Gangliosides as a potential new class of stem cell markers: the case of GD1a in human bone marrow mesenchymal stem cells

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Abstract  Owing to their exposure on the cell surface and the possibility of being directly recognized with specific antibodies, glycosphingolipids have aroused great interest in the field of stem cell biology. In the search for specific markers of the differentiation of human bone marrow mesenchymal stem cells (hBMSCs) toward osteoblasts, we studied their glycosphingolipid pattern, with particular attention to gangliosides. After lipid extraction and fractionation, gangliosides, metabolically 3H-labeled in the sphingosine moiety, were separated by high-performance TLC and chemically characterized by MALDI MS. Upon induction of osteogenic differentiation, a 3-fold increase of ganglioside GD1a was observed. Therefore, the hypothesis of GD1a involvement in hBMSCs commitment toward the osteogenic phenotype was tested by comparison of the osteogenic propensity of GD1a-highly expressing versus GD1a-low expressing hBMSCs and direct addition of GD1a in the differentiation medium. It was found that either the high expression of GD1a in hBMSCs or the addition of GD1a in the differentiation medium favored osteogenesis, providing a remarkable increase of alkaline phosphatase. It was also observed that ganglioside GD2, although detectable in hBMSCs by immunohistochemistry with an anti-GD2 antibody, could not be recognized by chemical analysis, likely reflecting a case, not uncommon, of molecular mimicry.—Bergante, S., E. Torretta, P. Creo, N. Sessarego, N. Papini, M. Piccoli, C. Fania, F. Cirillo, E. Conforti, A. Ghiroldi, C. Tringali, B. Venerando, A. Ibatici, C. Gelfi, G. Tettamanti, and L. Anastasia. Gangliosides as a potential new class of stem cell markers: the case of GD1a in human bone marrow mesenchymal stem cells. J. Lipid Res. 2014. 55: 549–560.

Supplementary key words  osteogenic differentiation • sphingolipids • stem cells characterization

Among adult stem cells, human bone marrow mesenchymal stem cells (hBMSCs) are the most widely studied, and they promise to be the closest ones to get into clinical applications for tissue engineering and repair (1). However, to date, the precise identification of surface markers that would unequivocally identify hBMSCs and distinguish them from other cells present in bone marrow (BM), particularly fibroblasts, is still missing (2). Furthermore, hBMSC preparations constitute a mixed population of bona fide stem cells and of terminally differentiated cells that cannot be directly induced to differentiate into other cell types. In addition, and importantly, the same stem cells contained in hBMSCs are likely a population of cells with different commitments (3). All of this called for a search for surface markers that would not only distinguish hBMSCs from differentiated cells, but also identify, and possibly serve to separate, the different subsets of progenitor cells with diverse proliferation and differentiation potential. Among possible new markers, glycosphingolipids, particularly gangliosides, represent an interesting class of components because: a) they are primarily exposed on the outer cell membrane in direct contact with the extracellular milieu (4); b) they are involved in the formation of lipid rafts (5, 6); c) they exhibit an extraordinarily large repertoire of different chemical structures (4–7); and d) it

 abbreviations: ALP, alkaline phosphatase; BM, bone marrow; Cer, ceramide; Gb3, globotriaosylceramide; GlcCer, glucosylceramide; hBMSC, human bone marrow mesenchymal stem cell; hDF, human dermal fibroblast; HPTLC, high-performance TLC; LacCer, lactosylceramide; RUNX2, runt-related transcription factor 2; TBST, TBS containing Tween 0.1%.†To whom correspondence should be addressed. e-mail: luigi.anastasia@unimi.it The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures.
is known that their distribution changes dramatically during development (7). Glycosphingolipids can be easily extracted from cell homogenates, separated into individual entities by high-performance TLC (HPTLC) (8), and chemically identified by mass spectrometric methods (9, 10). However, in the more general routine, glycosphingolipids have been identified only by the use of immunometric procedures employing specific anti-individual glycosphingolipid antibodies, quite useful for cytochemical investigations. Nonetheless, the specificity of anti-glycosphingolipid (particularly gangliosides) antibodies may often be poor, due to cross-reactivity (molecular mimicry) with other glycoconjugates, glycoproteins, glycosaminoglycans, and phosphatidyl glycosides (11–17). Still, as some glycosphingolipids, including gangliosides GM2, GD2, and GD3, are unequivocally expressed on the cell surface of several tumors (i.e., neuroblastoma, melanoma, and sarcoma), the possibility of specifically targeting them for an immunotherapeutic strategy has led to an increasing interest in developing new, and more specific, anti-ganglioside antibodies (18). Nevertheless, the recommendations to support and confirm the data obtained with immunoochemical procedures by the use of the analytical approaches mentioned above remain firm (12, 19).

The present investigation was undertaken with the purpose to identify, for possible further isolation, a subpopulation of hBMSCs having a marked propensity to osteogenic differentiation. We focused our attention on the glycosphingolipid patterns, particularly gangliosides, which were chemically defined by extraction, separation into the individual entities by HPTLC, and structural identification of each component by mass spectrometric procedures. The glycosphingolipid pattern was also inspected by immunoochemical methods using individual anti-glycosphingolipid antibodies. We observed, and herein report, that ganglioside GD1a appeared to be highly expressed by a sub-population of hBMSCs having a marked propensity to osteogenic determination and differentiation. We also noted that in hBMSCs, ganglioside GD2, which was positive by the use of an anti-GD2 monoclonal antibody, was chemically undetectable, likely as a result of a molecular mimicry condition.

**Cell isolation and culture**

hBMSCs were obtained from the stem cell bank at IRCCS Istituto Clinico Humanitas, and obtained from BM samples from healthy subjects donating BM to a sibling for allogenic hematopoietic stem cell transplantation, after obtaining written informed consent. Human dermal fibroblasts (hDFs) were purchased from ATCC. hBMSCs were grown in “basal medium” constituted of DMEM with low glucose (Sigma-Aldrich), 10% v/v FBS (Sigma-Aldrich), 4 mM L-glutamine, 1% antibiotic-antimycotic mixture (Euroclone) in a humidified atmosphere containing 5% CO₂ at 37°C. hBMSCs were grown in “basal medium” constituted of DMEM with high glucose (Sigma-Aldrich), 10% v/v FBS (Sigma-Aldrich), 4 mM L-glutamine, 1% antibiotic-antimycotic mixture (Euroclone) in a humidified atmosphere containing 5% CO₂ at 37°C.

