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]

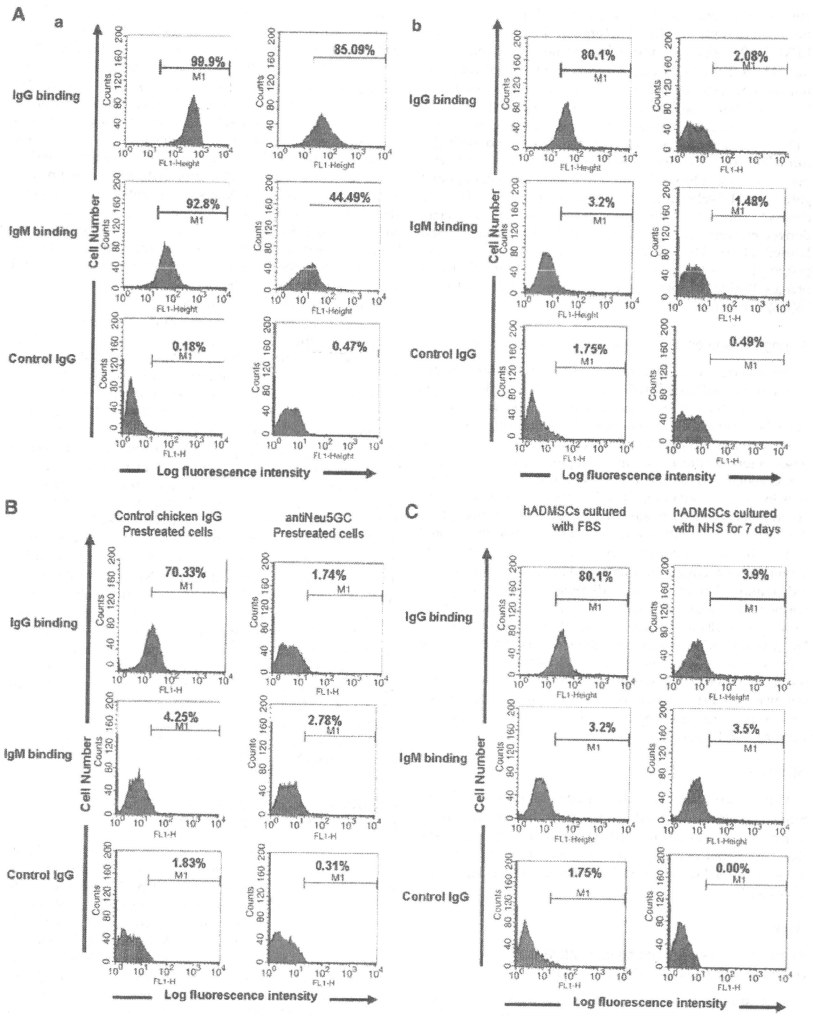
ADCC assay

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
b
IgG binding
IgM binding
Control IgG
99.9%
85.09%
92.8%
44.49%
0.18%
0.47%
80.1%
2.08%
3.2%
1.48%
1.75%
0.49%
Cell Number
Log fluorescence intensity
B
Control chicken IgG Prestreated cells
antiNeu5GC Prestreated cells
70.33%
1.74%
4.25%
2.78%
1.83%
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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,

