Human Bone Marrow Mesenchymal Stromal Cells Express the Neural Ganglioside GD2: A Novel Surface Marker for the Identification of MSCs

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Abstract

Mesenchymal stromal cells (MSCs) have enormous potential for the regeneration of bone, cartilage and other tissues derived from primitive mesoderm. Despite extensive research, there is still no single marker that reliably identifies MSCs within the bone marrow. Using immunocytochemistry and flow cytometry, we demonstrate here that the neural ganglioside GD2 is expressed by MSCs either newly isolated from bone marrow or expanded in tissue culture; this finding was supported by RT-PCR analysis showing expression of the mRNA for GD2 synthase, an essential enzyme for GD2 biosynthesis. GD2 was also expressed on MSCs isolated from adipose tissue, but not on foreskin fibroblasts. Importantly, MSCs were the only cells within normal marrow that expressed this marker. Thus, GD2 appears to be the first reported single surface marker that uniquely distinguishes MSCs from other marrow elements. GD2 may prove valuable to study MSC biology and for the preparation of MSCs for clinical applications.
Introduction

Human mesenchymal stromal cells (MSCs) are multipotent progenitors that can differentiate to bone, fat, cartilage and other mesenchymal tissues\textsuperscript{1,2}. Termed mesenchymal stem cells by some investigators, MSCs can be isolated from a variety of tissues, but those from bone marrow are the most widely studied and best characterized. Interest in MSCs for diverse applications has grown rapidly over the last decade\textsuperscript{3-6}; however, a single surface marker that would uniquely identify this population of cells within the bone marrow remains elusive\textsuperscript{2}.

The first monoclonal antibodies used to characterize MSCs were SH2 and SH3\textsuperscript{7}, which later were shown to recognize epitopes on CD105\textsuperscript{8} and CD73\textsuperscript{9}, respectively. While these antigens remain the cornerstone of human MSC identification\textsuperscript{2}, they are also expressed on hematopoietic and endothelial cells. GD2 is a disialoganglioside found mainly in the nervous system. Reports of neural antigen expression on MSCs\textsuperscript{10,11} led us to consider that these cells might express GD2, which could be a useful marker of MSCs, as its expression should not extend to hematopoietic cells or other normal marrow elements. Here, we demonstrate the expression of GD2 by MSCs but not other cells within the bone marrow, suggesting that this antigen might serve as a single definitive marker of marrow-derived MSCs.
Methods

Isolation of human MSCs. Bone marrow MSCs (from multiple donors) were isolated according to a protocol approved by the Institutional Review Board of St. Jude Children’s Research Hospital, Memphis, TN, as previously described\(^1\). Adipose-derived MSCs were obtained from tissue generously provided by Dr. Jeffrey Gimble according to a standard protocol described elsewhere\(^2\).

Immunocytochemistry. We performed immunocytochemical studies on formalin-fixed cells culture expanded on a sterile chamber slide, using a murine monoclonal antibody against GD2 (clone 14.G2A, BD Biosciences, San Jose, CA). The reaction was visualized with a biotinylated goat anti-mouse secondary antibody with ABC substrate and Nova Red as the chromogen (Vector Labs, Burlingame, CA). All slides were lightly counterstained with hematoxylin.

Reverse Transcription PCR. RNA was extracted with Trizol Reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Primers for GD2 synthase were forward 5’-CCAACTCAACAGGCAACTAC-3’, reverse 5’-GATCATAACGGAGGAAGGTC-3’ (230-bp product) and forward 5’-GACAAGCCAGAGCGCTTTTA-3’, reverse 5’-TACTTGGACACGGGCGAGT-3’ (99-bp product); primers for \(\beta_2\)-microglobulin (333 bp-product) were forward, 5’-CTCGCGCTACTCTCTCTTTTTG-3’, reverse 5’-GCTTACATGTCTCGATCCACTAA-3’. PCR conditions were denaturation at
95°C for 12 min, then 35 cycles at 95°C x 1 min, 59°C x 1 min, 72°C x 1 min, and then 72°C x 10 min.

**MSC Differentiation.** Culture-expanded MSCs were differentiated in vitro to osteoblasts\textsuperscript{14}, adipocytes\textsuperscript{15}, and chondroblasts\textsuperscript{16} as described. The differentiated osteoblasts were stained with Alizarin Red S\textsuperscript{17}, the adipocytes with Oil Red O\textsuperscript{18}, and the chondroblasts with Alcian Blue\textsuperscript{19} according to published protocols.