**Cell differentiation**

hBMSCs and hDFs were induced to differentiate toward osteoblasts, adipocytes, chondroblasts, and smooth muscle cells in vitro to test for their multi-lineage potential, according to standard procedures (20). Briefly: a) Differentiation toward osteoblasts: confluent cells were differentiated toward osteoblasts by culturing them in basal medium, with the addition of 10% FBS with 0.1 μM dexamethasone, 50 μg/ml l-ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (all reagents from Sigma-Aldrich) for 17 days. Alizarin Red solution (Millipore) was used to detect calcium deposition in derived osteoblasts according to the manufacturer’s instructions. b) Differentiation toward adipocytes: confluent cells were differentiated toward adipocytes using a mesenchymal stem cell adipogenesis kit (Millipore) for 21 days according to the manufacturer’s instructions. Oil Red O solution (Millipore) was utilized to stain lipid droplets of derived adipocytes. c) Differentiation toward smooth muscle cells: confluent cells were cultured in DMEM-low glucose with 1% FBS and 0.2 μl/ml TGFβ1 (Millipore) for 7 days. Immunofluorescence staining using FITC-conjugated anti-human α-actin (1:200; Sigma) was utilized to detect smooth muscle α-actin in differentiated cells. d) Differentiation toward chondrocytes: cells were maintained in a 3D culture by growing them in cell pellets in AdvanceSTEM chondrogenic differentiation medium (HyClone, Thermo Scientific), according to the manufacturer’s instructions. After 28 days of differentiation, matrix deposition by derived chondroblasts was detected with Alcian Blue staining (Sigma-Aldrich).

To evaluate the direct effect of ganglioside GD1a and GM1 on hBMSC differentiation toward osteoblasts, confluent cells were treated with 1–50 μM GD1a or GM1 (Santa Cruz Biotecno) and cultured for 5 days in basal medium or in osteogenic medium, as described above.

**RNA extraction and real-time PCR**

Total RNA was isolated using the RNeasy mini kit (Qiagen) and 0.8 μg of extracted RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (BioRad) according to the manufacturer’s instructions. Real-time PCR was performed with 10 ng of cDNA as template, 0.2 μM primers, and 1× iQ Custom SYBR Green Supermix (BioRad) in a 20 μl final volume using a 7900 HT Fast real-time PCR system instrument (Applied Biosystems).

The following primers were used to amplify the corresponding target genes: human alkaline phosphatase (ALP), forward 5′-GGCAGGAGATCTCTGACC-3′ and reverse 5′-GGCCACACCA-GATCTGTC-5′; human osteopontin (SPP1), forward 5′-TGCTAG-AGACGAGGACATCA-3′ and reverse 5′-GTATCCAGCTGACT-CGTTT-3′; human runt-related transcription factor 2 (RUNX2), forward 5′-CAGATGTTCAGAAAAACTTCTT-3′ and reverse 5′-TCACGTCGGTCATTGTCG-3′; human SOX9, forward 5′-GTCCTTCCA-CATGTTG-3′; and reverse 5′-GATGTGGT-3′ and reverse 5′-CGACCTA-AGAAAGGATACGCA-3′; and human tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide, forward 5′-GATCCC-CAATGCTCCAAAG-3′ and reverse 5′-TGCTTGTGACGATTCGAC-3′. The amplification program used was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 5 s each at 95°C and 30 s at 56°C. Relative quantification of target genes was performed in triplicate and was calculated by the equation 2^−ΔΔCt (21).

**MATERIALS AND METHODS**

**Cell isolation and culture**

hBMSCs were obtained from the stem cell bank at IRCCS Istituto Clinico Humanitas, and obtained from BM samples from healthy subjects donating BM to a sibling for allogenic hematopoietic stem cell transplantation, after obtaining written informed consent. Human dermal fibroblasts (hDFs) were purchased from ATCC. hBMSCs were grown in “basal medium” constituted of DMEM with low glucose (Sigma-Aldrich), 10% v/v FBS (HyClone, Thermo Scientific), 4 mM L-glutamine, 1% antibiotic-antimycotic mixture (Euroclone) in a humidified atmosphere containing 5% CO₂ at 37°C.
Flow cytometric analysis

Cells were detached and collected in PBS at a concentration of 2 × 10^6 cells/ml. Aspecific binding sites were blocked with a blocking solution (50% 1x PBS, 50% FBS) for 30 min at room temperature. Cells were stained with antibodies against human PE-conjugated CD105, PE-conjugated CD90, FITC-conjugated CD73, FITC-conjugated CD11b, PE-conjugated CD45, PerCP-eFluor 710-conjugated CD34, FITC-conjugated CD3, FITC-conjugated CD19, FITC-conjugated HLA-DR, PE-conjugated CD106 (BioLegend), PE-conjugated CD146 (BioLegend), and PerCP-eFluor710-conjugated CD166 (BioLegend) for 10 min at 4°C. The respective isotype antibodies were used as controls. Gangliosides GD2 and GD1a were also recognized incubating cells with the primary antibodies against human ganglioside GD2 (clone 14G2a; BD Pharmingen) or GD1a (clone GD1a-1; Millipore) for 10 min at 4°C and then with secondary antibodies PE-conjugated anti-mouse IgG (eBioscience) or Alexa Fluor 488-conjugated anti-mouse IgG (Jackson Immuno Research) for 10 min at 4°C. Cell samples were analyzed with a Navios flow cytometer (Beckman Coulter) equipped with Kaluza software (Beckman Coulter).

Immunofluorescence staining

To this purpose, cells were preliminarily rinsed twice with PBS and then fixed using a solution of 4% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. Aspecific binding sites were blocked with a blocking solution (PBS with 1% BSA) for 1 h at room temperature. Cells were then incubated with FITC-conjugated smooth muscle α-actin antibody (Sigma-Aldrich) at dilution of 1:200 in blocking solution for 75 min at room temperature. Cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich) at dilution of 1:500 in blocking solution containing 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature.