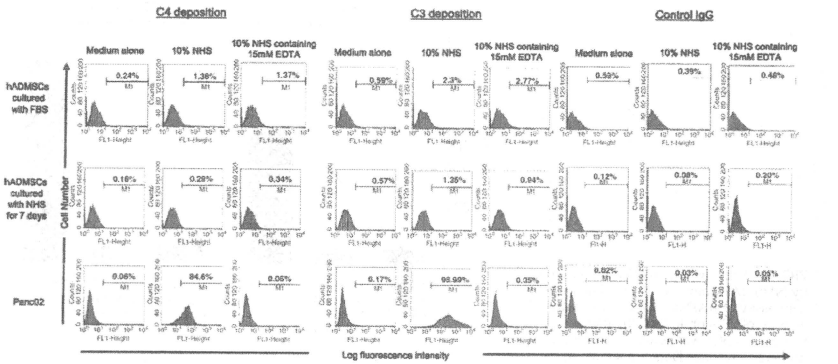

FIG. 3. Complement deposition onto hADSCs/MSCs by NHS. The cells were exposed to medium alone, 10% NHS, or 10% NHS containing 15 mM EDTA, followed by an analysis of deposition of complement fragments C4 and C3. The percentage of cells that stained positive is indicated in the upper right corner of each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

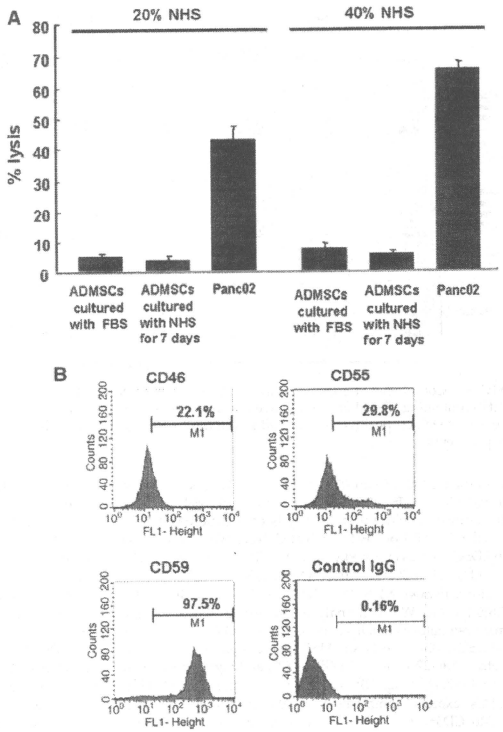

FIG. 4. Sensitivity of hADMSCs to lysis by NHS. (A) Complement-mediated cytotoxicity assay of hADSCs/MSCs. The cytotoxic activity of 20% or 40% NHS against hADSCs/MSCs was tested by lactate dehydrogenase release. Data are shown as mean ± standard deviation. (B) Complement regulatory proteins on hADSCs/MSCs 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-

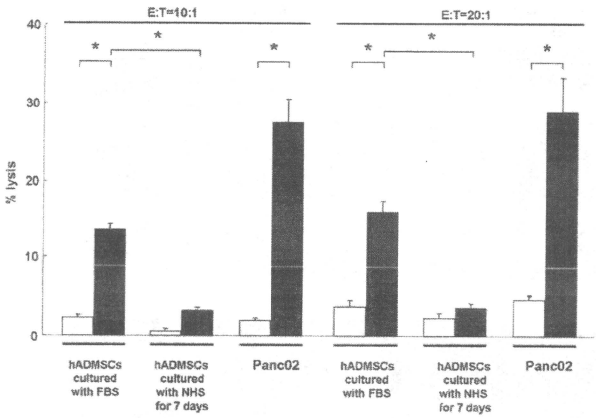

FIG. 5. Antibody-dependent cell-mediated cytotoxicity assay of hADSCs/MSCs. The cytotoxic activity of peripheral blood mononuclear cells against hADSCs/MSCs in the absence (white bar) or presence (black bar) of 10% NHS was tested by measuring lactate dehydrogenase release into medium (Effector:Target [*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).

