Reduction of N-Glycolylneuraminic Acid Xenoantigen on Human Adipose Tissue-Derived Stromal Cells/Mesenchymal Stem Cells Leads to Safer and More Useful Cell Sources for Various Stem Cell Therapies

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Adipose tissue is an attractive source for somatic stem cell therapy. Currently, human adipose tissue-derived stromal cells/mesenchymal stem cells (hADSCs/MSCs) are cultured with fetal bovine serum (FBS). Recently, however, not only human embryonic stem cell lines cultured on mouse feeder cells but also bone marrow-derived human MSCs cultured with FBS were reported to express N-glycolylneuraminic acid (Neu5Gc) xenoantigen. Human serum contains high titers of natural preformed antibodies against Neu5Gc. We studied the presence of Neu5Gc on hADSCs/MSCs cultured with FBS and human immune response mediated by Neu5Gc. Our data indicated that hADSCs/MSCs cultured with FBS expressed Neu5Gc and that human natural preformed antibodies could bind to hADSCs/MSCs. However, hADSCs/MSCs express complement regulatory proteins such as CD46, CD55, and CD59 and are largely resistant to complement-mediated cytotoxicity. hADSCs/MSCs cultured with FBS could be injured by antibody-dependent cell-mediated cytotoxicity mechanism. Further, human monocyte-derived macrophages could phagocytose hADSCs/MSCs cultured with FBS and this phagocytic activity was increased in the presence of human serum. Culturing hADSCs/MSCs with heat-inactivated human serum for a week could markedly reduce Neu5Gc on hADSCs/MSCs and prevent immune responses mediated by Neu5Gc, such as binding of human natural preformed antibodies, antibody-dependent cell-mediated cytotoxicity, and phagocytosis. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with heat-inactivated human serum were not less than that of those cultured with FBS. For stem cell therapies based on hADSCs/MSCs, hADSCs/MSCs that presented Neu5Gc on their cell surfaces after exposure to FBS should be cleaned up to be rescued from xenogeneic rejection.

Introduction

Adipose tissue is an attractive source for somatic cell therapy, because it is safe and abundant and many investigators have reported that the stromal cells derived from adipose tissue (adipose tissue-derived stromal cells [ADSCs]) could differentiate into various cell types.1-4 ADSCs are also referred to as adipose tissue-derived mesenchymal stem cells (MSCs). Human ADSCs (hADSCs)/MSCs are very similar to bone marrow (BM)-derived human MSCs (hMSCs) and therefore reveal differentiation potential similar to BM-derived hMSCs.5-7

For stem cell therapies based on hMSCs including hADSCs/MSCs, it is essential that stem cells are handled and cultured in a manner that guarantees the efficacy and safety of the cellular therapy product. One such aspect is the choice...
of cell culture medium and supplements. In principle, most investigators agree that all animal materials should be avoided to maximize product safety. Currently, however, hADSCs/MSCs are cultured with fetal bovine serum (FBS), and the clinical efficacy of BM-derived hMSCs in human disease has been investigated using hMSCs cultured with FBS in a number of clinical trials.1-12

Recently, not only human embryonic stem cell (hESC) lines cultured on mouse feeder cells but also BM-derived hMSCs cultured with FBS were reported to express N-glycine/uraminic acid (Neu5Gc) xenotransient.13,14 The so-called Hananmutzu-Deitcher antigen.15 Humans are incapable of synthesizing the common mammalian sialic acid, Neu5Gc, because of an AIV transposon-mediated inactivation of the cytidine monophosphate (CMP)-N-acetylluronaminic acid hydroxylase gene.16,17 Despite this, both hESC lines and BM-derived hMSCs were reported to express the Neu5Gc, apparently originating from the mouse feeder layers, animal-derived components, and FBS.13,14 The significant levels of Neu5Gc found on the surface of hESCs and hMSCs evidently originate from a Trojan Horse pathway involving endocytosis of extracellular glycoconjugates, delivery to the lysosome, release of Neu5Gc by lysosomal sialidase, active transport to the cytoplasm through the lysosomal sialidase transporter, activation by CMP, and addition to nascent glycoproteins and glycolipids in the secretory pathway.18 It is also possible that amphiphilic molecules carrying Neu5Gc might be directly transferred into the hESC and hMSC plasma membranes.19 Human serum contains higher titers of natural preformed antibodies against Neu5Gc xenotransient.20-22 Thus, binding of these natural preformed antibodies may lead to immune responses such as complement-mediated cytotoxicity (CMC) antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis. However, these immune responses mediated by natural preformed antibodies against human stem cells remain in controversy.13,23 This study was therefore undertaken to study the presence of Neu5Gc on hADSCs/MSCs cultured with FBS and the human immune responses mediated by Neu5Gc on hADSCs/MSCs.