Immunomagnetic separation of cell sub-populations enriched in GD2 or GD1a gangliosides

In order to ascertain the expression of GD2 and GD1a, hBMSCs or hDFs were separated with MACS technique (Miltenyi Biotech). Cells were trypsinized and collected in washing buffer (1× PBS, 0.5% BSA, 2 mM EDTA). 2.5 × 10^6 cells were incubated with 4 μl of primary antibody against GD2 (clone 14G2a; Millipore) or 4 μl of primary antibody against GD1a (clone GD1a-1; Millipore) for 15 min at 4°C followed by incubation with 30 μl of anti-mouse IgG microbeads (Miltenyi Biotech) for 15 min at 4°C. Both labeled and nonlabeled cells were collected using an octoMACS separator (Miltenyi Biotech) according to the manufacturer’s instructions.

Quantification of ALP

ALP was quantified in highly positive GD1a (GD1a⁺) and poorly positive GD1a (GD1a⁻) hBMSCs differentiated to osteoblasts, and in nondifferentiated hBMSCs as control, using a quantitative ALP ES characterization kit (Millipore) according to the manufacturer’s instructions. Briefly, cells were washed and harvested in PBS by scraping, collected in 1× wash solution for counting and centrifuged at 1,200 rpm for 6 min. Forty-five thousand cells for each sample were resuspended in 150 μl of β-nitrophosphyl phosphate buffer and transferred to three wells of a 96-well plate (15,000 cells/well). The enzymatic reaction was performed by incubating each sample for 20 min at room temperature in the dark in the presence of 50 μl of 2× β-nitrophosphyl phosphate substrate solution. The reaction was then stopped by adding 50 μl of a “reaction stop solution” contained in the kit. The amount of released β-nitrophosphyl was measured reading the absorbance at 405 nm using a Victor 3 plate reader (Perkin Elmer).

Metabolic labeling of cell sphingolipids

The metabolic labeling of cell sphingolipids was accomplished according to Riboni, Viani, and Tettamanti (22). To this purpose, cells (hBMSCs and hDFs) were plated in a tissue culture dish at the concentration of 3,600 cells/cm². After 24 h, [3-3H] sphingosine (D-erythro >97%, 50 μCi, 1.85 MBq; Perkin Elmer) dissolved in ethanol was transferred into a glass sterile tube and dried under a nitrogen stream; the residue was then dissolved in 5 ml of DMEM-low glucose with 10% FBS to obtain a final concentration of 2.4 nM sphingosine and an amount of radioactivity of 110,000 dpm/ml. The medium was added to the cells, and after 2 h of incubation (pulse) it was replaced with DMEM-low glucose with 10% FBS not containing radioactive sphingosine for 48 h (chase) in order to reach a metabolic steady state. These conditions were established as optimal after testing the chase time from 24 to 72 h. At the end of the chase, cells were washed and harvested in ice-cold PBS by scraping. The cell suspensions were frozen and lyophilized.

Extraction and chromatographic separation of radioactive sphingolipids

Total lipids from lyophilized cells, metabolically labeled as described above, were extracted twice with 20:1:1 (v/v/v) chloroform/methanol/water, dried under a nitrogen stream, and subjected to a two-phase partitioning in chloroform/methanol 2:1 (v/v) and 20% (v/v) water (8, 23). Radioactive lipids of the aqueous and organic phases obtained after partitioning were separated into individual entities and analyzed by HPTLC utilizing the solvent system chloroform/methanol/water 110:40:6 (v/v/v) for the organic phases and chloroform/methanol/0.2% aqueous CaCl₂ 60:40:9 (v/v/v) and chloroform/methanol/0.2% aqueous CaCl₂ 60:40:12 (v/v/v) for the aqueous phases (8, 23). Radioactive sphingolipids were visualized with a Beta Imager 2000 (Biospace) and the gangliosides identified by comparison with titrated standards prepared in our laboratory, according to consolidated procedures (24). The radioactivity associated with individual lipids was determined with the specific Beta Vision software (Biospace).

Determination of the sphingolipid patterns of hBMSCs and hDFs not submitted to metabolic labeling

To this purpose, a total of 6.5 million hBMSCs were plated in 10 × 150 cm² dishes (BD Falcon) and cultured for 3 days, reaching about 80% confluence. Then cells were washed and harvested in ice-cold PBS by scraping. The cell suspensions were frozen and lyophilized. Total lipids from lyophilized hBMSCs were extracted twice with chloroform/methanol/water 20:1:1 (v/v/v) and dried under a nitrogen stream, using the same procedure described above. Lipid extracts were dissolved in chloroform/methanol 2:1 (v/v) and subjected to a two-phase partitioning in chloroform/methanol 2:1 (v/v) and 20% (v/v) water to separate the organic phase from the aqueous phase. Lipids of the organic phase were submitted to an alkaline treatment to remove glycerophospholipids (25). Briefly, the dried organic phase was resuspended in 100 μl of CHCl₃ and 100 μl of 0.6 N NaOH in methanol and allowed to stand at 37°C for 1 h. The reaction was blocked by adding 120 μl of 0.5 M HCl in methanol. The sample was then submitted to another phase separation and the new organic phase was used for HPTLC analysis as described above. The aqueous and the organic phases were then loaded on the HPTLC plate for chromatographic separation. To this purpose, a portion of the extracted aqueous and organic phases corresponding to 4 and 2 mg of total protein of the original cell sample, respectively, was loaded on each HPTLC lane. For ganglioside analysis, the HPTLC was developed in chloroform/methanol/0.2% aqueous CaCl₂ 60:40:9 (v/v/v) and the
gangliosides visualized using Ehrlich reagent, while for the organic phase the HPTLC was developed in chloroform/methanol/water 110:40:6 (v/v/v) and the separated sphingolipids visualized using a periodate reagent solution (26).

