Bioorganic & Medicinal Chemistry 18 (2010) 3059–3065

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Dodecaborate lipid liposomes as new vehicles for boron delivery system of neutron capture therapy

Manabu Ueno [a], Hyun Seung Ban [a], Kei Nakai [b], Ryu Inomata [a], Yasufumi Kaneda [c], Akira Matsumura [b], Hiroyuki Nakamura [a,*]

[a] Department of Chemistry, Faculty of Science, Gakushuin University, Mejiro, Toshima-ku, Tokyo 171-8588, Japan
[b] Department of Neurosurgery, Institute of Clinical Medicine, University of Tsukuba, Tenno-dai, Tsukuba 305-8575, Japan
[c] Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 12 February 2010
Revised 17 March 2010
Accepted 18 March 2010
Available online 27 March 2010

Keywords:
Boron neutron capture therapy (BNCT)
Boron delivery system (BDS)
Boronated liposomes
closo-Dodecaborate lipids

ABSTRACT

closo-Dodecaborate lipid liposomes were developed as new vehicles for boron delivery system (BDS) of neutron capture therapy. The current approach is unique because the liposome shell itself possesses cytocidal potential in combination with neutron irradiation. The liposomes composed of *closo*-dodecaborate lipids DSBL and DPBL displayed high cytotoxicity with thermal neutron irradiation. The *closo*-dodecaborate lipid liposomes were taken up into the cytoplasm by endocytosis without degradation of the liposomes. Boron concentration of 22.7 ppm in tumor was achieved by injection with DSBL-25% PEG liposomes at 20 mg B/kg. Promising BNCT effects were observed in the mice injected with DSBL-25% PEG liposomes: the tumor growth was significantly suppressed after thermal neutron irradiation (1.8×10^{12} neutrons/cm^2).

1. Introduction

The cytotoxic effect of boron neutron capture therapy (BNCT) is due to the nuclear reaction of two essentially nontoxic species, boron-10 and thermal neutrons (Eq. 1):[1]

$$^{10}B + {}^{1}n \rightarrow {}^{4}He(\alpha) + {}^{7}Li + 2.4\ MeV \quad (1)$$

The resulting α-particle and Li nuclei are high linear energy transfer (LET) particles that exert the cytotoxic effect. The fact that LET particles travel a short range (approximately 10 μm) limits radiation-induced damage to cells containing boron-10. Therefore, the high accumulation and selective delivery of boron-10 into tumor tissue are the most important requirements to achieve efficient BNCT of cancers.[2–5] The amount of boron-10 necessary to realize fatal tumor cell damage is 20–35 μg/g tumor tissue.[6] At the same time, boron concentration in surrounding normal tissues and blood should be kept low to minimize damage to those tissues. Although mercaptoundecahydrododecaborate (BSH; $Na_2B_{12}H_{11}SH$)[7,8] and L-*p*-boronophenylalanine (L-BPA)[9,10] have been utilized for BNCT, the development of new boron-10 carriers that deliver an adequate concentration of boron-10 atoms to a tumor is still an important task to achieve effective cancer therapy.[11–13]

Recently much attention has been focused on the liposomal boron delivery system (BDS). Liposomes are efficient drug delivery vehicles because they can transport their contents to the interior of various tumors in a manner that is essentially independent of their contents. Therefore, boron compounds-encapsulated liposomes are attractive vehicles to deliver adequate quantities of boron to the tumor cells for BNCT. Various boron compounds-encapsulated BDSs have been developed including passive targeting liposomes[14–16] and/or active targeting liposomes by conjugating tumor specific ligands, such as mAb,[17–19] folate,[20] epidermal growth factor,[21] and transferrin (TF).[22,23] However, in order to deliver therapeutic quantities of boron to the tumor cells, concentrated aqueous solutions of polyhedral borane salts must be encapsulated. This causes osmotic problem of the liposome production.

In contrast, the development of lipophilic boron compounds embedded within the liposome bilayer is an attractive means to increase the overall incorporation efficiency of boron-containing species, as well as to raise the gross boron content of the liposome in the formation. Selective boron delivery to tumors by lipophilic species incorporated in the membranes of unilamellar liposomes was first demonstrated by Hawthorne and co-workers.[24,25] We previously reported the first synthesis of *nido*-carborane lipid (**1**) having a double-tailed moiety conjugated with *nido*-carborane as a hydrophilic moiety and its vesicle formation from **1** (Fig. 1).[26] Furthermore, we investigated the possibility of actively targeting boron liposomes to solid tumor by conjugating TF to the surface of the liposomes. Boron concentration of 22 μg ^{10}B/g tumor was observed

* Corresponding author. Tel.: +81 3 3986 0221; fax: +81 3 5992 1029.
E-mail address: hiroyuki.nakamura@gakushuin.ac.jp (H. Nakamura).

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doi:10.1016/j.bmc.2010.03.050

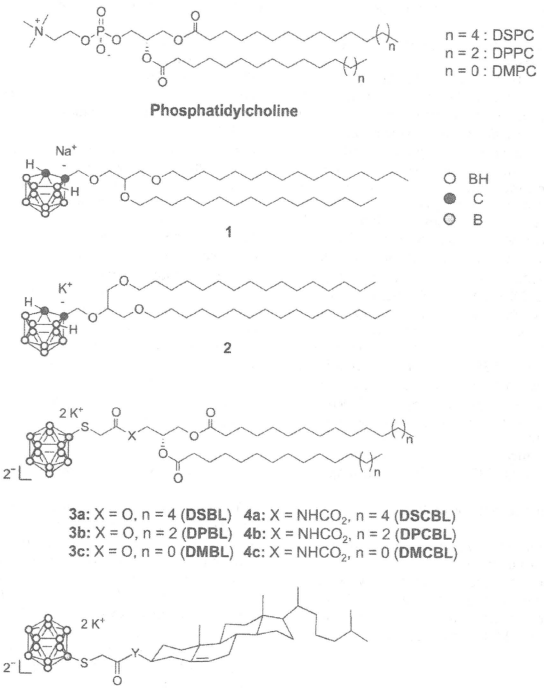


Figure 1. Structures of phosphatidylcholine, boron lipids (**1–4**).

in mice injected with the boron liposomes at 7.2 mg ^{10}B/kg body weight. However, the injection of a higher boron concentration (14 mg ^{10}B/kg body weight) resulted in acute toxicity to the mice.[27] Hawthorne and co-workers also recently reported similar acute toxicity in mice injected with *nido*-carborane lipid (**2**).[28] We surmised that this high toxicity may be caused by the *nido*-carborane structure, although the mechanism of *nido*-carborane cytotoxicity has not been studied in detail.

In order to overcome this drawback, we focused on BSH as an alternative hydrophilic boron cluster for boron lipids. BSH is a water-soluble divalent '*closo*-type' anion cluster that has significantly low toxicity. Because of this property, BSH has been utilized for BNCT. We were the first to synthesize *closo*-dodecaborate lipids (**3** and **4**) that possess the $B_{12}H_{11}S$ moiety as the hydrophilic function and have similar chirality to natural phospholipids, such as DSPC, in their lipophilic tails (Fig. 1).[29,30] We also focused on cholesterol, which is also one of the important contents for liposome formation, and synthesized *closo*-dodecaborate-conjugated cholesterols.[31] As we surmised, the liposomes prepared from the current *closo*-dodecaborate lipids and cholesterols did not show acute toxicity at 20 mg ^{10}B/kg body weight in healthy mice.[32] Recently, various boron compounds embedded within the liposome bilayer have been reported for BDS,[24,28,33–35] however their BNCT effects have not been reported yet. We found the significant BNCT effects of the mice treated with the current *closo*-dodecaborate lipid liposomes after neutron irradiation.[32] In this paper, we provide a full account of our BDS studies using *closo*-dodecaborate lipid liposomes.

2. Materials and methods

2.1. Chemicals

DSPC (MC-8080) and DSPE-PEG (SUNBRIGHT DSPE-020CN) were purchased from Nippon Oil and Fats (Japan). Cholesterol (Chol) was purchased from Kanto Chemical (Japan). $Na_2{}^{10}B_{12}H_{11}SH$ was kindly supplied by Stella Chemifa (Japan). DSBL (**3a**), DPBL (**3b**), DMBL (**3c**), DSCBL (**4a**), DPCBL (**4b**), DMCBL (**4c**), and BC (**5**) were synthesized as previously described[29–31] and transformed into sodium forms by an ion-exchange resin (Amberlite IR-120). PKH Linker Kit (MINI67-1KT) was purchased from Sigma (USA). All other chemicals were of the highest grade commercially available.