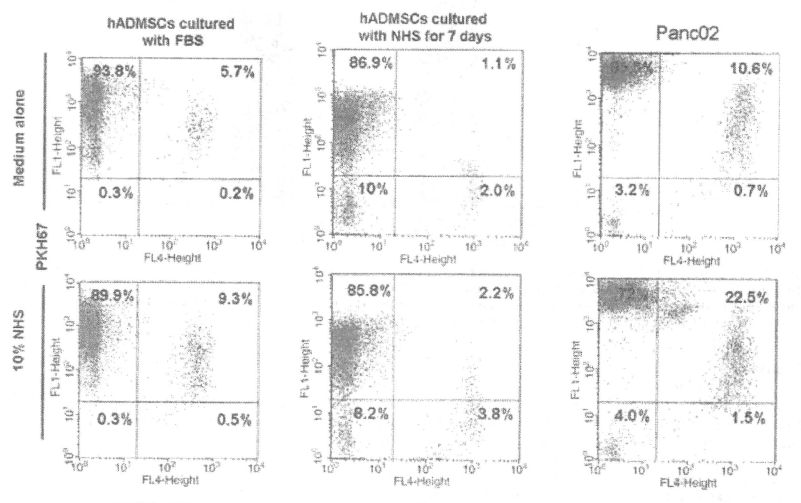

FIG. 6. Representative flow cytometry profiles of phagocytosis assay of hADSCs/MSCs. Upper left quadrant: Region of residual target cells. Upper right quadrant: Region of phagocytosed target cells. Percentages represent those of total cells in each region. Data are representative of three independent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

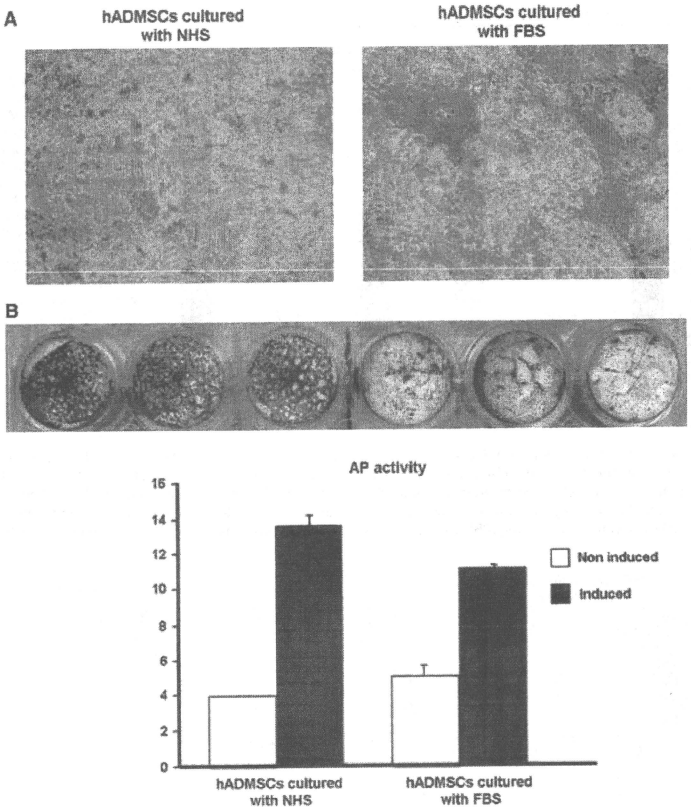

FIG. 7. Adipogenic and osteogenic differentiation potentials of hADSCs/MSCs cultured with FBS and NHS. (**A**) The efficiency of adipogenesis of hADSCs/MSCs cultured with NHS was similar to that of hADSCs/MSCs cultured with FBS. (**B**) The efficacy of osteogenic differentiation and alkaline phosphatase 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.

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Disclosure Statement

No competing financial interests exist.

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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

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Cardiomyoblast-like Cells Differentiated from Human Adipose Tissue-Derived Mesenchymal Stem Cells Improve Left Ventricular Dysfunction and Survival in a Rat Myocardial Infarction Model