Materials and Methods

Cells

hADSCs/MSCs were prepared as described previously1,2 with modifications.3,4 Adipose tissue was dissected during plastic surgery in five human subjects (four men and one woman; age, 20-60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue was collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of the Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and Foundation for Biomedical Research and Innovation. All subjects fasted for at least 10 h before surgery and none was being treated with steroids. The dissected excess adipose tissue was minced and then digested in Hank’s balanced salt solution (Gibco Invitrogen; Grand Island, NY) containing 0.075% collagenase type II (Sigma Aldrich, St. Louis, MO) at 37°C for 1 h. Digests were filtered with a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 g for 10 min. Erythrocytes were excluded using density gradient centrifugation with Lymphoprep (d = 1.077; Nycomed, Oslo, Norway). The cells were then plated using Dulbecco’s modified Eagle’s medium (DMEM; Gibco Invitrogen) with 10% defined FBS (Hyclone, Northumberland, United Kingdom) and incubated for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and treated with 0.2 g/L ethylenediaminetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan), and the resulting suspended cells were replated at a density of 10,000 cells/cm2 on human fibronectin-coated dishes (BD BioCoat, Franklin Lakes, NJ) in a medium containing 60% DMEM-low glucose, 40% MCDB-201 medium (Sigma Aldrich), 100 μM ascorbic acid 2-phosphate (Sigma Aldrich), 10 ng/mL epidermal growth factor (PeproTec, Rocky Hill, NJ), and 5% FBS. For analysis of the effects of human serum on Neu5Gc expression on hADSCs/MSCs, the cells were cultured for 7 days, where FBS was replaced by 5% heat-inactivated normal human pooled serum (NHS) from type AB blood. As control cells, a murine pancreatic cell line, Panc02, was cultured with RPMI 1640 medium (Gibco Invitrogen) supplemented with 10% FBS and 1% antibiotic/antimycotic solution.

Flow cytometry

Cells were detached from culture dishes and suspended in Dulbecco’s phosphate-buffered saline (D-PBS; Nacalai Tesque). Aliquots (5 x 10⁶ cells) were incubated for 30 min at 4°C with a chicken anti-Neu5Gc polyclonal antibody (a gift from Prof. N. Wakamiya, Asahikawa Medical College, Hokkaido, Japan).24 Cells incubated with D-PBS alone were used as negative control. After washing with D-PBS, cells were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken immunoglobulin G (IgG; Cappel, Cochranville, PA) as a second antibody. After staining, the cells were washed