2.2. Preparation and composition of boronated liposomes

Boronated liposomes and PEG boronated liposomes were prepared from boron lipids, DSPC, Chol (X:1 – X:1, molar ratio, $0 < X < 1$) and boron lipids, DSPC, Chol, DSPE-PEG (X:1 – X:1:0.11, molar ratio, $0 < X < 1$), respectively. These boronated liposomes were prepared according to the reverse-phase evaporation (REV) method.[36] Total lipids of 200 mg were dissolved in 6 mL of chloroform/diisopropyl ether mixture (1:1, v/v) and 3 mL of distilled water was added to form a w/o emulsion. The emulsion was sonicated for 3 min and then, the organic solvents were removed under reduced pressure in a rotary evaporator at 60 °C for 30 min to obtain a suspension of liposomes. The liposomes obtained were subjected to extrusion 10 times through a polycarbonate membrane filter of 100 nm pore size (Whatman, 110605, FILTER, 0.1UM, 25MM, Gentaur Molecular Products, Belgium), using an extruder device (LIPEX™ Extruder, Northern Lipids, Canada) thermostated at 60 °C. Purification was accomplished by ultracentrifugation (himac cp 80 wx, Hitachi Koki, Japan) at 200,000g for 60 min at 4 °C, and the pellets obtained were resuspended in 0.9% NaCl solution or PBS. Particle size distribution of the boronated liposomes was measured with an electrophoretic light scattering spectrophotometer (Nano-ZS, Sysmex, Japan). The compositions of boron lipids and DSPC in liposomes were calculated from data obtained by the simultaneous measurement of boron and phosphorus concentrations by inductively coupled plasma atomic emission spectroscopy (ICP-AES, HORIBA, Japan).

2.3. Transmission electron microscopy analysis

An aliquot of the sample solution was applied to electron microscope carbon-coated grids covered with parlodion backing. The excess of the solution was blotted with filter paper. Grids were immediately negatively stained with 2.5% uranyl acetate for 10 min. The grids were examined in a Transmission Electron Microscope (JEM 2000FX) at an accelerating voltage of 200 kV.

2.4. Cell culture and neutron irradiation

The mouse colorectal carcinoma cell line, colon 26, was maintained at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, USA). For subsequent experiments, the cells were seeded at a density of 5×10^3 cells/well in a 96-well plate (Greiner, Germany) and incubated at 37 °C for 20 h. Neutron irradiation was carried out in the Japan Research Reactor No. 4 (JRR4) of Japan Atomic Energy Agency.

Method A (no wash): The cells were incubated for 30 min in the presence of various concentrations of boron lipid-25% liposomes prepared from DSPC, ^{10}B-enriched boron lipids, and Chol (0.75:0.25:1, molar ratio) in medium. The cells were irradiated with thermal neutrons in the JRR4 for 30 min ($3.8–5.0 \times 10^{11}$ neutrons/cm^2). After irradiation, the cells were washed with PBS and incubated for 3 days in fresh medium. Cell viability was determined by the MTT assay.

Method B (wash): The cells were incubated for 30 min in the presence of various concentrations of boron lipid-25% liposomes (^{10}B-enriched) in medium. After the medium was exchanged with a fresh one, the cells were incubated for another 30 min and then irradiated with thermal neutrons in the JRR4 for 30 min ($3.8–5.0 \times 10^{11}$ neutrons/cm^2). After irradiation, the cells were washed with PBS and incubated for 3 days in fresh medium. Cell viability was determined by the MTT assay.

2.5. In vitro fluorescence imaging

PKH67-labeled boronated liposomes were prepared according to the conventional cell membrane labeling method (Sigma, PKH67 Green Fluorescent Cell Linker Kit). Briefly, pellets of boronated liposomes were dissolved in 250 μL of Diluent C, and then the boronated liposome solution was added dropwise into 1 μL of PKH67 dye stock solution. The mixture was maintained at 20 °C for 5 min and free PKH67 was removed by ultracentrifugation at 200,000g for 60 min at 4 °C. The obtained PKH67-labeled boronated liposomes were resuspended in PBS.

Colon 26 cells were maintained at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. For subsequent experiments, the cells were seeded at a density of 5×10^4 cells in a 35 mm diameter dish (Greiner) and incubated at 37 °C for 20 h. The cells were incubated at 37 °C or 4 °C in the presence of NaN_3 (1 mM)[37] for another 3 h in the presence of PKH67-labeled boronated liposomes in medium. To prevent pH changes under CO_2-free condition, Leibovitz's L-15 medium (Invitrogen, USA) were used. After incubation, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, and coverslipped using Vectashield mounting medium (Vector Lavoratories, USA) for further analysis under a fluorescent confocal microscope (FV100D IX81, OLYMPUS, Japan).

2.6. Biodistribution of boronated liposomes in mice

Tumor-bearing mice (female, 5–6 weeks old, 16–20 g, Sankyo Labo Service, Japan) were prepared by injecting subcutaneously (s.c.) a suspension (2.5×10^6 cells/mouse) of colon 26 cells directly into the right thigh. The mice were kept on a regular chow diet and water and maintained under 12 h light/dark cycle in an ambient atmosphere. Biodistribution experiments were performed when the tumor diameter was 7–9 mm. The tumor-bearing mice were injected via the tail vein with 200 μL of BSH (6000 ppm B) in 0.9% NaCl solution or DSBL-25% and -50% PEG liposomes (2000 ppm B). At selected time intervals after administration, the mice were lightly anesthetized and blood samples were collected from the retro-orbital sinus. The mice were then sacrificed by cervical dislocation and dissected. Liver, spleen, kidney, heart, brain, lung, muscle, and tumor were excised, washed with 0.9% NaCl solution, and weighed. The excised tissues were digested with 2 mL of concd HNO_3 (ultratrace analysis grade, Wako, Japan) at 90 °C for 1–3 h, and then the digested samples were diluted with distilled water. After filtering through a hydrophobic filter (13JP050AN, ADVANTEC, Japan), boron concentration was measured by ICP-AES.

2.7. BNCT for tumor-bearing mice

Liposomes (DSBL-25%) were prepared from ^{10}B-enriched DSBL (**3a**), DSPC, Chol, and DSPE-PEG (0.25:0.75:1:0.11, molar ratio) and injected into colon 26 tumor bearing mice (female, 6–7 weeks old, 16–20 g) via the tail vein at a dose of 20 mg ^{10}B/kg (2000 ppm of ^{10}B concentration; 200 μL of boronated liposome solution). The mice were anesthetized with isoflurane (Forane, Abbott, Japan) and placed in an acrylic mouse holder 24 h after iv injection. The mice were irradiated in the JRR4 for 30 min at a rate of 1.8×10^{12} neutrons/cm^2. The BNCT effects were evaluated on the basis of the changes in tumor volume of the mice. Mortality was monitored daily and tumor volume was measured at intervals of a few days. To determine tumor volume, two perpendicular diameters of the tumor were measured with a slide caliper and calculation was carried out using the formula 0.5 ($A \times B^2$), where A and B are the longest and shortest dimensions of the tumor in

millimeters, respectively. All protocols were approved by the Institutional Animal Care and Use Committee of Gakushuin University.

3. Results and discussion

3.1. Characterization of boronated liposomes

The boronated liposomes were prepared from DSBL (**3a**), DSPC, Chol (X:1 − X:1, molar ratio, X<1) and DSBL, DSPC, Chol, DSPE-PEG (X: 1 − X:1:0.11, molar ratio, X<1) by the REV method and particle sizes were measured with an electrophoretic light scattering spectrophotometer. As shown in Table 1, particle sizes (diameter) were distributed in the range of 95–105 nm. The boronated liposomes from each boron lipid (**3b–c** and **4a–c**) also displayed similar particle size distribution (data not shown). DSBL/DSPC lipid ratios in the liposomes were calculated from the concentrations of boron and phosphorus determined by ICP-AES. The mixing ratio of DSBL to DSPC (X value) in the preparation of the liposomes is plotted on the abscissa and the DSBL/DSPC ratio in the liposomes obtained is plotted on the ordinate, as shown in Figure 2. It was revealed that the DSBL/DSPC ratio in the liposomal membrane is proportional to the mixing ratio in the preparation. The formation of DSBL-25% liposomes was analyzed under a transmission electron microscope by the negative staining method after extrusion through a 100 nm filter. As shown in Figure 3, boronated liposomes were formed as unilamellar particles measuring 100 nm in diameter.