Hanayuki Okura, M.S.,[1–4] Akifumi Matsuyama, M.D., Ph.D.,[1] Chun-Man Lee, M.D., Ph.D.,[1,2]
Ayami Saga, M.S.,[1] Aya Kakuta-Yamamoto, B.S.,[1] Anna Nagao, B.S.,[1,2] Nagako Sougawa, D.M.D., Ph.D.,[1]
Naosumi Sekiya, M.D.,[3] Kazuhiro Takekita, M.S.,[1,2] Yashuhiro Shudo, M.D.,[3]
Shigeru Miyagawa, M.D., Ph.D.,[3] Hiroshi Komoda, M.D., Ph.D.,[1,5]
Teruo Okano, Ph.D.,[6] and Yoshiki Sawa, M.D., Ph.D.[2,3]

Adipose tissue–derived mesenchymal stem cells (ADMSCs) are multipotent cells. Here we examined whether human ADMSCs (hADMSCs) could differentiate into cardiomyoblast-like cells (CLCs) by induction with dimethylsulfoxide and whether the cells would be utilized to treat cardiac dysfunction. Dimethylsulfoxide induced the expression of various cardiac markers in hADMSCs, such as α-cardiac actin, cardiac myosin light chain, and myosin heavy chain; none of which were detected in noncommitted hADMSCs. The induced cells were thus designated as hADMSC-derived CLCs (hCLCs). To confirm their beneficial effect on cardiac function, hCLC patches were transplanted onto the Nude rat myocardial infarction model, and compared with noncommitted hADMSC patch transplants and sham operations. Echocardiography demonstrated significant short-term improvement of cardiac function in both the patch-transplanted groups. However, long-term follow-up showed rescue and maintenance of cardiac function in the hCLC patch-transplanted group only, but not in the noncommitted hADMSC patch-transplanted animals. The hCLCs, but not the hADMSCs, engrafted into the scarred myocardium and differentiated into human cardiac troponin I-positive cells, and thus regarded as cardiomyocytes. Transplantation of the hCLC patches also resulted in recovery of cardiac function and improvement of long-term survival rate. Thus, transplantation of hCLC patches is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

Introduction

END-STAGE HEART FAILURE remains a major cause of death worldwide, with most cases due to ischemia. This is despite the remarkable progress in recent years in both medical and surgical treatments for heart failure. Cardiac transplantation and mechanical support using implantation of the left ventricular assist system were established as the ultimate means of support for these patients.[1,2] However, these treatment entities have limitations including donor shortage, rejection, and left ventricular assist system durability, and alternative strategies are needed in such circumstances.

Cellular cardiomyoplasty was developed as a new approach to restore impaired heart function,[3,4] using a variety of cell types, with encouraging initial results.[3–5] Mesenchymal stem cells (MSCs) seem particularly advantageous for cellular therapy in general because they are multipotent, potentially immune privileged,[6] and expand easily *ex vivo*. MSCs also proliferate rapidly, induce angiogenesis, and can differentiate into cardiomyogenic cells.[7–10] An MSC population was recently isolated from human adipose tissue, which is abundantly available and can be resected easily and safely in most patients.[11,12] These adipose tissue-derived cell lineages showed cardiomyocytic differentiation and rescued

[1]Department of Somatic Stem Cell Therapy, Foundation for Biomedical Research and Innovation, Kobe, Japan.
[2]Medical Center for Translational Research, Osaka University Hospital, Suita, Japan.
[3]Department of Surgery, Osaka University Graduate School of Medicine, Suita, Japan.
[4]Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan.
[5]Department of Internal Medicine, National Hospital Organization Chiba Medical Center, Chiba, Japan.
[6]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan.

cardiac dysfunction in a myocardial infarction (MI) animal model. Thus, the adipose tissue is a convenient and preferred source of stem cell recovery for cardiac therapy. Recently, transplantation of monolayered adipose tissue-derived MSCs (ADMSCs) into MI rats reversed wall thinning in the scarred area and improved cardiac function in a short term, with the engrafted sheet of cells forming a thick stratum containing newly formed vessels and scattered cardiomyocytes derived from the implanted cells.[13] As patients with severe heart failure desire sustained and long-term recovery of cardiac function after treatment rather than short-term improvement, continued efforts should be made to develop cell transplants from ADMSCs that survive and differentiate into cardiomyocytes *in vivo* for subsequent engraftment onto scarred myocardium.