MS

To this purpose, HPTLC plates were loaded and developed as for the HPTLC analysis of hBMSC and hDF sphingolipids not submitted to metabolic radiolabeling (see above). Then the HPTLC plates were placed into an ImagePrep device (Bruker Daltonics) for matrix deposition. DBH mixture (Sigma-Aldrich) in water/acetone was employed as parameters of number of cycles, spray power, modulation, spray time, incubation, and dry time were adjusted to optimize signal to noise ratio. Subsequently, HPTLCs were mounted on the MTP TLC adaptor (Bruker Daltonics) and transferred to a MALDI mass spectrometer. Spectra were acquired in reflector negative mode using an Ultraflex III mass spectrometer equipped with Smartbeam laser (frequency of 100 Hz; Bruker Daltonics) and FlexControl software version 3.3 (Bruker Daltonics). Spectrometer settings were: ion source 1, 20 kV; ion source 2, 17.2 kV; lens, 7.4 kV; reflector, 21 kV; reflector 2, 11 kV; deflection, mass suppression up to m/z 800; pulsed ion extraction, 200 ns; detector gain voltage, 1,552 V; electronic gain, 100 mV/full scale; sample rate, 1 GS/s; and laser attenuator offset, 80%. TLC MALDI software was used for automatic data acquisition using the following parameters: X-step, 0.5 mm; 3, number of Y-spots for summing; lane width, 5 mm; and total laser shots, 600 (200 shots per raster position). Spectra were externally calibrated using calibration standard mixture peaks, achieved by loading one or more glycosphingolipid standards on HPTLC. SurveyViewer software version 1.1 (Bruker Daltonics) was employed for data analysis. This software presents all spectra in a 2D density plot where the glycosphingolipid mass to charge and position on HPTLC can be easily visualized. Spectra of interest were then analyzed by FlexAnalysis software version 3.3 (Bruker Daltonics). Correct attribution of glycosphingolipids was made with LIPID MAPS structure database (27) and with a glycosphingolipid MS precursor ion analysis tool (28).

Immunoblotting

Cells (hBMSCs and hDFs) were harvested in ice-cold PBS by scraping and centrifuged at 1,400 rpm for 10 min. Cell pellet was resuspended in ice-cold PBS with complete protease inhibitors (Roche) and lysed by sonication. The amount of protein was measured using a Pierce BCA protein assay kit (Thermo Scientific).

Protein (40 μg) was subjected to SDS/PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). After blocking with 5% (w/v) of nonfat dry milk in TBS containing Tween 0.1% (TBST) for 1 h at room temperature, the membrane was immunoblotted with anti-human CD2 primary antibody diluted 1:500 in 5% (w/v) of nonfat dry milk in TBST overnight at 4°C. The membrane was then washed in TBST three times and incubated with HRP-conjugated anti-mouse secondary antibody diluted 1:2,000 in 5% (w/v) of nonfat dry milk in TBST for 1 h at room temperature. After three washes in TBST, the membrane was developed using the ECL detection system (GE Healthcare, Amersham).

RESULTS

Characterization of hBMSCs

hBMSCs were cultured to passage three, and then subjected to immunophenotyping by flow cytometry, revealing positivity for mesenchymal antigens CD73, CD90, CD105, CD166, CD106, and CD146 and negativity for hematopoietic and endothelial antigens CD3, CD11b, CD19, CD34, CD45, and HLA-DR, as expected (Fig. 1A) (29). To confirm their plasticity, isolated hBMSCs were induced to differentiate in vitro into osteoblasts, adipocytes, chondrocytes, or smooth muscle cells by treatment with the proper differentiation media (Fig. 1B–D). Osteogenic differentiation was induced for 17 days and acknowledged with ALP activity staining (Fig. 1B). Adipogenic differentiation was...
also induced for 3 weeks and Oil Red O staining revealed the formation of mature adipocytes (Fig. 1C). Smooth muscle cell differentiation was induced for 7 days and positively detected with α-actin staining (Fig. 1D). Chondrogenesis was induced for 28 days in cell pellets and recognized with Alcian Blue staining (Fig. 1E). hDFs were used as controls for both immunophenotyping and differentiation (supplementary Fig. I). As shown in supplementary Fig. I, the immunophenotype of hDFs does not differ significantly from that of hBMSCs, whereas the capability of the same cells to be induced to osteogenic, adipogenic, chondrogenic, and smooth muscle cells is almost null, as expected for terminally differentiated cells.

**Sphingolipid pattern analysis of hBMSCs**

hBMSCs and hDFs were subjected to sphingolipid pattern analysis using the analytical approaches described in the Materials and Methods: a) metabolic radiolabeling of cell sphingolipids; b) sphingolipid extraction and fractionation into an organic phase for hydrophobic sphingolipids, and an aqueous phase for hydrophilic sphingolipids (gangliosides); and c) separation of the individual sphingolipids contained in each phase with HPTLC.

**hBMSCs.** The HPTLC patterns of the neutral (essentially hydrophobic) and acidic (essentially hydrophilic) sphingolipids of hBMSCs, obtained after metabolic radiolabeling (Fig. 2), revealed the following average distribution of the incorporated radioactivity into hydrophobic sphingolipids, contained in the organic phase: ceramide (Cer) 5.90 ± 0.74%, glucosylceramide (GlcCer) 8.50 ± 2.22%, lactosylceramide (LacCer) 4.70 ± 1.04%, globotriosylsphingosine (Gb3, Gb2, Gb1, 4GlcC) 13.4 ± 3.43%, and SM plus globoside (Gb4: GalNAcβ1,3Galα1,4Galβ1,4Glc-Cer) together (as they could not be separated under these experimental conditions) 67.5 ± 5.65% (Fig. 2B, C).

On the other hand, the aqueous phase contained the following gangliosides, as percent of incorporated radioactivity: GM3 70.5 ± 3.68%, GM2 9.8 ± 2.54%, GM1 1.6 ± 0.4%, GD3 12.2 ± 5.52%, and GD1a 5.9 ± 2.1% (Fig. 2E, F).