FIG. 1. Expression of Neu5Gc on hADSCs/MSCs. (A) Specificity of anti-Neu5Gc antibody; Panc02, a cell line derived from murine pancreatic carcinomas, expressed Neu5Gc. Flow cytometric analysis showed that chicken anti-Neu5Gc polyclonal antibody bound to the surfaces of Panc02, but Neu5Gc-preadsorbed anti-Neu5Gc polyclonal antibody could not react, showing specificity of the anti-Neu5Gc antibody. The percentage of cells that stained positive is indicated in the upper right corner of each panel. (B) Expression of Neu5Gc xenotransient on hADSCs/MSCs. Fresh hADSCs/MSCs did not express Neu5Gc on their cell surface. In accordance with passage numbers, the population of Neu5Gc-positive cells increased by cultivation with FBS. The percentage of cells that stained positive is indicated in the upper right corner of each panel. (C) Reduction of Neu5Gc xenotransient by chelating cultivation with human serum. After cultivaition of hADSCs/MSCs with heat-inactivated NHS but not FBS, the percentages of Neu5Gc-positive cells have decreased in accordance with culture duration. The decrement manners of second passaged hADSCs/MSCs and fifth passaged ones have been in a similar fashion. The percentage of cells that stained positive is indicated in the upper right corner of each panel. Data are representative of four independent experiments. Neu5Gc, N-glycolylneuraminic acid; hADSCs/MSCs, human adipose tissue-stromal cells/mesenchymal stem cells; FBS, fetal bovine serum; NHS, normal human pooled serum; IgG, immunoglobulin G; M1, marked positive area 1; FL1, fluorescence1.
and resuspended in D-PBS with 150 ng/mL 7-AAD (BD Pharmingen) to eliminate dead cells. The cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ). Data shown in figures are gated for live cells by excluding cells that remained positive for 7-AAD. Percentage of positive cells was defined against a 99% negative control exclusion gate. For detection of binding of human natural preformed antibodies, the cells were exposed to 10% fresh NHS or 5 mM Neu5Gc-preadsorbed NHS in D-PBS containing 15 mM EDTA for 30 min at 4°C. After washing, the cells were stained with FITC-conjugated goat anti-human IgG or IgM antibody (Cappel), or control goat IgG, respectively. To examine the blocking effects of anti-Neu5Gc antibody onto the surface of hADSCs/MSCs, hADSCs/MSCs cultured with FBS were precoated with anti-Neu5Gc antibody, exposed to 10% fresh NHS containing 15 mM EDTA, and then applied for flow cytometric analysis. Stained cells were washed and resuspended in D-PBS with 7-AAD and analyzed by a FACSCalibur flow cytometer. For detection of human complement regulatory proteins, cells were stained with FITC-conjugated mouse monoclonal antibodies to human CD46 (membrane cofactor protein), CD55 (decay accelerating factor), CD59, or control IgG (all from BD Pharmingen) and analyzed by a FACSCalibur flow cytometer as well.

Detection of complement deposition

The amounts of C4 and C3 fragments deposited on the cell surface were also analyzed by flow cytometry. The cells were detached by 0.25% trypsin/EDTA and subsequently incubated with 10% fresh NHS in DMEM for 30 min at 37°C. Cells incubated with DMEM alone or 10% fresh NHS in DMEM containing 15 mM EDTA was used as negative control. After washing with cold D-PBS three times, the cells were stained with FITC-conjugated rabbit anti-human C4c or C3c antibody (Dako, Cambridgeshire, United Kingdom). After staining, the cells were washed and resuspended in 500 μL of D-PBS with 7-AAD and analyzed by a FACSCalibur flow cytometer as well.

CMC assay

CMC was evaluated by measuring lactate dehydrogenase (LDH) release in media, using MTT-LDH kit (Kyoritsu Pharm, Tokyo, Japan) in accordance with the manufacturer's instructions. Target cells (hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, or Panc02) were plated at a concentration of 1 × 10^6 cells/well in a 96-well culture plate. Then, DMEM with 20% or 40% fresh NHS was added. The plates were incubated for 2 h at 37°C, and LDH release was determined. All assays included maximal release controls (1% Triton X), controls with medium and target cells, with medium containing fresh NHS, and with medium alone.

Isolation of effector cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats from healthy volunteers using density gradient centrifugation with Lymphoprep (Nycomed). Cell viability was more than 98%, as determined by trypan blue exclusion. Human monocyte-derived macrophages were isolated and cultured as reported previously.75

ADCC assay

ADCC was also determined by measuring LDH release into medium. Target cells (hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, or Panc02) were plated in 96-well culture plates as described earlier. Then, 1 × 10^5 or 2 × 10^5 PBMCs in DMEM alone or with 10% heat-inactivated NHS were added. The plates were incubated for 4 h at 37°C, and LDH release was determined. All assays included maximal release controls (1% Triton X), controls with medium and target cells, with medium and effector cells, with medium containing 10% heat-inactivated NHS, and with medium alone.