**Flow Cytometry.** All analyses were performed on a BD LSR II flow cytometer, with antibodies from BD Biosciences, except that the monoclonal antibody against human fibroblasts, clone D7-FIB, was from Serotec (Raleigh, NC). The data was analyzed with Cell Quest Pro Software Version 5.2.1 (BD Biosciences).

**Immunoselection of MSCs.** GD2-expressing MSCs were isolated from freshly harvested bone marrow mononuclear cells using our murine monoclonal antibody against GD2 and an allophycocyanin-conjugated donkey anti-mouse secondary antibody with the corresponding magnetic beads on an AutoMACS\textsuperscript{®} device (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions.
Results and Discussion

Human MSCs isolated from bone marrow showed the characteristic features of spindle shape and plastic adherence (Fig. 1A, upper left panel). Flow cytometric analysis demonstrated the expression of distinguishing MSC antigens (CD105, CD73 and CD90), and the absence of hematopoietic and endothelial antigens (CD45, CD34, CD19, CD3, CD11b, and HLA DR) (Fig. 1A, upper right panel). The culture-expanded cells were capable of in vitro differentiation to osteoblasts as demonstrated by Alizarin Red staining; adipocytes, as demonstrated by Oil Red O staining; and chondroblasts as demonstrated by Alcian Blue staining (Fig. 1A, lower panels). Thus, the isolated cells met the essential criteria used to define MSCs.

Immunocytochemical staining of the marrow derived MSCs revealed striking expression of the neural ganglioside GD2. (Fig. 1B, upper left panel). Comparable staining was found on all MSCs, indicating a pancellular expression of GD2 among these bone marrow elements. Staining of MSCs without the primary antibody yielded negative results demonstrating the lack of nonspecific staining (Fig. 1B upper right panel). To assess GD2 expression by an alternative method, we analyzed culture-expanded cells by flow cytometry, finding high levels of GD2 surface expression (Fig. 1B, middle panel). Reverse-transcription PCR analysis using two different primer pairs, with a neuroblastoma cell line and blood mononuclear cells serving as positive and negative controls, respectively, showed that MSCs expressed the mRNA for GD2 synthase (Fig. 1B, lower panel).
panel), an essential enzyme for GD2 biosynthesis. These results provide crucial supportive evidence of genuine ganglioside expression on MSCs.

All cells analyzed in the above experiments had been expanded in culture (passage 2). Since GD2 can play a role in cellular adhesion we considered that plastic adherence of MSCs in vitro might induce or alter the expression of GD2 in a manner different from the normal regulation of the ganglioside. To address this possibility, we analyzed newly harvested bone marrow mononuclear cells (MNCs) by four-color flow cytometry, gating first on marrow MNCs that lacked CD45 expression (Fig. 1C, top, gate R1). These cells (R1) were then analyzed for CD105 and CD73 expression (Fig. 1C, middle). About 95% of the CD45-CD105+ CD73+ cells (gate R2) expressed GD2 (Fig. 1C, lower). Further, newly harvested marrow MNCs that expressed either CD271 or D7Fib, both of which have been suggested to represent MSCs, were found to co-express GD2 (data not shown). The intensity of GD2 expression exceeded that of either CD271, which is lost during culture, or D7Fib, which is also expressed on skin fibroblasts. Notably, the entire population of expanded MSCs continues to express GD2 at similar levels though 8 culture passages (Fig. 1D).

To determine if GD2 expression is unique to marrow-derived MSCs, we analyzed such cells from adipose tissue. As shown in Fig. 1E, the adipose-derived cells also expressed GD2 at approximately the same level as marrow-derived MSCs. Foreskin fibroblasts, by contrast, lacked GD2 expression (Fig. 1E).
Finally, flow cytometric analysis of marrow mononuclear cells obtained from cellular residua of a bone marrow harvest collection bag (10- to 100-fold enriched for MSCs, unpublished observation) revealed 0.3% GD2 expression (Fig. 1F). The broad pattern of expression spanning 2 to 3 log units of fluorescence intensity is consistent with the MSC expression pattern demonstrated in Figs. 1B-D. Our RNA analysis revealed the lack of GD2 synthase expression in circulating blood cells (Fig. 1B). Most importantly, immunohistochemical staining of normal bone marrow biopsy specimens (Fig. 1F) did not detect other GD2-expressing cells. Collectively, these data indicate the lack of hematopoietic GD2 expression, consistent with prior reports23,24.