3.2. In vitro cytotoxicity of boronated liposomes after neutron irradiation

We next examined the effect of boronated liposomes (^{10}B-enriched) on colon 26 cells at various ^{10}B concentrations with thermal neutron irradiation. Cell viability with or without thermal neutron irradiation was measured by the MTT assay. In the case of method A (no wash), thermal neutron irradiation of cells was carried out in the presence of boronated liposomes prepared from DSBL (**3a**), DPBL (**3b**), DMBL (**3c**), DSCBL (**4a**), DPCBL (**4b**), and DMCBL (**4c**), BC (**5**) in the cell medium. BSH and $B(OH)_3$ were used as controls. As shown in Figure 4A, except DSCBL liposome, all boronated liposomes as well as BSH and $B(OH)_3$ controls showed no cytotoxicity when not irradiated with thermal neutrons. After thermal neutron irradiation, however, significant cell damage was observed in a boron-dose-dependent manner in all cases. Figure 4B shows BNCT effects on cells irradiated with thermal neutrons for 30 min after the cell medium was replaced with a boron-free one. Compared with liposomes composed of DMCBL and BC and boron compounds $B(OH)_3$ and BSH that showed no cytotoxicity, liposomes composed of DSBL, DPBL, DMBL, DSCBL, and DPCBL showed boron-dose-dependent cytotoxicity, and DSBL and DPBL were found to be potential boron lipids for liposomal boron vehicles. The results indicate that the boronated liposomes were taken up by the tumor cells and remained there for certain periods, whereas $B(OH)_3$ and BSH were readily washed out from the cells. In the current experiments, a slight difference in thermal neutron dose ranging from 3.8 to 5.0 × 10^{11} neutrons/cm^2 was observed on the 96-well plates. However, the difference did not affect cell viability remarkably.

Table 1
Particle size, polydispersity index, and zeta potential of DSBL liposomes

DSBL X value	Particle size[a] (nm)	Polydispersity index[a]	Zeta potential (mV)
0	94.8 ± 0.62	0.050 ± 0.003	−2.4
0.05	100.5 ± 0.17	0.033 ± 0.011	−42.8
0.1	95.6 ± 1.01	0.022 ± 0.011	−45.7
0.15	99.0 ± 0.20	0.029 ± 0.010	−42.5
0.25	102.0 ± 0.44	0.048 ± 0.005	−45.8
0.5	104.3 ± 0.61	0.034 ± 0.014	−46.7

[a] Data are expressed as means ± sem.

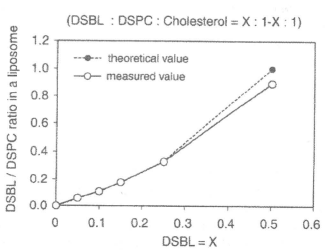


Figure 2. Composition of DSBL and DSPC in boronated liposome membranes. Boronated liposomes were prepared from DSBL, DSPC, and Chol (molar ratio is X:1 − X:1).

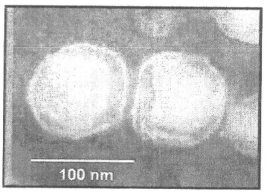


Figure 3. Transmission electron microscopy analysis of 25% DSBL liposomes.

3.3. Uptake of fluorescence-labeled boronated liposomes by endocytosis

Colon 26 cells were treated with DSBL-25% liposomes dyed green with PKH67. After 3 h incubation with the liposomes, the cells were washed with PBS and the PKH67-labeled DSBL liposomes were detected with a fluorescent confocal microscope. Figure 5A shows intracellular localization of PKH67-labeled DSBL liposomes at 37 °C. To confirm that the translocation of liposome is mediated by endocytosis, effects of endocytosis inhibitor sodium azide at low temperature.[37] As shown in Figure 5B, PKH67-labeled DSBL liposomes were localized on plasma membrane in the presence of sodium azide at 4 °C. These results indicate that DSBL liposomes were taken up into the cytoplasm by endocytosis without degradation of the liposomes.

3.4. Biodistribution of boronated liposomes in mice

Figure 6A and B show the time courses of boron concentrations in various tissues of tumor-bearing mice after injecting DSBL-25% PEG liposomes (20 mg B/kg) and BSH (60 mg B/kg). In the case of BSH, boron concentration in blood was 62.7 ppm 1 h after injection but dropped to 15.7 and 0.48 ppm 3 and 10 h after injection, respectively. Boron concentration in the other tissues decreased along with the disappearance of BSH in blood due to high renal clearance. Boron concentration of 37.8 ppm was observed in tumor with a tumor/blood ratio of ~0.6 at 1 h after injection. In contrast,

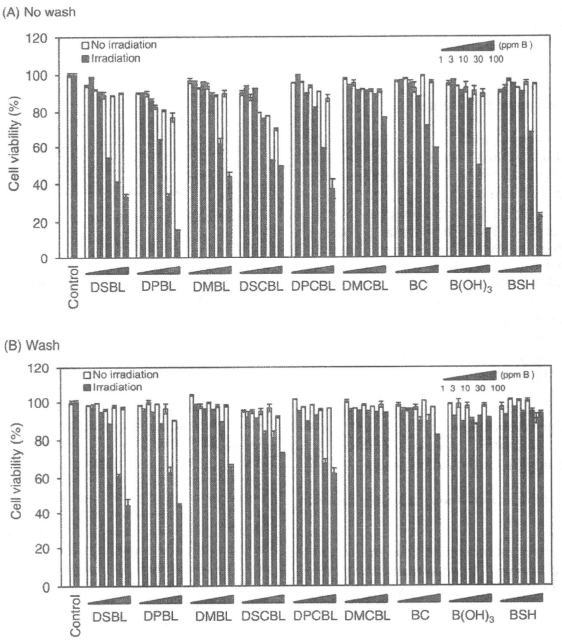


Figure 4. Effects of boronated liposomes on colon 26 cell viability after thermal neutron irradiation or no irradiation. All boronated liposomes were composed of 25% boron lipids or boron cholesterols. (A) Colon 26 cells were incubated in a 96-well microplate at 37 °C in 5% CO_2 in air for 30 min in the presence of boronated liposomes. The cells were irradiated with thermal neutrons for 30 min (no wash). Three days after irradiation, cell viability was determined by the MTT assay. (B) Colon 26 cells were incubated in a 96-well microplate at 37 °C in 5% CO_2 in air for 30 min in the presence of boronated liposomes. The cells were irradiated with thermal neutrons for 30 min after medium exchange (wash). Three days after irradiation, cell viability was determined by the MTT assay. Data are expressed as means ± sem (n = 3).

boron concentration of 22.7 ppm in tumor with a tumor/blood ratio of ~2 was observed 24 h after administration of DSBL-25% PEG liposomes, and boron concentration gradually decreased thereafter. High boron concentration was observed also in liver and spleen. The high boron concentration in spleen may be due to the instability of the DSBL-25% PEG liposomes present in blood. In general drug delivery systems, the high accumulation of drug-encapsulating or -attaching nanoparticles in other tissues, such as liver and spleen, sometimes induces side effects due to the cytotoxicity of the accumulated drugs. Current boron lipid liposomes displayed significantly low toxicity and were readily eliminated from the tissues within three weeks after injection. Therefore, it is considered that the high accumulation of boron in liver and spleen observed in Figure 6A would not have serious side effects unless thermal neutron irradiation is carried out on these tissues. In this regard, *BNCT is a double-targeting therapy that involves boron delivery to and neutron irradiation of cancers.*

3.5. BNCT effect of boronated liposomes on tumor-bearing mice

The cytotoxicity of DSBL-25% PEG liposomes was examined by irradiating colon 26 tumor bearing mice with thermal neutrons. When colon 26 cells transplanted into the left thighs of mice showed logarithmic growth, the mice were given saline as control and DSBL-25% PEG liposomes at a dose of 20 mg ^{10}B/kg. As the highest boron concentration in tumor (22.7 ppm at a dose of 20 mg B/kg) was observed 24 h after injecting DSBL-25% PEG liposomes, thermal neutron irradiation of the tumor-transplanted left thighs of mice was carried out 24 h after the injection while shielding bodies with the acrylic mouse holder. As shown in Figure 7, tumor volume in mice treated with DSBL-25% PEG liposomes was significantly inhibited after thermal neutron irradiation. The tumor volumes were ~20% of those of control mice two weeks after the neutron irradiation.