Phagocytosis assay

Target cells (hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, or Panc02) were stained with PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma Aldrich) according to the manufacturer's instructions. After labeling of target cells was terminated, the cells were washed and resuspended in RPMI medium. Then, 2 × 10^5 PKH67-labeled target cells were added into 24-well
culture plates and incubated with 2 \times 10^5 human monocyte-derived macrophages (Effector:Target \{E:T\} = 1:10) in 1 mL of RPMI 1640 medium alone or with 10% heat-inactivated NHS for 24 h at 37 °C. Following incubation, the target cells and human monocyte-derived macrophages were harvested with EDTA solution. The cells were counterstained with allophtalocyanin-conjugated mouse monoclonal antibodies to human CD11c (BD Pharmingen) and washed and fixed with 2% formaldehyde-PBS. Two-color flow cytometric analysis was performed with a FACSCalibur flow cytometer under optimal gating. PKH67-labeled target cells were detected in the FL-1 channel and allophtalocyanin-labeled human monocyte-derived macrophages were detected in the FL-4 channel. Dual-labeled cells (PKH67+ /CD11c+) were considered to represent phagocytosis of targets by human monocyte-derived macrophages. Residual target cells were defined as cells that were PKH67-/CD11c-

Adipogenic and osteogenic differentiation procedure

For adipogenic differentiation, cells were cultured in differentiation medium (Zen-Bio, Durham, NC). After 3 days, half of the medium was changed with adipocyte medium (Zen-Bio) every 2 days. Ten days after differentiation, characterization of adipocytes was confirmed by microscopic observation of intracellular lipid droplets by oil red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 mM dexamethasone, 50 mg/dL ascorbic acid 2-phosphate, 10 mM beta-glycerophosphate (Sigma, St. Louis, MO), and 10% FBS or heat-inactivated NHS. The differentiation was examined by alizarin red staining and alkaline phosphatase (AP) activity. For alizarin red staining, 7 or 18 days after differentiation, the cells were washed three times and fixed with dehydrated ethanol. After fixation, the cells were stained with 1% alizarin red S in 0.1% NH₄OH (pH 6.5) for 5 min and then washed with H₂O. AP activity was investigated at 2 weeks after differentiation using the procedure described previously. AP activity per cell was calculated based on the amount of DNA. DNA content was measured by a modification of the method of Labarca and Paigen.

Statistics

Values are given as the mean ± standard deviation. Student’s t-test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when p < 0.05. All statistical analyses were performed using the SPSS Statistics 17.0 package (SPSS, Chicago, IL).

Results

Presence of Neu5Gc and human natural preformed antibodies binding to hADSCs/MSCs

First, the specificity of chicken anti-Neu5Gc polyclonal antibody was examined (Fig. 1A). Flow cytometric analysis showed that chicken anti-Neu5Gc polyclonal antibody bound to the surfaces of Panc02, which constitutively expressed Neu5Gc, but Neu5Gc-preadsorbed anti-Neu5Gc polyclonal antibody could not react, indicating the anti-Neu5Gc antibody reacts to Neu5Gc specifically. Next, incorporation of Neu5Gc antigen via FBS-containing medium was examined (Fig. 1B). Fresh hADSCs/MSCs did not express Neu5Gc on their cell surface. In accordance with passage numbers, the population of Neu5Gc-positive cells has increased by cultivation with FBS (fresh: 0.33%; passage number 2: 19.77%; and passage number 5: 86.6%). Cultured with heat-inactivated NHS could markedly reduce Neu5Gc in human colon carcinoma cells, \(^{13}\) hESC, \(^{13}\) and hMSCs, \(^{14}\) apparently as the result of metabolic replacement by N-acetylneuraminic acid in the human serum. So, the reduction of incorporated Neu5Gc xenoreagent by chasing cultivation with human serum was examined (Fig. 1C). The Neu5Gc xenoreagent was reduced after cultivation of hADSC/MSCs with heat-inactivated NHS but not FBS. The percentages of Neu5Gc-positive cells have decreased in accordance with culture duration, and the decrement manners of second passaged hADSCs/MSCs and fifth passaged ones have been in a similar fashion.