Analysis of hBMSCs by HPTLC-MALDI MS (Fig. 3B) confirmed the data obtained with metabolic radiolabeling (Fig. 3A). In particular, no significant peaks corresponding to GD2 (or to any other sphingolipid) could be detected in the HPTLC plate lane area that was 14.5–19.0 mm from the sample deposition, which corresponded to the Rf value observed for the standard GD2 sample. Instead, GD2 could be detected and its mass spectra recorded to the presence of species with fatty acids of different chain length. Doubled spots in both standard and cellular sphingolipids correspond to the fatty acids that showed higher mobility in our solvent system (data not shown). The absence of GD2 was completely unexpected because the same ganglioside was reported to be expressed in hBMSCs (29), using an anti-GD2 antibody to detect this ganglioside. On this basis, we decided to investigate this issue in more detail. To this purpose, hBMSCs were treated with the same anti-GD2 antibody used by Martinez et al. (29), and then submitted to flow cytometric analysis, revealing that 19.5 ± 5% of hBMSCs stained positive for GD2 (Fig. 4A). Then, we enriched the GD2-positive population by cell sorting with magnetic immunobeads, using the same anti-GD2 antibody (Fig. 4B), obtaining two fractions, one 70.5 ± 10.8% different fatty acid composition, resulting in multiple bands for each ganglioside on the HPTLC plate, as we previously reported (23). In fact, each ganglioside species contained sphingosine (C18:1), and by HPTLC analysis was split in multiple bands containing palmitic acid (C16:0), which showed lower mobility in our solvent system, and C24:0, C24:1 fatty acids that showed higher mobility in our solvent system (data not shown). The absence of GD2 was completely unexpected because the same ganglioside was reported to be expressed in hBMSCs (29), using an anti-GD2 antibody to detect this ganglioside. On this basis, we decided to investigate this issue in more detail. To this purpose, hBMSCs were treated with the same anti-GD2 antibody used by Martinez et al. (29), and then submitted to flow cytometric analysis, revealing that 19.5 ± 5% of hBMSCs stained positive for GD2 (Fig. 4A). Then, we enriched the GD2-positive population by cell sorting with magnetic immunobeads, using the same anti-GD2 antibody (Fig. 4B), obtaining two fractions, one 70.5 ± 10.8%...
any of the samples (Fig. 4E, F). In addition, Western blot analysis of the above 70.5 and 9.7% seemingly positive and negative fractions by FACS analysis, which were both proven to be GD2 free by metabolic radiolabeling experiments, showed the presence of a number of components in the range 24–175 kDa recognized by the GD2 antibody, presumably of glycoprotein nature (Fig. 4G).
Gangliosides and hBMSCs sorting (supplementary Fig. IIIB), obviously in contrast with previously reported results, which showed that GD2 is not expressed by human fibroblasts (30). However, analysis of the sphingolipid pattern of GD2+ enriched hDFs revealed a “putative” GD2 fraction (8.09%) (supplementary Fig. IIIA), which could apparently be enriched to 24.05% by immunobead sorting (Fig. 4).

**Fig. 4.** Immunomagnetic sorting of hBMSCs with anti-GD2 antibody. A: Flow cytometric analysis of GD2 expression in hBMSCs using anti-GD2 antibody. B: Schematic image of immunomagnetic sorting protocol (MACS® by Miltenyi Biotech). Flow cytometric analysis of GD2 expression in GD2-positive (GD2+) (C) and GD2-negative (GD2−) (D) cell fractions obtained after immunobead sorting. E: Radiochromatoscanning image of metabolically radiolabeled gangliosides after HPTLC separation. Unsorted (Unsort.) hBMSCs (lane 1), GD2+ hBMSCs (lane 2), and GD2− hBMSCs (lane 3). F: Percentage distribution of radiolabeled gangliosides. Data are means ± SD of three different experiments. G: Western blot analysis of protein extracts of GD2+ and GD2− hBMSCs recognized by anti-GD2 antibody. Std, standard.

hDFs. FACS analysis of hDFs, which were used as controls and treated with anti-GD2 antibody, revealed a “putative” GD2 fraction (8.09%) (supplementary Fig. IIIA), which could apparently be enriched to 24.05% by immunobead sorting (supplementary Fig. IIIB), obviously in contrast with previously reported results, which showed that GD2 is not expressed by human fibroblasts (30). However, analysis of the sphingolipid pattern of GD2+ enriched hDFs
by metabolic radiolabeling unequivocally showed that GD2 could not be detected (supplementary Fig. IIIC). Hence, the putative positivity with the anti-GD2 antibody is not specific.

**Sphingolipid involvement in hBMSC differentiation**

The hBMSC sphingolipid pattern, assessed by metabolic radiolabeling, was analyzed before and after osteogenic differentiation induction (Fig. 5C, D). Osteogenic differentiation caused a drastic decrease (about 40%) of GM3 concentration, which passed from 67 to 41% of the total ganglioside content. Concomitantly, GM2, GM1, and GD1a underwent a 1.2-, 5-, and 3.3-fold increase, respectively, with GD1a becoming the ganglioside with the highest relative increase of expression during the differentiation process. Then, undifferentiated hBMSCs were analyzed by FACs using an anti-GD1a antibody, revealing a 25 ± 6% positivity for the ganglioside (Fig. 6A). Successive cell sorting with magnetic immunobeads, using the same anti-GD1a antibody, allowed us to obtain and separate a cell fraction 94 ± 6% highly positive for GD1a by cytofluorimetry, and a cell fraction poorly positive for GD1a, only for 13 ± 4.5% (Fig. 6A). Analysis of gangliosides by metabolic radiolabeling confirmed the enrichment in GD1a (Fig. 6B, C). To test whether highly positive GD1a (GD1a+) and poorly positive GD1a (GD1a−) hBMSCs differed in terms of stemness, the expression of mesenchymal markers CD106 and CD146 was analyzed in the two cell populations, as these two markers were expressed only by a fraction of the unsorted hBMSC population (Fig. 1A). Cytofluorimetric analysis showed that both CD106+ and CD146+ fractions were mostly negative for GD1a (Fig. 6D–F). Analysis of osteogenic markers by real-time PCR revealed significantly higher mRNA levels of ALP, osteopontin, and RUNX2 in GD1a+ as compared with GD1a− hBMSCs (Fig. 7A–C). Moreover, when cells were subjected to osteogenic differentiation for 17 days, GD1a+ hBMSCs showed significantly higher levels of the same osteogenic markers by real-time PCR, as well as higher levels of ALP activity as compared with GD1a− negative cells (Fig. 7D–G). Unsorted hBMSCs were then cultured in the presence of GD1a at different concentrations (1–50 μM) in normal or osteogenic medium. It was found that while...
GD1a addition did not significantly influence ALP expression in normal culturing medium (Fig. 7H), addition of the same ganglioside at the lowest tested concentration (1 μM) increased the expression of ALP upon osteogenic differentiation, while the high concentration (50 μM) seemed to inhibit the process (Fig. 7I). To test whether a high concentration of GD1a decreased ALP expression because it induced cells to differentiate toward other cell phenotypes, cells were treated with 50 μM GD1a and tested for their differentiation toward chondroblasts and smooth muscle cells (supplementary Fig. IVA, B). It was found that the expression of chondrogenic differentiation marker SOX9 and smooth muscle marker α-actin-2 did significantly decrease as compared with controls (supplementary Fig. IVA, B). As gangliosides GM2 and GM1 also increased during hBMSC differentiation, the effect of their addition to the osteogenic medium was also tested (supplementary Fig. IV). It was found that addition of 1–50 μM GM2 or GM1 to the differentiation medium did not increase osteogenesis, as the expression of ALP did not increase as compared with cells treated with normal osteogenic medium, with a slight decrease at 50 μM, which was significant only in the case of GM2 treatment (supplementary Fig. IVC, D).