4. Conclusion

We newly prepared the *closo*-dodecaborate lipids containing liposomes as BDS vehicles for neutron capture therapy. The current approach is unique because *the liposome shell itself possesses cytocidal potential in combination with neutron irradiation.* The boronated liposomes composed of DSBL or DPBL, in particular, displayed high cytotoxicity with thermal neutron irradiation in colon 26 cells. The efficient in vitro BNCT effects were due to the uptake of the boro-

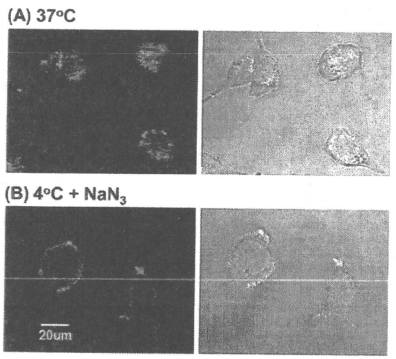


Figure 5. Intracellular localization of PKH-labeled boronated liposomes. (A) PKH67-labeled DSBL-25% liposomes were incubated at 37 °C for 3 h in colon 26 cells, and visualized under a fluorescent confocal microscope. (B) The cells were incubated at 4 °C for 3 h with PKH67-labeled DSBL-25% liposomes in the presence of NaN_3 (1 mM).

nated liposomes in the cytoplasm by endocytosis, which was observed in the fluorescence experiments using PHK67-labeled DSBL-25% liposomes. The previously developed boronated liposomes composed of *nido*-carborane lipid **1** showed acute toxicity within one day at a dose of 14 mg B/kg,[27] however the boronated liposomes described in this paper did not show acute toxicity toward healthy mice at a dose of 20 mg B/kg. Furthermore, the boronated liposomes composed of *closo*-dodecaborate lipids were readily eliminated from the body, whereas BSH-conjugated cholesterols BC and BBC were not eliminated and remained in tissues even after three weeks. Boron concentration of 22.7 ppm in tumor was achieved by injection with DSBL-25% PEG liposomes at 20 mg B/kg in tumor-bearing mice. As described, 20–35 ppm boron concentrations are required to realize fatal tumor cell damage, therefore the concentration observed by injection with DSBL-25% PEG liposomes in tumor-bearing mice is expected to result in the fatal tumor cell damage with BNCT. In fact, significant suppression of tumor growth was observed after thermal neutron irradiation. This is the first promising BNCT of tumor-bearing mice with the boron lipid liposomes, although various boron compounds embedded within the liposome bilayer have been reported. As the internal aqueous core of the examined boronated liposomes is still vacant, drugs, including boron compounds, can be encapsulated in it. In this regard, boron-10 and drugs may be simultaneously delivered

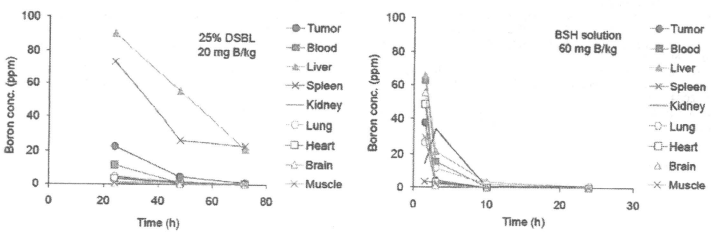


Figure 6. Time course of biodistribution of (A) DSBL-25% PEG liposomes (20 mg B/kg) and (B) BSH solution (60 mg B/kg). Each sample was injected into tumor-bearing mice (Balb/c, female, 6 weeks old, 14–20 g) via the tail vein.

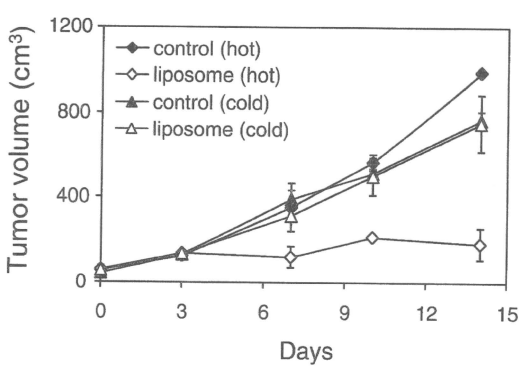


Figure 7. Tumor volume in mice (Balb/c, female, 6 weeks old, 14–20 g) bearing colon 26 solid tumor with thermal neutron irradiation (hot) for 30 min (1.8×10^{12} neutrons/cm^2) or without irradiation (cold). The irradiation was performed at 24 h after iv injection of DSBL-25% PEG liposomes (20 mg B/kg). Data are expressed as means ± sem ($n = 3$).

to a tumor, realizing combination therapy consisting of BNCT and chemotherapy. Investigation for further efficient BDS including active targeting to tumor by functionalization of the boronated liposomes are going on in our laboratory.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas 'Cancer Therapy' from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and notes

1. Locher, G. L. *Am. J. Roentgenol.* **1936**, *36*, 1.
2. Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F. G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998**, *98*, 1515.
3. Barth, R. F.; Coderre, J. A.; Vicente, M. G.; Blue, T. E. *Clin. Cancer Res.* **2005**, *11*, 3987.
4. Hawthorne, M. F. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 950.
5. Yinghuai, Z.; Cheng Yan, K.; Maguire, J. A.; Hosmane, N. S. *Curr. Chem. Biol.* **2007**, *1*, 141.
6. Barth, R. F.; Soloway, A. H.; Fairchild, R. G.; Brugger, R. M. *Cancer* **1992**, *70*, 2995.
7. Soloway, A. H.; Hatanaka, H.; Davis, M. A. *J. Med. Chem.* **1967**, *10*, 714.
8. Nakagawa, Y.; Hatanaka, H. *J. Neurooncol.* **1997**, *33*, 105.
9. Snyder, H. R.; Reedy, A. J.; Lennarz, W. J. *J. Am. Chem. Soc.* **1958**, *80*, 835.
10. Mishima, Y.; Ichihashi, M.; Hatta, S.; Honda, C.; Yamamura, K.; Nakagawa, T. *Pigment Cell Res.* **1989**, *2*, 226.
11. Kato, I.; Ono, K.; Sakurai, Y.; Ohmae, M.; Maruhashi, A.; Imahori, Y.; Kirihata, M.; Nakazawa, M.; Yura, Y. *Appl. Radiat. Isot.* **2004**, *61*, 1069.
12. Aihara, T.; Hiratsuka, J.; Morita, N.; Uno, M.; Sakurai, Y.; Maruhashi, A.; Ono, K.; Harada, T. *Head Neck* **2006**, *28*, 850.
13. Suzuki, M.; Sakurai, Y.; Hagiwara, S.; Masunaga, S.; Kinashi, Y.; Nagata, K.; Maruhashi, A.; Kudo, M.; Ono, K. *Jpn. J. Clin. Oncol.* **2007**, *37*, 376.
14. Shelly, K.; Feakes, D. A.; Hawthorne, M. F.; Schmidt, P. G.; Krisch, T. A.; Bauer, W. F. *PNAS* **1992**, *89*, 9039.
15. Feakes, D. A.; Shelly, K.; Knobler, C. B.; Hawthorne, M. F. *PNAS* **1994**, *91*, 3029.
16. Martini, S.; Ristori, S.; Pucci, A.; Bonechi, C.; Becciolini, A.; Martini, G.; Rossi, C. *Biophys. Chem.* **2004**, *111*, 27.
17. Yanagie, H.; Fujii, Y.; Takahashi, T.; Tomita, T.; Fukano, Y.; Hasumi, K.; Nariuchi, H.; Yasuda, T.; Sekiguchi, M.; Uchida, H. *Human Cell* **1989**, *2*, 290.
18. Yanagie, H.; Tomita, T.; Kobayashi, H.; Fujii, Y.; Takahashi, T.; Hasumi, K.; Nariuchi, H.; Sekiguchi, M. *Br. J. Cancer* **1991**, *63*, 522.
19. Pan, X.; Wu, G.; Yang, W.; Barth, R. F.; Tjarks, W.; Lee, R. J. *Bioconjugate Chem.* **2007**, *18*, 101.
20. Pan, X. Q.; Wang, H.; Shukla, S.; Sekido, M.; Adams, D. M.; Tjarks, W.; Barth, R. F.; Lee, R. J. *Bioconjugate Chem.* **2002**, *13*, 435.
21. Kullberg, E. B.; Carlsson, J.; Edwards, K.; Capala, J.; Sjoberg, S.; Gedda, L. *Int. J. Oncol.* **2003**, *23*, 461.
22. Maruyama, K.; Ishida, O.; Kasaoka, S.; Takizawa, T.; Utoguchi, N.; Shinohara, A.; Chiba, M.; Kobayashi, H.; Eriguchi, M.; Yanagie, H. *J. Controlled Release* **2004**, *98*, 195.
23. Yanagie, H.; Ogura, K.; Takagi, K.; Maruyama, K.; Matsumoto, T.; Sakurai, Y.; Skvarc, J.; Illic, R.; Kuhne, G.; Hisa, T.; Yoshizaki, I.; Kono, K.; Furuya, Y.; Sugiyama, H.; Kobayashi, H.; Ono, K.; Nakagawa, K.; Eriguchi, M. *Appl. Radiat. Isot.* **2004**, *61*, 639.
24. Feakes, D. A.; Shelly, K.; Hawthorne, M. F. *PNAS* **1995**, *92*, 1367.
25. Watson-Clark, R. A.; Banquerigo, M. L.; Shelly, K.; Hawthorne, M. F.; Brahn, E. *PNAS* **1998**, *95*, 2531.
26. Nakamura, H.; Miyajima, Y.; Takei, T.; Kasaoka, S.; Maruyama, K. *Chem. Commun.* **2004**, 1910.
27. Miyajima, Y.; Nakamura, H.; Kuwata, Y.; Lee, J.-D.; Masunaga, S.; Ono, K.; Maruyama, K. *Bioconjugate Chem.* **2006**, *17*, 1314.
28. Li, T.; Hamdi, J.; Hawthorne, M. F. *Bioconjugate Chem.* **2006**, *17*, 15.
29. Lee, J.-D.; Ueno, M.; Miyajima, Y.; Nakamura, H. *Org. Lett.* **2007**, *9*, 323.
30. Nakamura, H.; Lee, J.-D.; Ueno, M.; Miyajima, Y.; Ban, H. *NanoBioTechnology* **2007**, *3*, 1551.
31. Nakamura, H.; Ueno, M.; Lee, J.-D.; Ban, H. S.; Justus, E.; Fan, P.; Gabel, D. *Tetrahedron Lett.* **2007**, *48*, 3151.
32. Nakamura, H.. In *Methods Enzymol*; Nejat, D., Ed.; Academic Press, 2009; Vol. 465, p 179.
33. Justus, E.; Awad, D.; Hohnholt, M.; Schaffran, T.; Edwards, K.; Karlsson, G.; Damian, L.; Gabel, D. *Bioconjugate Chem.* **2007**, *18*, 1287.
34. Shirakawa, M.; Yamamto, T.; Nakai, K.; Aburai, K.; Kawatobi, S.; Tsurubuchi, T.; Yamamoto, Y.; Yokoyama, Y.; Okuno, H.; Matsumura, A. *Appl. Radiat. Isot.* **2009**, *67*, S88.
35. Thirumamagal, B. T.; Zhao, X. B.; Bandyopadhyaya, A. K.; Narayanasamy, S.; Johnsamuel, J.; Tiwari, R.; Golightly, D. W.; Patel, V.; Jehning, B. T.; Backer, M. V.; Barth, R. F.; Lee, R. J.; Backer, J. M.; Tjarks, W. *Bioconjugate Chem.* **2006**, *17*, 1141.
36. Szoka, F. J.; Papahadjopoulos, D. *PNAS* **1978**, *75*, 4194.
37. Mudhakir, D.; Akita, H.; Tan, E.; Harashima, H. *J. Controlled Release* **2008**, *125*, 164.