Because human serum contains high titers of natural preformed antibodies against the Neu5Gc xenoreagent, \(^{15,16}\) we assessed whether such antibodies could recognize Neu5Gc-containing epitopes on hADSCs/MSCs cultured with FBS (Fig. 2). Panc02 cultured with FBS and exposed to 10% fresh NHS containing 15 mM EDTA showed high human IgG (99.9%) and IgM (92.8%) binding (Fig. 2Aa). hADSCs/MSCs cultured with FBS and treated with fresh NHS also showed high human IgG binding (80.1%), but human IgM binding was very low (3.2%) (Fig. 2Ab). Preincubation of fresh NHS with Neu5Gc resulted in significant decrease in human IgG binding on hADSCs/MSCs cultured with FBS (80.1% to 2.0%). Further, pretreatment of hADSCs/MSCs with anti-Neu5Gc polyclonal antibody also resulted in reduction of human IgG binding (70.3% to 1.74%; Fig. 2B). Culturing hADSCs/MSCs with heat-inactivated NHS, which decreased Neu5Gc expression of hADSCs/MSCs effectively, reduced human IgG binding on hADSCs/MSCs when exposed to fresh NHS (Fig. 2C). Taken together, these data indicate that the hADSCs/MSCs cultured with FBS expressed Neu5Gc and the human natural preformed antibodies could bind to hADSCs/MSCs. This binding of human natural preformed antibodies on hADSCs/MSCs was related to the amount of Neu5Gc on hADSCs/MSCs. Culture with heat-inactivated NHS could markedly reduce IgG binding on hADSCs/MSCs when exposed to fresh NHS (80.1% to 3.9%).

Complement fragment deposition on hADSCs/MSCs and CMC assay

Cell surface antibody binding may activate the classical complement pathway leading to cytotoxicity. We assessed whether the deposition of complement fragments on hADSCs/MSCs occurred after exposure to fresh NHS. Whether hADSCs/MSCs were cultured with FBS or heat-inactivated NHS, the amount of deposition of C4 and C3 fragments on hADSCs/MSCs after a short incubation period of 30 min was no different from negative control (cells incubated with DMEM alone or 10% fresh NHS in DMEM containing 15 mM EDTA) (Fig. 3). To control for the fresh NHS activity and variability, we tested the deposition of C4 and C3 fragments on Panc02. Both complement fragments were clearly deposited on Panc02 (C4: 84.6%; C3: 98.9%) and this deposition was abolished by adding 15% EDTA (Fig. 3). We next analyzed the CMC of hADSCs/MSCs cultured with FBS or heat-inactivated NHS. To control for CMC of fresh NHS,
we tested CMC of Panc02. CMC of Panc02 was clearly detected (20% NHS: 42.7% ± 4.7%; 40% NHS: 65.4% ± 2.4%). In contrast, significant specific lysis of hADSCs/MSCs cultured with FBS or heat-inactivated NHS was not detected (hADSCs/MSCs cultured with FBS + 20% NHS: 4.8% ± 1.3%; or 40% NHS: 7.4% ± 2.0%; hADSCs/MSCs cultured with heat-inactivated NHS: 20% NHS: 3.6% ± 1.6%; 40% NHS: 5.6% ± 1.6%). We then analyzed the expression of complement regulatory proteins such as CD46, CD55, and CD59 on hADSCs/MSCs. hADSCs/MSCs were weakly positive for both CD46 (22.1%) and CD55 (29.8%) and highly positive for CD59 (97.5%) (Fig. 4B). These data indicate that hADSCs/MSCs express complement regulatory proteins such as CD46, CD55, and CD59 and are largely resistant to killing by CMC mechanism.

