $p < 0.01$). The results were supported by QRT-PCR analysis which showed the gene expression of these stem cell markers in GD2⁺ cells were much higher than unsorted cells (Fig. 4C; $p < 0.001$).

Multi-lineage differentiation assays

A) Osteogenic and adipogenic differentiation.

To compare the multi-lineage potential of GD2⁺ cells to unsorted cells, we cultured unsorted, GD2⁺ or GD2⁻ cells in adipogenic and osteogenic medium at the same density. Cells were grown in induction medium for 7, 14 and 21 days before analysis of the differentiating along

Fig. 5. Adipogenic and osteogenic differentiation capacity of unsorted, GD2⁺ and GD2⁻ cells. (A) Unsorted, GD2⁺ and GD2⁻ cells showed the different differentiation capability to adipocytes (Oil Red O stain) and osteoblasts (von Kossa stain). The unsorted cells were plated in expansion medium as the control. Scale bar=100μm. (B) Different expression of adipogenic and osteogenic specific genes in induced unsorted, GD2⁺ and GD2⁻ cells. After 21 days of induction, RT-PCR analyzed the expression of adipogenic specific genes (LPL, PPARr2) and osteogenic specific genes (ALP, BSP, OC) in three fractions. (C) Adipogenic and osteogenic specific genes expression of induced GD2⁺ and unsorted cells in a time-course study. Abbreviations: PPARr2, peroxisome proliferator-activated receptor 2; LPL, lipoprotein lipase; ALP, alkaline phosphatase; BSP, bone sialoprotein; OC, osteocalcin.

Fig. 6. Neuronal differentiation capacity of unsorted, GD2⁺ and GD2⁻ cells. (A) Nestin, NSE and GFAP-positive cells was detected by immunofluorescence. Scale bar=100μm. (B) Percentage of neural cells immunoreaction-positive for Nestin, NSE and GFAP in three fractions. Data represented mean ± SD (n=3). ***p*<0.01. (C) After 10 days of neural induction, RT-PCR analyzed the expression of neural specific genes (Nestin, NSE, GFAP and Musashi) in induced unsorted, GD2⁺ and GD2⁻ cells. Abbreviations: NSE: neuron specific enolase GFAP: fibrillary acidic protein.

adipocytes and osteoblasts lineages. After 21 days of induction, unsorted and GD2⁺ cells were stained positively with oil red O and von Kossa. However, lipid droplets and calcium deposits were not detected in GD2⁻ cells and control groups. Moreover, oil red O and von Kossa staining showed that GD2⁺ cells were able to form adipocytes and osteoblasts more readily than unsorted cells (Fig. 5A). The results were supported by RT-PCR analysis which showed in two lineages GD2⁺ cells have higher gene expression than unsorted cells but GD2⁻ cells had no expression of these genes (Fig. 5B). Furthermore, in adipogenesis, the expression of PPARr2 in GD2⁺ cells was significantly higher than

unsorted cells and the appearance of LPL in the former was earlier than the latter. In contrast to unsorted cells, ALP and OC expression was rather high in GD2⁺ cells and BSP appeared in GD2⁺ cells only after the 1st week of osteogenic induction Fig. 5C).

B) Neurogenic differentiation. Neural differentiation was identified by immunofluorescent (Fig. 6A) and RT-PCR (Fig. 6C). After 10 days of induction, GD2⁺ and unsorted cells were positive for Nestin (neural stem cells marker), NSE (neurons marker) and GFAP (astrocytes marker). The proportion of cells staining positively for Nestin (37.33±6.29% versus 12.87±2.35%), NSE (68.8±3.54% versus 36.1±5.17%) and GFAP (14.93±1.95% versus 4.6±1.73%) was higher in induced GD2⁺ cells than induced unsorted cells (Fig. 6B; $p<0.01$). RT-PCR analysis further confirmed the higher expression of Nestin, NSE and GFAP by the former than the latter. Notably, RT-PCR analysis demonstrated that Musashi, characteristically expressed in neural stem cells, was largely upregulated in induced GD2⁺ cells. Neither the immunofluorescent nor RT-PCR analysis showed expression of these specific markers in GD2⁻ cells and control groups.

Discussion

We have previously established a method to readily isolate MSCs from human umbilical cord (UC-MSCs) with a 100% success rate [20]. However, the lack of definitive markers does not simply allow the identification and purification of these cells in early-passage culture. Here, our study demonstrated that a novel surface marker neural ganglioside GD2, the first reported single surface marker uniquely distinguishing MSC from other bone marrow elements, could be used as an ideal definitive marker for the identification and purification of human UC-MSCs.