**DISCUSSION**

Glycosphingolipids represent a very attractive class of compounds, as they a) are exposed primarily on the outer

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**Fig. 7.** GD1a role in osteogenesis. A–C: Gene expression of osteogenic differentiation markers (ALP, osteopontin, and RUNX2) in GD1a+ and GD1a− hBMSCs compared with unsorted (Unsort.) cells. D–F: Gene expression of osteogenic differentiation markers by quantitative (q)PCR (ALP, osteopontin, and RUNX2) in GD1a+ and GD1a− hBMSCs differentiated toward osteoblasts compared with unsorted undifferentiated hBMSCs. G: Graph showing ALP activity in GD1a+ and GD1a− hBMSCs differentiated toward osteoblasts and in unsorted undifferentiated hBMSCs as control. Data are means ± SD of three different experiments; statistical differences were determined by one-way ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with respective controls. H: Gene expression of ALP by qPCR in unsorted hBMSCs treated with 1, 10, and 50 μM GD1a for 5 days compared with untreated hBMSCs. I: Gene expression of ALP by qPCR in unsorted hBMSCs treated with 1, 10, and 50 μM GD1a in osteogenic medium for 5 days. Untreated cells cultured in osteogenic medium for 5 days were used as control. Data are means ± SD of three different experiments; statistical differences were determined by one-way ANOVA. ***P < 0.001.
With a radiochromatoscanner (23, 33) (Fig. 2B, E). The use of sphingolipids that can be detected on the HPTLC plate explains the biosynthesis of radiolabeled glycosphingolipids by adding [3H]sphingosine in the culture medium, resulting in the formation of radiolabeled sphingolipids that appear, in particular, to serve as molecular markers for recognizing sub-classes of multipotent stem cells, like hBMSCs. This ability depends on the fact that they can be addressed by specific monoclonal antibodies, thus providing a tool for cell recognition and separation (7, 14). The interest in this approach also prompted the search for carbohydrate-mimicking peptides that have a more pronounced immunogenicity than gangliosides and induce the formation of more resistant antibodies (18). However, the use of antibodies alone to identify glycosphingolipids, gangliosides in particular, should be done with caution, because these compounds share similar sugar moieties with many other molecules present on the cell membrane (i.e., glycoproteins, glycosaminoglycans, phosphatidyl glycosides, etc.) and this could cause cross-reactivity (molecular mimicry), hence ambiguous results (11–17). Actually, molecular mimicry may also occur in the case of carbohydrate mimicking peptides (31). Therefore, when recognizing glycosphingolipids, gangliosides in particular, by immunocchemical procedures, it is highly recommended to support and confirm the data obtained by the analytical procedures suitable for chemical identification of the compounds (12, 19).

In this work, we adopted different analytical methodologies to determine the sphingolipid expression pattern of in vitro cultures of hBMSCs, which were first shown to meet the indispensable criteria (3) used to define the stem cell potential of hBMSCs (Fig. 1). Glycosphingolipids have been analyzed by separation of cell lipid extracts into aqueous and organic phases containing acidic glycosphingolipids (gangliosides) and neutral sphingolipids, respectively. The separation of the different components of the two phases was accomplished by HPTLC and the individual compounds were identified by comparison with commercially available standards. In this study, we used two different techniques to visualize cell sphingolipids after the chromatographic steps: the first one uses Ehrlich’s or p-anisaldehyde/sulphuric acid staining for sphingolipid detection on the plate (32) (Fig. 2A, D), while the second one requires an initial metabolic radiolabeling of all sphingolipids by adding [3H]sphingosine in the culture medium, resulting in the biosynthesis of radiolabeled sphingolipids that can be detected on the HPTLC plate with a radiochromatoscanner (23, 33) (Fig. 2B, E). The use of metabolic radiolabeling greatly increases the sensitivity of the method, allowing reduction of the number of stem cells required for each analysis, and, of course, warrants for the sphingolipid nature of the analyzed glycoconjugates, as only those incorporating radiolabeled sphingosine in their structure can be detected. Surprisingly, we found that ganglioside GD2, which was previously identified as a novel hBMSC surface marker (29), could not be detected in any of the hBMSC samples that were analyzed. In fact, no spots could be detected on the HPTLC plate with Rf values corresponding to that of a GD2 standard that was used as a control sample (Fig. 2E, GD2 lane). To further confirm these results, cell sphingolipids were also analyzed by HPTLC coupled to MALDI MS. This analytical methodology allows the mass spectrometer laser to be pointed to a specific area of the HPTLC plate, and the mass spectra of the sample extracted from the matrix to be recorded. This rigorous analytical approach also confirmed that GD2 could not be detected in any of the analyzed hBMSC samples. As these results excluded the presence of any significant amounts of GD2 in hBMSCs, they raised serious concerns about the possibility of electing this ganglioside as a new specific marker of this class of adult stem cells, as was previously proposed (29). Actually, cross-reactivity of anti-GD2 mAb 14G2a with glycoprotein CD166, which is also highly expressed in hBMSCs, was recently reported (31). Nonetheless, in order to exclude that the absence of GD2 resulted from differences in hBMSC isolation and culturing procedures, hBMSCs were analyzed by cytofluorimetry using the same anti-GD2 antibody that was used in the previous study (29), revealing positivity for GD2 that could be increased by immunomagnetic cell sorting (Fig. 4). Successive analysis by metabolic radiolabeling of putative GD2-enriched cells revealed that GD2 could never be detected in any of the samples, supporting the hypothesis that the antibody is recognizing something different for GD2 as the result of molecular mimicry (Fig. 4E, F). Actually, many glycoproteins share the same sugar moiety of gangliosides, and could compete with GD2 in binding its antibody, especially when GD2 expression is under detectable levels, as it appears to be in the case of hBMSCs. This was confirmed by Western blot analysis of GD2-positive and -negative hBMSC protein extracts, revealing that the anti-GD2 antibody recognizes a variety of proteins in the 24–175 kDa range (Fig. 4G). To further confirm these results, hDFs were used as controls in all experiments, and the results compared with those of hBMSCs. hDFs were chosen as control cells because they are often isolated together with hBMSCs as they possess a similar surface marker expression to that of hBMSCs (supplementary Fig. IA). On the other hand, hDFs alone do not possess the stem cell capability of being induced to trans-differentiate toward other cell types (supplementary Fig. IB–E) and they are also known not to express ganglioside GD2. Surprisingly, we found that the results for hDFs were partially positive (8.09%) for GD2 when analyzed with the same anti-GD2 antibody that was used for hBMSCs (supplementary Fig. III). Moreover, immunobead sorting with anti-GD2 antibody seemed to allow the separation of a hDF sub-population enriched in GD2 (up to 24%) (supplementary Fig. IIIIB). However, GD2 could never be detected in hDFs by metabolic radiolabeling, as in the case of hBMSCs (supplementary Fig. IIC).