ADCC of hADSCs/MSCs mediated by human natural preformed antibodies in NHS

IgG antibodies play an important role in ADCC. Our study demonstrated that natural preformed IgG antibodies could bind to hADSCs/MSCs cultured with FBS. Therefore, to evaluate the role of these IgG antibodies in cell-mediated cytotoxicity, ADCC assay was performed with hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, or Panc02 as targets and human PBMCs as effector cells, using E:T ratios of 10:1 and 20:1, and 4-h incubation periods. PBMCs in the absence of heat-inactivated NHS caused no significant lysis of hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, and Panc02 (hADSCs/MSCs cultured with FBS: E:T= 10:1, 2.37% ± 0.35%; E:T= 20:1, 3.78% ± 0.85%; hADSCs/MSCs cultured with heat-inactivated NHS: E:T= 10:1, 0.57% ± 0.36%; E:T= 20:1, 2.34% ± 0.67%; Panc02: E:T= 10:1, 1.98% ± 0.35%; E:T= 20:1, 4.72% ± 0.54%; Fig. 5, white bar). The cytotoxicity of Panc02 in the presence of heat-inactivated NHS was significantly greater than that in the absence of heat-inactivated NHS (in the presence of NHS vs. in the absence of heat-inactivated NHS: E:T = 10:1, 27.4% ± 3.1% vs. 1.98% ± 0.35%, p < 0.05; E:T = 20:1, 28.9% ± 4.6% vs. 4.72% ± 0.54%, p < 0.05), which proved the effective use of PBMCs (Fig. 5). A significant increase of cytotoxicity of the hADSCs/MSCs cultured with FBS was also evident in the presence of heat-inactivated NHS (in the presence of heat-inactivated NHS vs. in the absence of heat-inactivated NHS: E:T = 10:1, 13.5% ± 0.82% vs. 2.37% ± 0.35%, p < 0.05; E:T = 20:1, 16.0% ± 1.5% vs. 3.78% ± 0.85, p < 0.05; Fig. 5). In contrast, no increase of cytotoxicity of the hADSCs/MSCs cultured with heat-inactivated NHS was detected in the presence of heat-inactivated NHS (in the presence of heat-inactivated NHS vs. in the absence of heat-inactivated NHS: E:T = 10:1, 3.23% ± 0.52% vs. 0.57% ± 0.36%; E:T= 20:1, 3.75% ± 0.51% vs. 2.34% ± 0.67%; Fig. 5). In addition, the cytotoxicity the hADSCs/MSCs cultured with FBS was significantly greater than that of hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible amount of Neu5Gc (hADSCs/MSCs cultured with FBS vs. hADSCs/MSCs cultured with heat-inactivated NHS: E:T = 10:1, 13.5% ± 0.82% vs. 3.23% ± 0.52%, p < 0.05; E:T = 20:1, 16.0% ± 1.5% vs. 3.75% ± 0.51, p < 0.05; Fig. 5). Taken together, these data indicate that the hADSCs/MSCs cultured with FBS are injured by ADCC mechanism. In contrast, hADSCs/MSCs cultured with NHS are less sensitive to ADCC.

Phagocytosis of hADSCs/MSCs by human monocyte-derived macrophages

hADSCs/MSCs cultured with FBS, hADSCs/MSCs cultured with heat-inactivated NHS, or Panc02 were stained with fluorescent PKH67, respectively. Labeled cells were cocultured with human monocyte-derived macrophages in the presence or absence of heat-inactivated NHS for 24 h. After counterstaining with monoclonal antibodies to human CD11c, two-color flow cytometric analysis was performed.
(Fig. 6). Phagocytosis of target cells by human monocyte-derived macrophages could be identified as dual-labeled cells (PKH67⁺/CD11c⁺, right upper panel). Similar results were obtained in three independent experiments. Phagocytosis of Panc02 was clearly detectable (10.6%) and increased twofold in the presence of heat-inactivated NHS, which proved the effective use of human monocyte-derived macrophages. Phagocytosis of hADSCs/MSCs cultured with NHS by human monocyte-derived macrophages was somewhat detectable (5.7%) and also increased in the presence of heat inactivated human serum (9.3%). In contrast, human monocyte-derived macrophages could not phagocytose hADSCs/MSCs cultured with heat-inactivated NHS neither in the absence nor in the presence of heat-inactivated NHS (medium alone: 1.1%; 10% heat-inactivated NHS: 2.2%; Fig. 6). Thus, human monocyte-derived macrophages phagocytosed hADSCs/MSCs cultured with FBS and this phagocytic activity increased when hADSCs/MSCs cultured with FBS were opsonized by the natural preformed antibodies in the presence of heat-inactivated NHS. In contrast, hADSCs/MSCs cultured with heat-inactivated NHS were resistant to phagocytosis either in the absence or in the presence of heat-inactivated NHS.

**Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with FBS and heat-inactivated NHS**

To compare the *in vitro* differentiation potential of hADSCs/MSCs cultured with FBS or heat-inactivated NHS, cells were differentiated toward the adipogenic and osteogenic lineages. Adipogenic differentiation was induced by culture with differentiation medium containing 1-methyl-3-isobutylxanthine, peroxisome proliferator-activated receptor (PPAR)-gamma agonist, dexamethasone, and insulin. The acquisition of the adipogenic phenotype was determined by staining the cell monolayers with oil red O (Fig. 7A). The efficiency of adipogenesis of hADSCs/MSCs cultured with heat-inactivated NHS was similar to that of hADSCs/MSCs cultured with FBS (Fig. 7A). Both hADSCs/MSCs showed multiple intracellular lipid-
filled droplets in 35–50% of cells after adipogenic induction. Osteogenic differentiation was induced by treating cells with low concentrations of dexamethasone, ascorbic acid, and betaglycerophosphate. Calcium deposition was demonstrated by staining monolayers with alizarin red (Fig. 7B). hADSCs/MSCs cultured with heat-inactivated NHS and those cultured with FBS showed similar potential toward osteogenic differentiation. High AP activity was detected in hADSCs/MSCs cultured with heat-inactivated NHS and those cultured with FBS in response to osteogenic induction after 2 weeks (Fig. 7B).