TISSUE ENGINEERING: Part A
Volume 16, Number 4, 2010

DOI: 10.1089/ten.tea.2009.0386

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

Hiroshi Komoda, M.D., Ph.D.,[1,2,*] Hanayuki Okura, M.S.,[1,3,4,*] Chun Man Lee, M.D., Ph.D.,[1,5] Nagako Sougawa, D.M.D., Ph.D.,[1] Tomoaki Iwayama, D.M.D.,[6] Tomoko Hashikawa, D.M.D., Ph.D.,[6] Ayami Saga, M.S.,[1] Aya Yamamoto-Kakuta, B.S.,[1] Akihiro Ichinose, M.D., Ph.D.,[7] Shinya Murakami, D.M.D., Ph.D.,[6] Yoshiki Sawa, M.D., Ph.D.,[3,5] and Akifumi Matsuyama, M.D., Ph.D.[1]

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

[1]Department of Somatic Stem Cell Therapy, Foundation for Biomedical Research and Innovation, Kobe, Hyogo, Japan.
[2]Department of Internal Medicine, National Hospital Organization Chiba Medical Center, Chiba, Japan.
[3]Department of Surgery, Osaka University Graduate School of Medicine, Suita, Osaka, Japan.
[4]Japan Society for the Promotion of Science, Tokyo, Japan.
[5]Medical Center for Translational Research, Osaka University Hospital, Suita, Osaka, Japan.
[6]Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Osaka, Japan.
[7]Department of Plastic Surgery, Kobe University Hospital, Kobe, Hyogo, Japan.
*These authors contributed equally to this work.

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.[8–12]

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*-glycolylneuraminic acid (Neu5Gc) xenoantigen,[13,14] the so-called Hanganutziu–Deicher antigen.[15] Humans are incapable of synthesizing the common mammalian sialic acid, Neu5Gc, because of an *Alu* transposon-mediated inactivation of the cytidine monophosphate (CMP)-*N*-acetylneuraminic 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 sialic acid transporter, activation by CMP, and addition to nascent glycoproteins and glycolipids in the secretory pathway.[18] It is also possible that amphipathic molecules carrying Neu5Gc might be directly transferred into the hESC and hMSC plasma membranes.[19] Human serum contains high titers of natural preformed antibodies against Neu5Gc xenoantigen.[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 controversary.[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

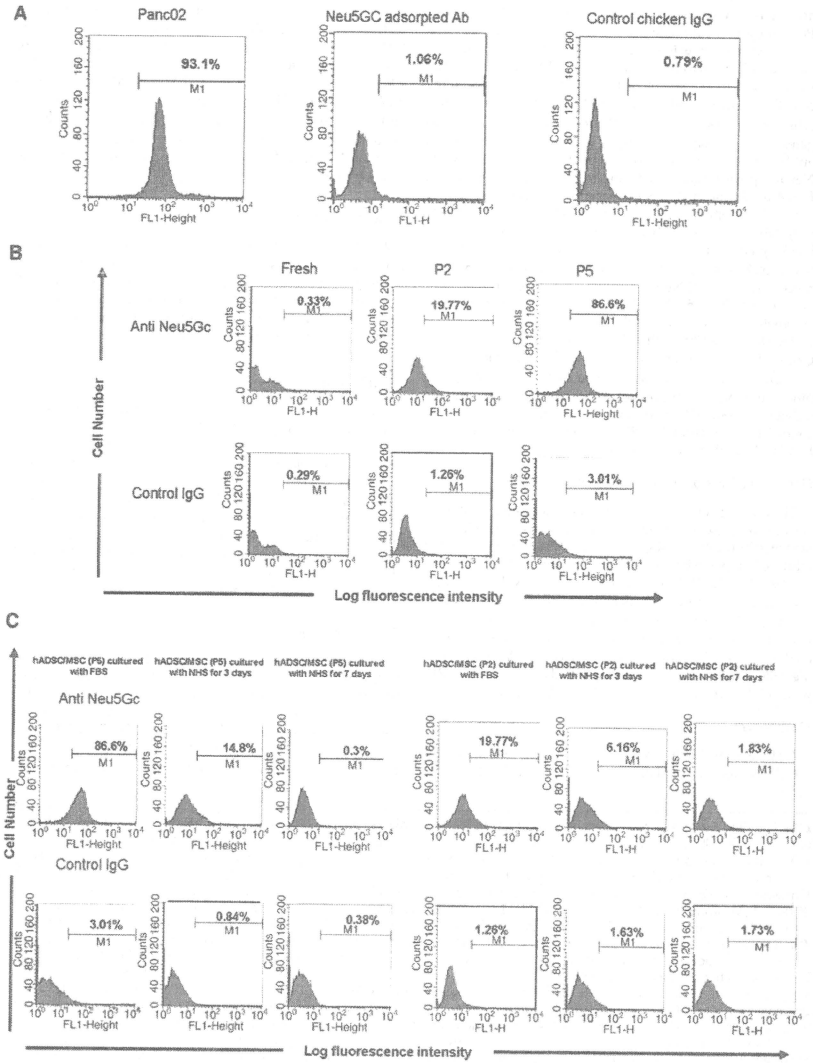
Cells

hADSCs/MSCs were prepared as described previously[1,2] with modifications.[3,4] Adipose tissue was resected 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 resected 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 ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan), and the resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin-coated dishes (BD BioCoat, Franklin Lakes, NJ) in a medium containing 60% DMEM-low glucose, 40% MCDB-201 medium (Sigma Aldrich), 1×insulin–transferrin–selenium (Gibco Invitrogen), 1 nM dexamethasone (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 (5×10^5 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 xenoantigen 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 xenoantigen by chasing cultivation with human serum. After cultivation 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.


A
Panc02
93.1%
M1
Neu5GC adsorpted Ab
1.06%
M1
Control chicken IgG
0.79%
M1
Counts
FL1-Height
FL1-H
B
Fresh
P2
P5
Anti Neu5Gc
0.33%
19.77%
86.6%
Control IgG
0.29%
1.26%
3.01%
Cell Number
Log fluorescence intensity
C
hADSC/MSC (P5) cultured with FBS
hADSC/MSC (P5) cultured with NHS for 3 days
hADSC/MSC (P5) cultured with NHS for 7 days
hADSC/MSC (P2) cultured with FBS
hADSC/MSC (P2) cultured with NHS for 3 days
hADSC/MSC (P2) cultured with NHS for 7 days
Anti Neu5Gc
86.6%
14.8%
0.3%
19.77%
6.16%
1.83%
Control IgG
3.01%
0.84%
0.38%
1.26%
1.63%
1.73%
Cell Number
Log fluorescence intensity

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 stained 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.