This study investigated the differentiation of human ADMSCs (hADMSCs) into cardiomyoblast-like cells (CLCs) *in vitro*, analyzed the functional and histological regeneration of damaged myocardium after transplantation of CLCs *in vivo*, and examined the effects of such transplantation on long-term patient survival.

Materials and Methods

Adipose tissues from human subjects

Excess omental adipose tissues were resected from the gastro-omental artery during coronary artery bypass graft surgery and gastrectomy in 10 subjects [4 men and 6 women; age, 55 ± 5 years, mean ± standard error of mean (SEM); range, 40–60 years]. All subjects provided informed consent, and the Review Board for Human Research of Osaka University Graduate School of Medicine approved all protocols. All subjects fasted for at least 10 h before surgery and none was on steroid therapy at the time of surgery. Ten to 50 grams of adipose tissue was obtained from each subject.

Isolation of hADMSCs and differentiation into CLCs

hADMSCs were obtained as reported previously, with modification.[11,14] Briefly, the resected excess adipose tissue was minced and then digested at 37°C for 1 h in Hank's balanced salt solution (Gibco–Invitrogen, Grand Island, NY) containing 0.075% collagenase type II (Sigma–Aldrich, St. Louis, MO). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800 *g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep ($d = 1.077$; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (Gibco–Invitrogen) with 10% defined fetal bovine serum (Hyclone, Logan, UT) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/L ethylenediaminetetraacetate solution (Nacalai Tesque, Kyoto, Japan). 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 60% Dulbecco's modified Eagle's medium-low glucose, 40% MCDB-201 medium (Sigma–Aldrich), 1×insulin-transferring 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% fetal bovine serum. After passaging five to six times in the same medium, the hADMSCs were used for transplantation. Cardiomyocytic differentiation was achieved by inducing hADMSCs with 0.1% dimethylsulfoxide (DMSO) for 48 h, resulting in a population named CLCs.

Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from hADMSCs and cardiomyoblasts using an RNAeasy kit (Qiagen, Hilden, Germany). As a control, excess human myocardium was resected during maze surgery from 10 matched subjects (4 men and 6 women; age, 55 ± 5 years, mean ± SEM; range, 40–60 years) with informed consent. Control subjects also fasted for at least 10 h before surgery, and none was taking steroids. Approximately 1 g of myocardium was obtained from each subject, and the same protocol was performed to obtain total RNA. After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen, Carlsbad, CA). The absence of DNA contamination in RNA samples was confirmed with polymerase chain reaction (PCR) primers flanking an intron. Primers and the reaction conditions are described in Table 1. The PCR products were fractionated by 2% agarose gel electrophoresis.

TABLE 1. PRIMERS USED IN REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Primer		*Sequence*	*No. of cycles*	*Annealing temperature (°C)*
GAPDH	Forward	GTCAGTGGTGGACCTGACCT	35	60
	Reverse	AGGGGAGATTCAGTGTGGTG		
Islet-1	Forward	TGATGAAGCAACTCCAGCAG	35	60
	Reverse	GGACTGGCTACCATGCTGTT		
Nkx2.5	Forward	GGTGGAGCTGGAGAAGACAGA	35	60
	Reverse	CGACGCCGAAGTTCACGAAGT		
GATA-4	Forward	ACCAGCAGCAGCGAGGAGAT	35	60
	Reverse	GAGAGATGCAGTGTGCTCGT		
α-Cardiac actin	Forward	GGAGTTATGGTGGGTATGGGTC	35	60
	Reverse	AGTGGTGACAAAGGAGTAGCCA		
Myosin light chain-2v	Forward	5′-GCGCCAACTCCAACGTGTTCT	35	60
	Reverse	5′-GTGATGATGTGCACCAGGTTC		
Myosin heavy chain	Forward	GGGGACAGTGGTAAAAGCAA	35	60
	Reverse	TCCCTGCGTTCCACTATCTT		