FIG. 5. Antibody-dependent cell-mediated cytotoxicity assay of hADSCs/MSCs. The cytotoxic activity of peripheral blood mononuclear cells against hADSCs/MSCs in the absence (white bar) or presence (black bar) of 10% NHS was tested by measuring lactate dehydrogenase release into medium (Effector:Target [E:T] = 10:1 or 20:1). Data are shown as mean ± standard deviation (*p < 0.05) and are representative of three independent experiments.

FIG. 6. Representative flow cytometry profiles of phagocytosis assay of hADSCs/MSCs. Upper left quadrant: Region of residual target cells. Upper right quadrant: Region of phagocytosed target cells. Percentages represent those of total cells in each region. Data are representative of three independent experiments.
FIG. 7. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with FBS and NHS. (A) The efficiency of adipogenesis of hADSCs/MSCs cultured with NHS was similar to that of hADSCs/MSCs cultured with FBS. The efficacy of osteogenic differentiation and alkaline phosphatase activity was similar between cultures with NHS and FBS in response to osteogenic induction. Data are representative of four independent experiments.

Discussion

Previous studies have reported that hESCs and BM-derived hMSCs are capable of efficient Neu5Gc uptake from culture media components. Human serum contains high titers of natural preformed antibodies against Neu5Gc xenoreagent and binding of these natural preformed antibodies may lead to immune responses. Importantly, this may be reflected in the published results of human clinical trials using BM-derived hMSCs cultured with FBS. Further, in human clinical trials with FBS-grown hMSCs, antibodies against FBS have been detected. However, these immune responses against human stem cells mediated by natural preformed antibodies remain in controversy.

In this study, because of the usefulness of hADSCs/MSCs as an alternative source of stem cells, we assessed the presence of Neu5Gc in hADSCs/MSCs cultured with FBS and the human immune response mediated by Neu5Gc xenoreagent.

Our study using a chicken anti-Neu5Gc polyclonal antibody showed that most of the hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoreagent. This result is similar to the previous study that hESCs and BM-derived hMSCs express Neu5Gc. In addition, our data suggested...
that human natural preformed antibodies could bind to hADSCs/MSCs after exposure to fresh NHS. The subtype of natural preformed antibodies was mainly IgG, not IgM. This human IgG binding was related to the amount of Neu5Gc on the hADSCs/MSCs, because hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc showed negligible levels of IgG binding when exposed to fresh NHS. This result is also consistent with the previous study that anti-Neu5Gc antibodies constitute the majority of natural preformed xenoreactive antibodies besides anti-galactose-alpha 1,3-galactose (Gal) antibodies, particularly in the IgG subclass.22,23 In effect, hADSCs/MSCs cultured with FBS may seem like xenogeneic cells to the human immune systems.

When xenogeneic grafts are transplanted into humans, binding of natural preformed antibodies that recognize xenoantigens, including Gal and Neu5Gc, mediates two types of rejection response, hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR).19 HAR begins with binding of natural preformed antibodies to the xenogeneic epitopes on donor endothelial cells, including Gal and Neu5Gc xenoantigens, leading to complement activation by mainly classical pathway.19 The graft is rejected within minutes to hours. Therefore, we analyzed the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen, using fresh NHS. However, we could not confirm the existence of CMC. The deposition of C4 and C3 fragments on hADSCs/MSCs after a short incubation with fresh NHS could not also be detected. In this issue, there are no reports describing the CMC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Martin et al. reported that binding of natural preformed antibodies to Neu5Gc on hESCs mediated complement activation leading to cell death.19 In contrast, Cerdan et al. reported that complement activation by anti-Neu5Gc antibody does not mediate killing of hESCs.25 Several reasons for this discrepancy have been supposed. One is the difference of procedures used for testing cell cytotoxicity. Previous two reports detected cell cytotoxicity by propidium iodide or 7-AAD exclusion using flow cytometry. Single-cell suspension required for this procedure may cause extensive cell death even under controlled conditions. We detected cell cytotoxicity by conventional LDH release assay, which is often used in cytotoxicity assays.26,27 The other and more possible reason is the biological difference among the human stem cells, including hESCs and hMSCs. We assessed the expression of complement regulatory proteins such as CD46, CD55, and CD59 on hADSCs/MSCs. hADSCs/MSCs were weakly positive for both CD46 and CD55 and highly positive for CD59. It is reported that HAR could be prevented by inhibiting complement activation, using transgenic animals bearing transgenes encoding human complement regulatory proteins.28,29 Thus, it is supported that hADSCs/MSCs express complement regulatory proteins and may be largely resistant to killing by CMC mechanism. However, the expression of complement regulatory proteins on other human stem cells such as hESCs remains uncertain and further investigation is needed.