Cells isolated from freshly harvested bone marrow by selection for GD2 expression using immunomagnetic beads demonstrated typical MSC morphology and plastic adherence (Fig. 2). The culture-expanded cells differentiated to osteoblasts, adipocytes, and chondroblasts (Fig. 2) as expected for MSCs.

GD2 is the first candidate marker of MSCs to be consistently expressed at a high level on all cells of this population, whether freshly isolated or ex vivo expanded; hence, GD2 selection may offer an improved approach for MSC identification and isolation. Immune recognition of the GD2-expressing MSCs in the marrow microenvironment may underlie the hematopoietic suppression observed when anti-GD2 antibodies are used as immunotherapy for neuroblastoma.

A critical question in MSC biology is whether this cell population possesses a relatively uniform differentiation capability, and responds to specific
signals to generate diverse mesenchymal lineages, or is comprised of biologically distinct subsets of progenitors committed to differentiate in particular pathways. The availability of a marker antigen such as GD2 would be useful in resolving this issue, much in the way that CD34 has helped to discriminate among subsets of hematopoietic progenitors.
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Figure Legends.

Figure 1. GD2 expression on marrow MSCs. (A) Photomicrograph of undifferentiated MSCs (upper left panel) showing the characteristic spindle shape and adherent properties of the cells. Original magnification, 40x. Flow cytometry histograms (upper right panel) demonstrating the typical expression pattern of MSC antigens (—), isotype control (---) and other measured antigen (—) as indicated. Immunocytochemical staining (lower panel) demonstrating the differentiation of MSCs into osteoblasts (Alizarin Red stain), adipocytes (Oil Red O stain), and chondroblasts (Alcian Blue stain). (B) Immunocytochemical staining of culture-expanded MSCs for GD2 (upper left panel) and staining without the primary anti-GD2 antibody as a negative control (upper right panel). Flow cytometry histogram (middle panel) showing GD2 expression (bold line) by MSCs and the isotype control (thin line). Reverse transcription PCR for GD2 synthase (lower panel). Results using primers generating a 230-bp product are shown at the top, primers generating a 99-bp product are in the middle, and β2-microglobulin as a control for the quality and quantity of RNA is at the bottom. No RNA, a complete reaction omitting the RNA sample; NB, RNA from neuroblastoma cells (positive control), MSC, culture-expanded after marrow derivation; MNCs, RNA from blood mononuclear cells (negative control); No RT, reaction with MSC RNA, but omitting reverse transcriptase. (C) Flow cytometry histogram (upper panel) of bone marrow cells for CD45 expression. The R1 gate indicates CD45- cells. Analysis of the CD45- cells from the R1 gate for CD105
and CD73 expression (middle panel). The R2 gate indicates the double-positive cells. Analysis of the CD45- CD105+ CD73+ cells from the R2 gate for GD2 expression (lower panel). These cells, MSCs, from freshly harvested bone marrow, were never in tissue culture. (D) Flow cytometry histograms of GD2 expression on MSCs after serial passage in tissue culture. The experimental and control curves are as indicated in Fig. 1B. (E) Immunocytochemical staining of adipose-derived MSCs (upper left) and a negative control (upper right) in which the primary anti-GD2 antibody was omitted. The specimens were lightly counterstained with hematoxylin. Original magnification, 4x. Immunocytochemical staining of foreskin fibroblasts (lower left) and a negative control (lower right) as for the adipose-derived MSCs. Original magnification, 4x. (F) Flow cytometric histograms (upper panel) showing GD2-expressing (positive control) and nonexpressing (isotype control) cells and (middle panel) GD2 expression in bone marrow mononuclear cells obtained from bone marrow harvest bag residua (see text). Anti-GD2 immunohistochemical staining of a bone marrow biopsy specimen (lower left) and MSCs (lower right, positive control). Both specimens were counterstained with hematoxylin.

**Figure 2. Ex vivo expansion and trilineage differentiation of GD2 selected MSCs.** Photomicrograph of undifferentiated, ex vivo expanded cells (upper panel) after isolation by GD2 selection. The characteristic spindle shape morphology and plastic adherence of MSCs is shown. Immunocytochemical staining (lower panel) demonstrates the differentiation of the GD2 selected MSCs
into osteoblasts (Alizarin Red stain), adipocytes (Oil Red O stain), and chondroblasts (Alcian Blue stain).
Figure 2

Undifferentiated GD2-selected MSCs

Osteoblasts
Adipocytes
Chondroblasts