Firstly, cultured UC-MSCs expressing GD2 were demonstrated by flow cytometry and immunocytochemistry. These results were further supported by RT-PCR analysis which showed UC-MSCs also expressed the mRNA for GD2 synthase, a key enzyme involved in GD2 synthesis. More importantly, UC-MSCs were the only cells within umbilical cord that expressed this marker. Other cells such as HUVEC, fibroblasts or cord blood mononuclear cells, which all are possible contamination within UC-MSCs at early passages, did not express GD2 and the mRNA for GD2 synthase. In addition, our results demonstrated that UC-MSCs cultured in the medium with human AB serum expressed obviously higher level of GD2 than FBS. Flow cytometric analysis of FBM-MSCs and ABM-MSCs all showed the same results. A recent study also demonstrated that AB serum could act as a safe and ideal alternative to FBS for the

expansion of MSCs in vitro [26]. At present a lot of MSCs research on cell therapy and tissue engineering has entered into the clinical transplantation setting. Taking into consideration the safety of transplantation, we should choose the optimal culture conditions for clinical-scale production of human MSCs. In order to avoid potential risk of infection as well as immunological reaction, all animal-derived products should be excluded from the culture system of MSCs.

In contrast to cultured MSCs, only little information is learned about the exact nature and localization of MSCs in vivo. Whether any marker, expressed on cultured MSCs, exists in vivo is in confusion in the MSCs field [27-30]. However, our study demonstrated that not only cultured UC-MSCs expressed GD2 but also GD2-positive MSCs were present in vivo. Using immunohistochemistry and immunofluorescence in situ, we found that GD2 expressing cells extended from perivascular and intervascular areas to subamniotic areas, which suggested that MSCs existed in all umbilical cord regions. These findings supported and extended our previous studies which demonstrated that the largest number of MSCs could be isolated from umbilical cord without tissue dissection. In contrast to other groups, who obtained UC-MSCs from Wharton's jelly or the subendothelial layer of umbilical cord vein [7-10], we used the whole umbilical cord to isolate UC-MSCs and our previous studies had proved that these cell populations met the minimal criteria proposed by ISCT for defining MSCs.

Furthermore the flow cytometric analysis showed that the GD2 expression level of unsorted and GD2⁺ cells decreased gradually with the passage, whereas the reverse situation did not occur to GD2⁻ cells. This strongly suggested a differentiation from the GD2⁺ to the GD2⁻ phenotype. On the basis of these observations, we speculated that GD2 might identify very early precursor cells from a heterogeneous pool of UC-MSCs. To confirm this hypothesis, we further analyzed whether there is the difference in the phenotypic and functional characteristics between unsorted and sorted cells. The results showed that GD2⁺-sorted cells displayed significantly higher level of CFU than unsorted cells and had a higher proliferation rate compared to that of unsorted cells at P2, P6 and P10. However, GD2⁻ cells never showed any residual CFU-F activity and grew very slowly. Further studies showed that GD2⁺-sorted cells maintained high level of SSEA-4 (68.90±5.36%), an embryonic stem cells marker [31], which was reported to identify BM-MSCs [18]. However the unsorted cells and GD2⁻ cells only expressed 28.99±3.72% and 0.47±0.14% respectively. We further

detected whether GD2⁺-sorted cell enhanced the expression of Oct-4, Sox-2 and Nanog, the key transcription factors for the maintenance of the undifferentiated state of ES. All three transcription factors are expressed in high levels in embryonic stem cells and are considered markers of pluripotent stem cells [32-34]. The flow cytometric and QRT-PCR analysis showed that GD2⁺-sorted cell expressed much higher levels of Oct-4, Sox-2 and Nanog at the cellular and molecular level in comparison to unsorted cells. However, we could not detect the expression of these transcription factors in GD2⁻ cells. These studies strongly suggested that GD2 marked very early precursor cells from UC-MSCs. It was further supported by the later results which demonstrated GD2⁺ cells have significantly stronger multi-differentiation potentials than unsorted cells. Oil Red O and von Kossa staining revealed that GD2⁺ cells were able to form adipocytes and osteoblasts more readily than the unsorted cells. However GD2⁻ cells did not show these capabilities at all. Further studies demonstrated that there was a large up-regulation of adipogenic and osteogenic specific genes in induced GD2⁺ cells. Moreover the immunofluorescent and RT-PCR analysis showed that GD2⁺ cells have higher neural differentiation capacity than do the unsorted cells. After 10 days of neural induction,

the percent of Nestin, NSE and GFAP-positive cells observed in induced GD2⁺ cells was higher than the unsorted cells. RT-PCR analysis also demonstrated the expression level of neural specific genes in induced GD2⁺ cells is obviously higher than induced unsorted cells.

In conclusion, these results support GD2 identifies primitive UC-MSCs with high specificity and describe a strategy for selecting the most multipotential cells from a heterogeneous pool of MSCs. These findings may have a significant impact on the studies of isolation and purification of MSCs and help in establishing a standard method for the isolation and purification of UC-MSCs. In the clinical setting, transplantation of pure primitive MSCs rather than stromal cells at different maturation stages may prove to be more advantageous and efficient.

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