AHXR occurs when HAR is prevented, and it can be induced by low levels of natural preformed antibodies.20 The binding of natural preformed antibodies to xenogeneic endothelial cells results in ADCC by natural killer cells, macrophages, and neutrophils, endothelial cell activation, thrombosis, and vasoconstriction.20 It is reported that AHXR could be mediated by natural preformed antibodies against non-Gal xenoantigen,29,30 particularly Neu5Gc xenoantigen.29 Therefore, we analyzed the ADCC of hADSCs/MSCs cultured with FBS that express Neu5Gc xenoantigen. Our data indicated the clear existence of ADCC of hADSCs/MSCs cultured with FBS. This ADCC is supposed to be mediated by preformed natural antibodies that recognize Neu5Gc because ADCC of hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc could not be detected. We also analyzed the antibody-mediated cell phagocytosis of hADSCs/MSCs cultured with FBS by monocyte-derived macrophage because macrophages can target opsonized cells. However, in our study, a low level of phagocytic activity of hADSCs/MSCs cultured with FBS even in the absence of NHS was detected and this phagocytic activity clearly increased in the presence of NHS. Ide et al. reported that human macrophages could phagocytose porcine cells in an antibody- and complement-independent manner and elimination of Gal on porcine cells that expressed Neu5Gc did not prevent this phagocytic activity.31 Our data indicated that hADSCs/MSCs cultured with heat-inactivated NHS which expressed negligible levels of Neu5Gc were resistant to phagocytosis mediated by human macrophages in the presence or absence of fresh NHS. Accordingly, human macrophages may be able to recognize Neu5Gc xenoantigen and phagocytose hADSCs/MSCs.

We showed here that hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoantigen and that binding of natural preformed antibodies led to immune response. Based on current data, it is clear that hADSCs/MSCs should be chased without animal materials. Yamaguchi et al. have tried xenofree techniques on hematopoietic stem cells by growing them on human stromal cells and using medium containing NHS.41 To eliminate Neu5Gc on hADSCs/MSCs, we cultured them in a medium in which FBS was replaced by heat-inactivated NHS for a week after culturing with FBS. The expression of Neu5Gc on these hADSCs/MSCs was extremely reduced. Heiskanen et al. described that BM-derived hMSCs became decontaminated after 2 weeks of culture in a medium in which FBS was replaced by NHS, but complete decontamination was difficult to achieve by changing culture conditions.42 Therefore, hADSCs/MSCs may not be completely decontaminated with Neu5Gc by culturing with heat-inactivated NHS for a week. However, our data suggested that human immune responses mediated by Neu5Gc on hADSCs/MSCs, such as ADCC and phagocytosis, were nearly completely prevented by this culture condition. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with heat-inactivated NHS were not less than that of those cultured with FBS. This work implies that the culture conditions avoiding renewed exposure to animal materials can reduce the expression of Neu5Gc on hADSCs/MSCs and consequently prevent human immune responses against hADSCs/MSCs. Although major complications have not been reported in the clinical trials with hMSCs cultured with FBS, human immune responses mediated by Neu5Gc may potentially influence the survival and efficacy of the transplanted cells and thus bias the published results. For clinical application of stem cell therapies based on hADSCs/MSCs, hADSCs/MSCs that presented Neu5Gc on their cell surfaces after
exposure to fBS should be cleaned up by chasing without Neu5Gc condition and thus might be rescued from xenogeneic rejection.

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