CMC assay

CMC was evaluated by measuring lactate dehydrogenase (LDH) release in media, using MTX-LDH kit (Kyokuto 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 1×10^4 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.[25]

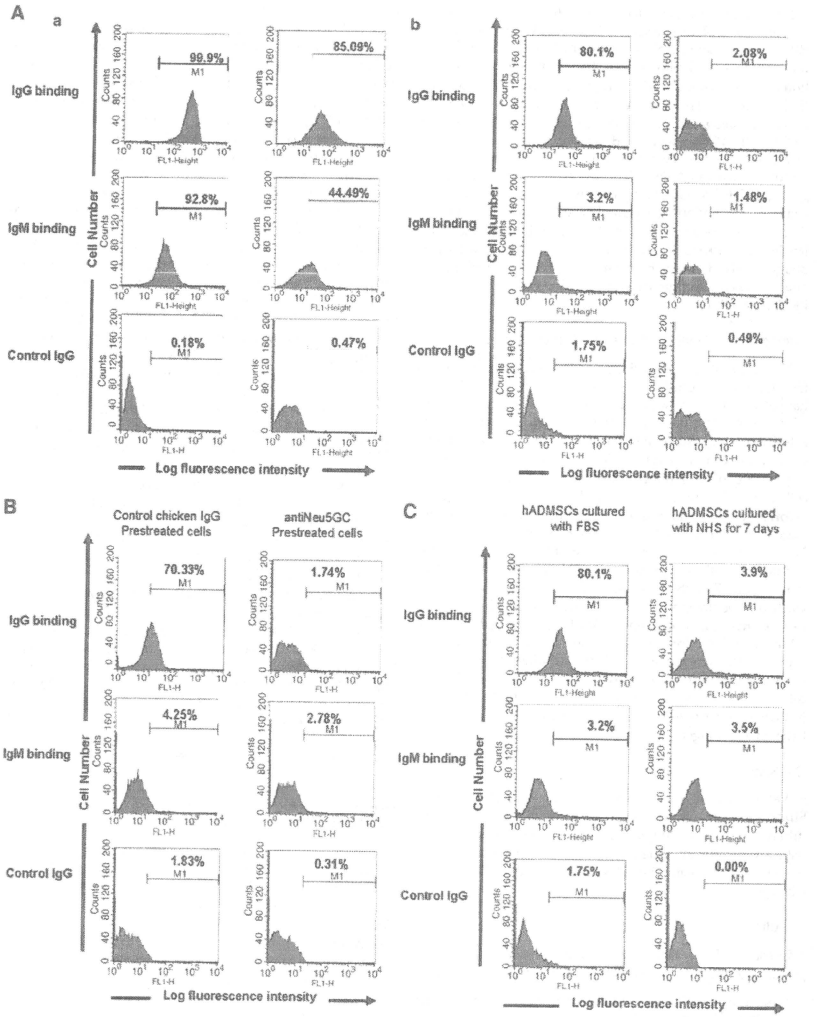
ADCC assay

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, 2×10^6 PKH67-labeled target cells were added into 24-well

FIG. 2. Binding of natural preformed antibodies to hADSCs/MSCs. (**A**) Binding of natural preformed antibodies to Panc02 and hADSCs/MSCs. (**a**) Murine pancreatic carcinoma cell line Panc02 was exposed to 10% fresh NHS containing 15 mM EDTA, then stained with secondary FITC-conjugated goat anti-human IgG or IgM antibody, and studied by flow cytometry to demonstrate the binding of IgG and IgM. The natural performed antibodies human IgG and IgM bound onto Panc02. Exposition of Neu5Gc-preadsorbed NHS could reduce the natural performed antibody binding (IgG binding: 99.95% to 85.09%; IgM binding: 92.8% to 44.49%). (**b**) hADSCs/MSCs were cultured with FBS, exposed to 10% fresh NHS containing 15 mM EDTA, and then stained with secondary FITC-conjugated goat anti-human IgG or IgM antibody, or control goat IgG. The natural performed antibodies human IgG and IgM bound onto hADSCs/MSCs, and exposition of Neu5Gc-preadsorbed NHS could reduce IgG binding (80.01% to 2.08%). The percentage of cells that stained positive is indicated in the upper right corner of each panel. Data are representative of four independent experiments. (**B**) Anti-Neu5Gc antibody pretreatment suppressed the binding of natural preformed antibodies onto hADSCs/MSCs. hADSCs/MSCs cultured with FBS were precoated with anti-Neu5Gc antibody and then exposed to 10% fresh NHS containing 15 mM EDTA. The natural performed antibody human IgG bound onto hADSCs/MSCs, and exposition of anti-Neu5Gc antibody could reduce IgG binding (70.33% to 1.74%). The percentage of cells that stained positive is indicated in the upper right corner of each panel. Data are representative of three independent experiments. (**C**) Decrement of binding of natural preformed antibodies onto hADSCs/MSCs by chase with NHS. After cultivation of hADSCs/MSCs with heat-inactivated NHS but not FBS, the percentages of human IgG-positive cells decreased. The percentage of cells that stained positive is indicated in the upper right corner of each panel. Data are representative of four independent experiments. hADMSCs, adipose-tissue derived mesenchymal stem cells; EDTA, ethylenediaminetetraacetate; FITC, fluorescein isothiocyanate.


A
a
IgG binding
IgM binding
Control IgG
99.9%
85.09%
92.8%
44.49%
0.18%
0.47%
Cell Number
Log fluorescence intensity
b
80.1%
2.08%
3.2%
1.48%
1.75%
0.49%
B
Control chicken IgG Prestreated cells
antiNeu5GC Prestreated cells
70.33%
1.74%
4.25%
2.78%
1.83%
0.31%
C
hADMSCs cultured with FBS
hADMSCs cultured with NHS for 7 days
80.1%
3.9%
3.2%
3.5%
1.75%
0.00%

culture plates and incubated with 2×10^5 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 allophycocyanin-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 allophycocyanin-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 nM 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% NH_4OH (pH 6.5) for 5 min and then washed with H_2O. AP activity was investigated at 2 weeks after differentiation using the procedure described previously.[26] 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.[27]

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%). Culture with heat-inactivated NHS could markedly reduce Neu5Gc in human colon carcinoma cells,[22] hESCs,[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 xenoantigen by chasing cultivation with human serum was examined (Fig. 1C). The Neu5Gc xenoantigen was reduced after cultivation of hADSCs/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 xenoantigen,[20–22] 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 2.08%). Further, pretreatment of hADSCs/MSCs with anti-Neu5Gc polyclonal antibody also resulted in reduction of human IgG binding (70.33% 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 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: 98.99%) 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,

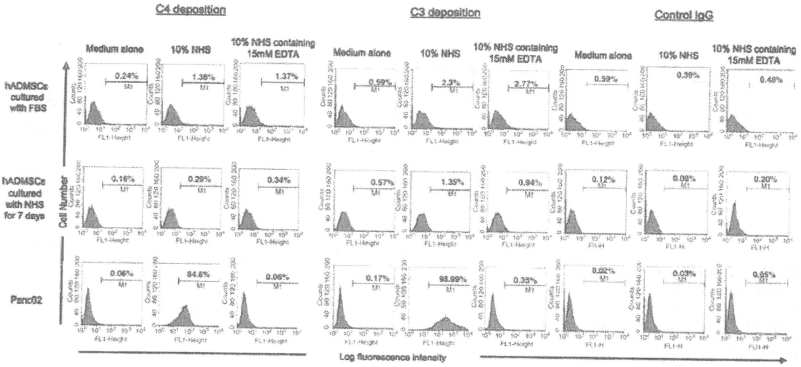


FIG. 3. Complement deposition onto hADSCs/MSCs by NHS. The cells were exposed to medium alone, 10% NHS, or 10% NHS containing 15 mM EDTA, followed by an analysis of deposition of complement fragments C4 and C3. The percentage of cells that stained positive is indicated in the upper right corner of each panel. Data are representative of four independent experiments.

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.[28] 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, 4.7% ± 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. 4.7 ± 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

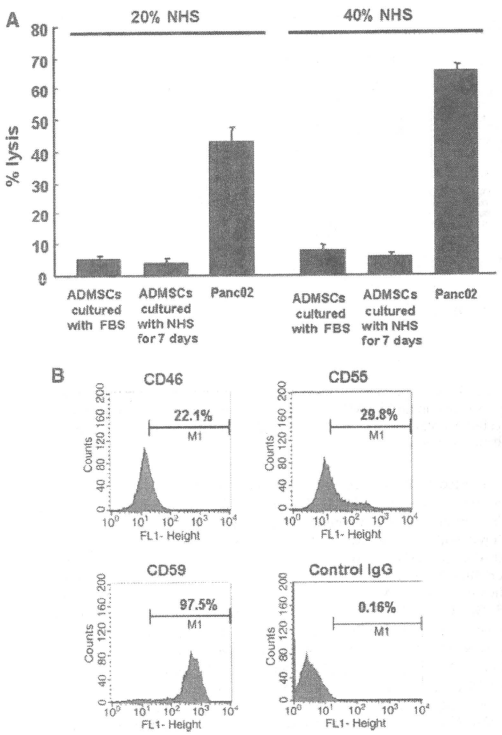


FIG. 4. Sensitivity of hADMSCs to lysis by NHS. (**A**) Complement-mediated cytotoxicity assay of hADSCs/MSCs. The cytotoxic activity of 20% or 40% NHS against hADSCs/MSCs was tested by lactate dehydrogenase release. Data are shown as mean ± standard deviation. (**B**) Complement regulatory proteins on hADSCs/MSCs were studied by flow cytometry using FITC-conjugated antibodies to human CD46, CD55, CD59, or control IgG. Data are representative of three independent experiments.