Analyses of the ganglioside patterns of hBMSCs obtained from five different donors revealed expression of gangliosides GM3, GM2, GD3, and GD1a (Fig. 5A, B). The pattern was quite similar in the different donors, with a
marked prevalence of GM3 (about 67%). These data raised the question of whether it could be possible to distinguish sub-populations of hBMSCs on the basis of a different expression pattern of sphingolipids during the process of hBMSC differentiation. Along this line, a previous report on dental pulp progenitor cells showed that GD1a increased during osteogenic and neuronal differentiation, suggesting a possible role of this ganglioside in directing the process (34, 35). Preliminary, we differentiated hBMSCs under osteogenic conditions, and confirmed the ganglioside pattern before and after differentiation, observing marked differences: GM3 and GD3 diminished (~39 and 20%, respectively), whereas GM2, GM1, and GD1a underwent a marked increase, particularly GD1a. To assess whether ganglioside GD1a could play a role in hBMSC differentiation, hBMSCs were immunomagnetically sorted with anti-GD1a antibody (Fig. 6A), obtaining a hBMSC differentiation, hBMSCs were immunomagnetically sorted with anti-GD1a antibody (Fig. 6B, C). Cyttofluorimetric analysis showed that both CD106+ and CD146+ cell fractions highly positive for GD1a (GD1a+) and a cell fraction poorly positive for GD1a (GD1a−), as confirmed by metabolic radiolabeling (Fig. 6B, C). Cytofluorimetric analysis showed that both CD106+ and CD146+ fractions were mostly negative for GD1a, supporting the notion that GD1a hBMSCs are more undifferentiated than GD1a− hBMSCs (Fig. 6D–F). Moreover, the GD1a+ cell fraction revealed a significantly higher expression of osteogenic markers than GD1a− hBMSCs, confirming their higher commitment toward the osteogenic phenotype (Fig. 7A–C), which turned into a significantly higher expression of the same osteogenic markers upon differentiation induction with the osteogenic medium (Fig. 7D–G). Finally, it was found that addition of GD1a increased the expression of ALP upon hBMSC differentiation in osteogenic medium (Fig. 7I), supporting the notion that GD1a plays a role in driving osteogenesis, as recently reported for dental pulp stem cells (35). These results, although to be further consolidated, seem to support the notion that glycosphingolipids, which are enriched in lipid rafts and can interact with many cell signaling sensors, appear to play key roles in stem cell biology, and they should definitely be investigated in further depth. In fact, the reported results support the hypothesis that they could potentially be used not only as stem cell markers, but also to direct stem cell differentiation. Further work in this direction is currently being done in our laboratories.