Model animals for MI

The left anterior descending coronary artery of rats with severe combined immunodeficiency was ligated. In brief, rats were anesthetized with nembutal (40 mg/kg), before being intubated and ventilated at a rate of 60 cycles/min with a tidal volume of 5 mL under room air supplemented with oxygen (2 L/min). The hearts were exposed through the fifth left-intercostal space and the left anterior descending was ligated. After 4 weeks, the hearts were again exposed through the fifth left-intercostal space, and the infarct area was identified visually based on surface scarring and abnormal wall motion. Cell sheets were subsequently implanted onto the infarcted myocardium. The control group was treated similarly, but no cell sheets were implanted. The Osaka University Graduate School of Medicine Standing Committee on Animals approved all experimental protocols.

Preparation of monolayered cell sheets

After four to five passages, the hADMSCs were trypsinized and then replated onto 35-mm temperature-responsive dishes (CellSeed, Tokyo, Japan) in 2 mL of expansion medium at 1×10^6 cells per dish. After culture at 37°C for 2 days, 0.1% DMSO was added to the medium on half of the dishes to differentiate the hADMSCs into cardiomyoblasts. After 2 days of culture, the cells were incubated again at 20°C. Within 20 min, the hADMSCs and CLC sheets detached spontaneously and floated up into the medium for use as monolayered cell grafts.[13,15–17]

Assessment of rat cardiac function

Cardiac ultrasound studies were performed before ligation, before implantation, and at 2, 4, 8, 10, 12, 14, and 16 weeks after implantation using a SONOS 7500 (Philips Medical Systems, Andover, MA). Plasma atrial natriuretic protein (ANP) level was analyzed using an ANP ELISA system (Phoenix Pharmaceuticals, Burlingame, CA) by following the instructions supplied by the manufacturer.

Histological analyses

The rat hearts were dissected out and immediately fixed overnight in 4% paraformaldehyde, washed in 70% alcohol, dehydrated through a graded ethanol series, cleared in xylene, and finally processed for embedding in paraffin wax. Paraffin sections were cut at 5 μm thickness, delineated on the microscope slide using a Dako pen (Dako, Glostrup, Denmark), deparaffinized in xylene, and then rehydrated through a graded ethanol series into distilled water. The sections were then immersed in Target Retrieval Solution (Dako) in distilled water and boiled, followed by cooling at room temperature for 20 min. The sections were then washed in two changes of Tris-buffered saline (TBS), pH 7.4, followed by 1% polyoxyethylene sorbitan monolaurate (Tween 20) in TBS (TBS-T), and then an overnight incubation with 10% Blocking One® (Nacalai Tesque) in TBS-T. The sections were then incubated in a humid chamber for 16 h at 4°C with mouse monoclonal antibodies to α-cardiac actin (α-CA) and human troponin I, diluted in the blocking solution, followed by Alexa Fluor 546-labeled donkey anti-goat IgG (Molecular Probes, Eugene, OR). The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan).

Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were analyzed for statistical significance by the Student's *t*-test using SPSS Statistics 17.0 (SPSS, Inc., Chicago, IL). A *p*-value less than 0.05 denoted a statistically significant difference. Survival curves were constructed by the Kaplan–Meier method and survival among groups was compared using the Log-Rank test (StatMate III for Windows; Atoms, Tokyo, Japan).

Results

Cardiac differentiation of hADMSCs into CLCs

The potential for hADMSCs to differentiate into CLCs was evaluated from the mRNA expression of several cardiac differentiation markers by reverse transcriptase-PCR before and after DMSO induction, as follows: *islet-1* is a cardiac stem cell marker; *Nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and α-*CA*, *myosin light chain*, and *myosin heavy chain* (*MHC*) are markers of cardiac differentiation (Fig. 1A). Preinduced hADMSCs expressed *islet-1* and *Nkx2.5* mRNA, but not that of *GATA-4*, α-*CA*, *myosin light chain*, or *MHC*. After induction by DMSO for 48 h, hADMSCs expressed all markers, indicating that DMSO treatment successfully differentiated hADMSCs into cells of the cardiac lineage, and these induced cells were named CLCs.