(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-

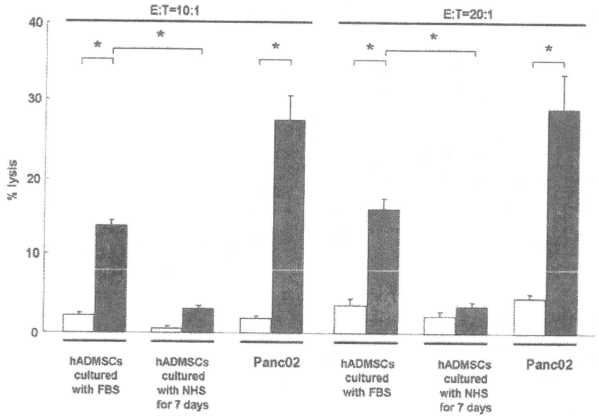


FIG. 5. Antibody-dependent cell-mediated cytotoxicity assay of hADSCs/MSCs. The cytotoxic activity of peripheral blood mononuclear cells against hADSCs/MSCs in the absence (white bar) or presence (black bar) of 10% NHS was tested by measuring lactate dehydrogenase release into medium (Effector:Target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

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 beta-glycerophosphate. 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).

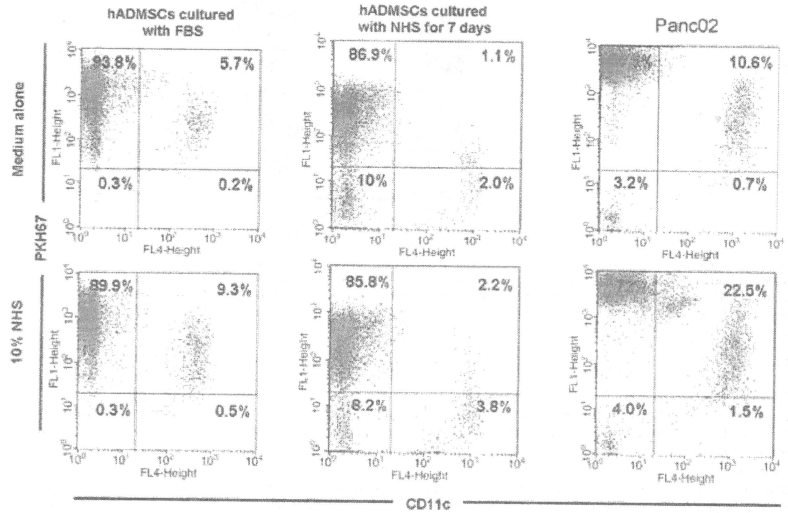


FIG. 6. Representative flow cytometry profiles of phagocytosis assay of hADSCs/MSCs. Upper left quadrant: Region of residual target cells. Upper right quadrant: Region of phagocytosed target cells. Percentages represent those of total cells in each region. Data are representative of three independent experiments.

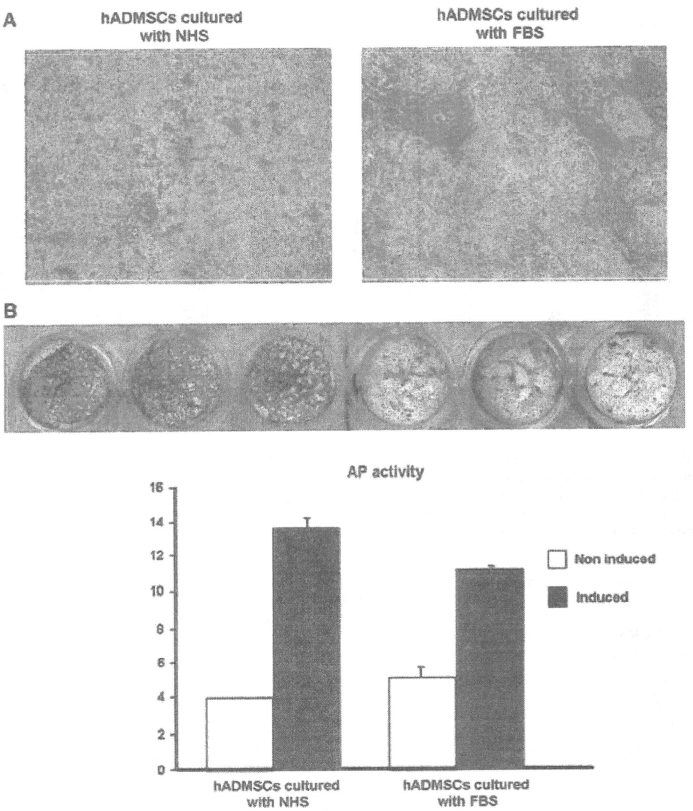


FIG. 7. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with FBS and NHS. (**A**) The efficiency of adipogenesis of hADSCs/MSCs cultured with NHS was similar to that of hADSCs/MSCs cultured with FBS. (**B**) The efficacy of osteogenic differentiation and alkaline phosphatase 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.[13,14] Human serum contains high titers of natural preformed antibodies against Neu5Gc xenoantigen[20–22] 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.[8–12] Further, in human clinical trials with FBS-grown hMSCs, antibodies against FBS have been detected.[12] However, these immune responses against human stem cells mediated by natural preformed antibodies remain in controversary.[13,23] 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 xenoantigen.

Our study using a chicken anti-Neu5Gc polyclonal antibody showed that most of the hADSCs/MSCs cultured with FBS expressed Neu5Gc xenoantigen. This result is similar to the previous study that hESCs and BM-derived hMSCs express Neu5Gc.[13,14] 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.[20,29] 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).[30] 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.[30] 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.[13] In contrast, Cerdan *et al.* reported that complement activation by anti-Neu5Gc antibody does not mediate killing of hESCs.[23] 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.[32,33] 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.[34–36] 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.[30] 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.[30] It is reported that AHXR could be mediated by natural preformed antibodies against non-Gal xenoantigen,[37,38] particularly Neu5Gc xenoantigen.[39] 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.[40] 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 xeno-free 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.[14] 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.

Acknowledgments

The authors thank Prof. Nobutaka Wakamiya, Department of Microbiology and Immunochemistry, Asahikawa Medical College, Hokkaido, Japan, for providing a chicken anti-Neu5Gc polyclonal antibody. This study was supported in part by a grant-in-aid to Akifumi Matsuyama from the Kobe Translational Research Cluster, the Knowledge Cluster Initiative, Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan.

Disclosure Statement

No competing financial interests exist.