REFERENCES

1. Mariani, E., and A. Facchini. 2012. Clinical applications and bio-safety of human adult mesenchymal stem cells. Curr. Pharm. Des. 18: 1821–1845.
2. Hametti, P. 2012. Mesenchymal stromal cells and fibroblasts: a case of mistaken identity? Cytotherapy. 14: 516–521.
3. Dominici, M., M. K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Derns, A. Keating, D. Prockop, and E. Horwitz. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytometry. 68: 315–317.
4. Hakomori, S. 2003. Structure, organization, and function of glycosphingolipids in membrane. Curr. Opin. Hematol. 10: 16–24.
5. Hakomori, S., K. Handa, K. Iwabuchi, S. Yamamura, and A. Prinetti. 1998. New insights in glycosphingolipid function: “glycosignaling domain,” a cell surface assembly of glycosphingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. Glycobiology. 8: xi–xii.
6. Sonnino, S., A. Prinetti, L. Mauri, V. Chigorno, and G. Tettamanti. 2006. Dynamic and structural properties of sphingolipids as driving forces for the formation of membrane domains. Chem. Rev. 106: 2111–2125.
7. Yanagisawa, M. 2011. Stem cell glycolipids. Neurochem. Res. 36: 1625–1635.
8. Yu, R. K., and T. Arita. 2000. Ganglioside analysis by high-performance thin-layer chromatography. Methods Enzymol. 312: 115–134.
9. Adams, J., and Q. Ann. 1993. Structure determination of sphingolipids by mass spectrometry. Mass Spec. Rev. 12: 51–83.
10. Taketomi, T., and E. Sugiyama. 2000. Extraction and analysis of multiple sphingolipids from a single sample. Methods Enzymol. 312: 80–101.
11. Wucherpfennig, K. W. 2001. Structural basis of molecular mimicry. J. Autoimmun. 16: 293–302.
12. Kaida, K., T. Arita, and R. K. Yu. 2009. Antiganglioside antibodies and their pathophysiological effects on Guillain-Barre syndrome and related disorders–a review. Glycobiology. 19: 676–692.
13. Tonegawa, Y., and S. I. Hakomori. 1977. “Ganglioprotein and globoprotein”: the glycoproteins reacting with anti-ganglioside and anti-globoside antibodies and the ganglioprotein change associated with transformation. Biochem. Biophys. Res. Commun. 76: 9–17.
14. Schwarz, A., and A. H. Futerman. 1997. Determination of the localization of gangliosides using anti-ganglioside antibodies: comparison of fixation methods. J. Histochem. Cytochem. 45: 611–618.
15. Agrawal, V., and A. F. Frankel. 2010. 14G2a anti-GD2 crossreactivity with the CD166 antigen. J. Immunother. 33: 1014–1015.
16. Yanagisawa, M., T. Arita, and R. K. Yu. 2006. Cholera toxin B subunit binding does not correlate with GD1 expression: a study using mouse embryonic neural precursor cells. Glyobiology. 16: 196–226.
17. Thomas, F. P., A. M. Lee, S. N. Romanas, and N. Latov. 1989. Monoclonal IgMs with anti-Gal(beta 1-3) GalNAc activity in lower motor neuron disease; identification of glycoprotein antigens in neural tissue and cross-reactivity with serum immunoglobulins. J. Neuromuscul. 23: 167–174.
18. Bolesta, E., A. Kowalczyk, A. Wierzbicki, P. Rotkiewicz, B. Bambach, C. Y. Tsao, I. Horwack, A. Kolinski, H. Rokita, M. Brecher, et al. 2005. DNA vaccine expressing the mimotope of GD2 ganglioside induces protective GD2 cross-reactive antibody responses. Cancer Res. 65: 3410–3418.
19. Usuki, S., M. Pajaniappan, S. A. Thompson, and R. K. Yu. 2007. Chemical validation of molecular mimicry: interaction of cholera toxin with Campylobacter lipooligosaccharides. Glycoconj. J. 24: 167–180.
20. Mosna, F., L. Sensebe, and M. Krampara. 2010. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. Stem Cells Dev. 19: 1440–1452.
21. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25: 402–408.
22. Riboni, L., P. Viani, and G. Tettamanti. 2000. Estimating sphingolipid metabolism and trafficking in cultured cells using radiolabeled compounds. Methods Enzymol. 311: 656–682.
23. Papini, N., L. Anastasia, C. Tringali, G. Croci, R. Brenci, K. Yamaguchi, T. Miyagi, A. Preti, A. Prinetti, S. Prioni, et al. 2004. The plasma membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions. J. Biol. Chem. 279: 16989–16995.
24. Sonnino, S., V. Chigorno, and G. Tettamanti. 2000. Preparation of radioactive gangliosides, 3H or 14C isotopically labeled at oligosaccharide or ceramide moieties. Methods Enzymol. 311: 659–656.
25. Scandroglio, F., N. Loberto, M. Valsecchi, V. Chigorno, A. Prinetti, and S. Sonnino. 2009. Thin layer chromatography of gangliosides. Glycocongl. J. 26: 961–973.
26. Friedl, B. and J. Schrama. 1996. Practical Thin-Layer Chromatography: A Multidisciplinary Approach. CRC Press, Boca Raton, FL.
27. Sud, M., E. Fahy, D. Cotter, A. Brown, E. A. Dennis, C. K. Glass, A. H. Merrill, Jr., R. C. Murphy, C. R. Raetz, D. W. Russell, et al. 2007. LMSD: LIPID MAPS structure database. Nucleic Acids Res. 35: D527–D532.
28. Fahy, E., M. Sud, D. Cotter, and S. Subramaniam, 2007. LIMPS: online tools for lipid research. Nucleic Acids Res. 35: W606–W612.
29. Martinez, C., T. J. Heaton, P. G. Marino, M. A. Dominici, and E. M. Horwitz. 2007. Human bone marrow mesenchymal stem cells.
express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. *Blood.* **109:** 4245–4248.

30. Valaperta, R., V. Chigorno, L. Basso, A. Prinetti, R. Bresciani, A. Preti, T. Miyagi, and S. Sonnino. 2006. Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts. *FASEB J.* **20:** 1227–1229.

31. Kozbor, D. 2010. Cancer vaccine with mimotopes of tumor-associated carbohydrate antigens. *Immunol. Res.* **46:** 23–31.

32. Varki, A., H. H. Freeze, and A. E. Manzi. 2009. Overview of glycoconjugate analysis. *Curr. Protoc. Protein Sci.* **57:** 12.1.1–12.1.10.

33. Scaringi, R., M. Piccoli, N. Papini, F. Cirillo, E. Conforti, S. Bergante, C. Tringali, A. Garatti, C. Gelfi, B. Venerando, et al. 2013. NEU3 sialidase is activated under hypoxia and protects skeletal muscle cells from apoptosis through the activation of the epidermal growth factor receptor signaling pathway and the hypoxia-inducible factor (HIF)-1α. *J. Biol. Chem.* **288:** 3153–3162.

34. Ryu, J. S., K. Ko, J. W. Lee, S. B. Park, S. J. Byun, E. J. Jeong, K. Ko, and Y. K. Choo. 2009. Gangliosides are involved in neural differentiation of human dental pulp-derived stem cells. *Biochem. Biophys. Res. Commun.* **387:** 266–271.

35. Yang, H. J., K. Y. Jung, D. H. Kwak, S. H. Lee, J. S. Ryu, J. S. Kim, K. T. Chang, J. W. Lee, and Y. K. Choo. 2011. Inhibition of ganglioside GD1a synthesis suppresses the differentiation of human mesenchymal stem cells into osteoblasts. *Dev. Growth Differ.* **53:** 323–332.