References

1. Björntorp, P., Karlsson, M., Gustafsson, L., Smith, U., Sjöström, L., Cigolini, M., Storck, G., and Pettersson, P. Quantitation of different cells in the epididymal fat pad of the rat. J Lipid Res **20,** 97, 1979.
2. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell **13,** 4279, 2002.
3. Okura, H., Matsuyama, A., Lee, C.M., Saga, A., Kakuta-Yamamoto, A., Nagao, A., Sougawa, N., Sekiya, N., Takekita, K., Shudo, Y., Miyagawa, S., Komoda, H., Okano, T., and Sawa, Y. Cardiomyoblast-like cells differentiated from human adipose tissue-derived mesenchymal stem cells improve left ventricular dysfunction and survival in a rat myocardial infarction model. Tissue Eng Part C Methods July 22, 2009. [Epub ahead of print].
4. Okura, H., Fumimoto, Y., Komoda, H., Yanagisawa, T., Nishida, T., Noguchi, S., Sawa, Y., and Matsuyam, A. Transdifferentiation of human adipose tissue-derived stromal cells into insulin-producing clusters. J Artif Organs **12,** 123, 2009.
5. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
6. Kern, S., Eichler, H., Stoeve, J., Klüter, H., Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells **24,** 1294, 2006.
7. Romanov, Y.A., Darevskaya, A.N., Merzlikina, N.V., and Buravkova, L.B. Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. Bull Exp Biol Med **140,** 138, 2005.
8. Sotiropoulou, P.A., Perez, S.A., Salagianni, M., Baxevanis, C.N., and Papamichail, M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells **24,** 462, 2006.
9. Mazzini, L., Fagioli, F., Boccaletti, R., Mareschi, K., Oliveri, G., Olivieri, C., Pastore, I., Marasso, R., and Madon, E. Stem cell therapy in amyotrophic lateral sclerosis: a methodological approach in humans. Amyotroph Lateral Scler Other Motor Neuron Disord **4,** 158, 2003.
10. Bang, O.Y., Lee, J.S., Lee, P.H., and Lee, G. Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol **57,** 874, 2005.
11. Chen, S.L., Fang, W.W., Ye, F., Liu, Y.H., Qian, J., Shan, S.J., Zhang, J.J., Chunhua, R.Z., Liao, L.M., Lin, S., and Sun, J.P. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol **94,** 92, 2004.
12. Horwitz, E.M., Gordon, P.L., Koo, W.K., Marx, J.C., Neel, M.D., McNall, R.Y., Muul, L., and Hofmann, T. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Acad Sci USA **99,** 8932, 2002.
13. Martin, M.J., Muotri, A., Gage, F., and Varki, A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med **11,** 228, 2005.
14. Heiskanen, A., Satomaa, T., Tiitinen, S., Laitinen, A., Mannelin, S., Impola, U., Mikkola, M., Olsson, C., Miller-Podraza, H., Blomqvist, M., Olonen, A., Salo, H., Lehenkari, P., Tuuri, T., Otonkoski, T., Natunen, J., Saarinen, J., and Laine, J. *N*-Glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells **25,** 197, 2007.
15. Nowak, J.A., Jain, N.K., Stinson, M.W., and Merrick, J.M. Interaction of bovine erythrocyte *N*-glycolylneuraminic acid-containing gangliosides and glycoproteins with a human Hanganutziu-Deicher serum. Mol Immunol **23,** 693, 1986.
16. Chou, H.H., Takematsu, H., Diaz, S., Iber, J., Nickerson, E., Wright, K.L., Muchmore, E.A., Nelson, D.L., Warren, S.T., and Varki, A. A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc Natl Acad Sci USA **95,** 11751, 1998.
17. Hayakawa, T., Satta, Y., Gagneux, P., Varki, A., and Takahata, N. *Alu*-mediated inactivation of the human CMP-*N*-acetylneuraminic acid hydroxylase gene. Proc Natl Acad Sci USA **98,** 11399, 2001.
18. Bardor, M., Nguyen, D.H., Diaz, S., and Varki, A. Mechanism of uptake and incorporation of the non-human sialic acid *N*-glycolylneuraminic acid into human cells. J Biol Chem **280,** 4228, 2005.
19. Lanctot, P.M., Gage, F.H., and Varki, A.P. The glycans of stem cells. Curr Opin Chem Biol **11,** 373, 2007.
20. Zhu, A., and Hurst, R. Anti-*N*-glycolylneuraminic acid antibodies identified in healthy human serum. Xenotransplantation **9,** 376, 2002.
21. Nguyen, D.H., Tangvoranuntakul, P., and Varki, A. Effects of natural human antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. J Immunol **175,** 228, 2005.
22. Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., and Muchmore, E. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. Proc Natl Acad Sci USA **100,** 12045, 2003.
23. Cerdan, C., Bendall, S.C., Wang, L., Stewart, M., Werbowetski, T., and Bhatia, M. Complement targeting of nonhuman sialic acid does not mediate cell death of human embryonic stem cells. Nat Med **12,** 1113, 2006.
24. Asaoka, H., Nishinaka, S., Wakamiya, N., Matsuda, H., and Murata, M. Two chicken monoclonal antibodies specific for

heterophil Hanganutziu-Deicher antigens. Immunol Lett **32,** 91, 1992.
25. Matsuyama, A., Yamashita, S., Sakai, N., Maruyama, T., Okuda, E., Hirano, K., Kihara, S., Hiraoka, H., and Matsuzawa, Y. Identification of a GPI-anchored type HDL-binding protein on human macrophages. Biochem Biophys Res Commun **272,** 864, 2000.
26. Hashikawa, T., Takedachi, M., Terakura, M., Yamada, S., Thompson, L.F., Shimabukuro, Y., and Murakami, S. Activation of adenosine receptor on gingival fibroblasts. J Dent Res **85,** 739, 2006.
27. Labarca, C., and Paigen, K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem **102,** 344, 1980.
28. Schaapherder, A.F., Daha, M.R., te Bulte, M.T., van der Woude, F.J., and Gooszen, H.G. Antibody-dependent cell-mediated cytotoxicity against porcine endothelium induced by a majority of human sera. Transplantation **57,** 1376, 1994.
29. Ezzelarab, M., Ayares, D., and Cooper, D.K. Carbohydrates in xenotransplantation. Immunol Cell Biol **83,** 396, 2005.
30. Yang, Y.G., and Sykes, M. Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol **7,** 519, 2007.
31. Roos, A., and Daha, M.R. Antibody-mediated activation of the classical complement pathway in xenograft rejection. Transpl Immunol **9,** 257, 2002.
32. Miyagawa, S., Kubo, T., Matsunami, K., Kusama, T., Beppu, K., Nozaki, H., Moritan, T., Ahn, C., Kim, J.Y., Fukuta, D., and Shirakura, R. Delta-short consensus repeat 4-decay accelerating factor (DAF: CD55) inhibits complement-mediated cytolysis but not NK cell-mediated cytolysis. J Immunol **173,** 3945, 2004.
33. Komoda, H., Miyagawa, S., Kubo, T., Kitano, E., Kitamura, H., Omori, T., Ito, T., Matsuda, H., and Shirakura, R. A study of the xenoantigenicity of adult pig islets cells. Xenotransplantation **11,** 237, 2004.
34. Diamond, L.E., Quinn, C.M., Martin, M.J., Lawson, J., Platt, J.L., and Logan, J.S. A human CD46 transgenic pig model system for the study of discordant xenotransplantation. Transplantation **71,** 132, 2001.
35. Schuurman, H.J., Pino-Chavez, G., Phillips, M.J., Thomas, L., White, D.J., and Cozzi, E. Incidence of hyperacute rejection in pig-to-primate transplantation using organs from hDAF-transgenic donors. Transplantation **73,** 1146, 2002.
36. Zhou, C.Y., McInnes, E., Copeman, L., Langford, G., Parsons, N., Lancaster, R., Richards, A., Carrington, C., and Thompson, S. Transgenic pigs expressing human CD59, in combination with human membrane cofactor protein and human decay-accelerating factor. Xenotransplantation **12,** 142, 2005.
37. Chen, G., Qian, H., Starzl, T., Sun, H., Garcia, B., Wang, X., Wise, Y., Liu, Y., Xiang, Y., Copeman, L., Liu, W., Jevnikar, A., Wall, W., Cooper, D.K., Murase, N., Dai, Y., Wang, W., Xiong, Y., White, D.J., and Zhong, R. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. Nat Med **11,** 1295, 2005.
38. Chen, G., Sun, H., Yang, H., Kubelik, D., Garcia, B., Luo, Y., Xiang, Y., Qian, A., Copeman, L., Liu, W., Cardella, C.J., Wang, W., Xiong, Y., Wall, W., White, D.J., and Zhong, R. The role of anti-non-Gal antibodies in the development of acute humoral xenograft rejection of hDAF transgenic porcine kidneys in baboons receiving anti-Gal antibody neutralization therapy. Transplantation **81,** 273, 2006.
39. Saethre, M., Baumann, B.C., Fung, M., Seebach, J.D., and Mollnes, T.E. Characterization of natural human anti-non-gal antibodies and their effect on activation of porcine gal-deficient endothelial cells. Transplantation **84,** 244, 2007.
40. Ide, K., Ohdan, H., Kobayashi, T., Hara, H., Ishiyama, K., and Asahara, T. Antibody- and complement-independent phagocytotic and cytolytic activities of human macrophages toward porcine cells. Xenotransplantation **12,** 181, 2005.
41. Yamaguchi, M., Hirayama, F., Wakamoto, S., Fujihara, M., Murahashi, H., Sato, N., Ikebuchi, K., Sawada, K., Koike, T., Kuwabara, M., Azuma, H., and Ikeda, H. Bone marrow stromal cells prepared using AB serum and bFGF for hematopoietic stem cells expansion. Transfusion **42,** 921, 2002.

Address correspondence to:
Akifumi Matsuyama, M.D., Ph.D.
Department of Somatic Stem Cell Therapy
Institute of Biomedical Research and Innovation
Foundation for Biomedical Research and Innovation
1-5-4 TRI 305
Minatojima-Minamimachi
Chuo-ku
Kobe 650-0047
Japan

E-mail: akifumi-matsuyama@umin.ac.jp

Received: June 9, 2009
Accepted: October 27, 2009
Online Publication Date: December 18, 2009