Preparation and transplantation of hADMSC-derived CLC patches

To evaluate the potential therapeutic usefulness of CLCs, we designed an experimental rat model of coronary ligated infarction to assess cardiac function after transplantation of CLC patches. CLC and control hADMSC patches were prepared from cell sheets, as described earlier (Fig. 1B). These patches were transplanted onto the scarred area of the left ventricular wall in the MI model Nude rats, whose left anterior descending artery had been ligated 4 weeks before graft implantation (Fig. 1C, D). Sham transplantations were also performed.

Effects of CLC transplantation on cardiac function and survival rate

Cardiac function was assessed by echocardiography at preligation, pretransplantation, and every 2 weeks after transplantation (Fig. 1D). Sixteen weeks after transplantation, the treated animals were sacrificed and cardiac tissues prepared for histological examination. Four weeks after graft implantation, wall motion was improved in both control and CLC patch-implanted hearts. However, the wall motion of control and noncommitted hADMSC patch-transplanted heart tissue was exacerbated at 16 weeks after transplantation, while improved motion was maintained with the CLC patch transplants (Fig. 2A). In the early phases of the post-transplantation period, left ventricular diastolic dimension was significantly reduced in both the transplanted groups, but by 8 weeks after implantation this parameter increased in the control hADMSC patch-transplanted group, whereas it remained unchanged in those animals that received CLC patch transplants (Fig. 2B). Likewise, left ventricular ejection

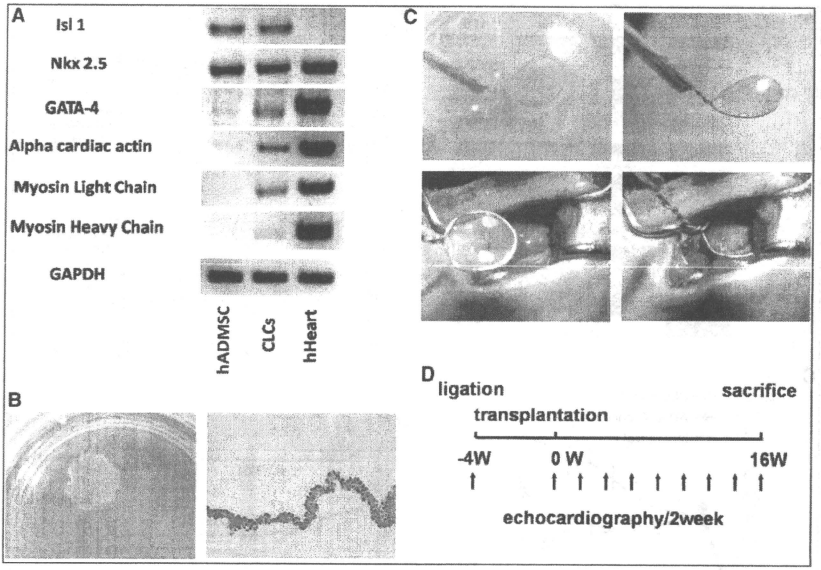

FIG. 1. Preparation and transplantation of human adipose tissue-derived mesenchymal stem cell (hADMSC)-derived cardiomyoblast-like cell (hCLC) patches. (**A**) Treatment with dimethylsulfoxide (DMSO) transformed hADMSCs into CLCs. The mRNA expressions of *islet-1*, *Nkx2.5*, *GATA-4*, *α-cardiac actin* (*α-CA*), *myosin light chain* (*MLC*), and *MHC* were analyzed by reverse transcriptase–polymerase chain reaction. The mRNAs for *islet-1* and *Nkx2.5*, with trace levels of *GATA-4* and *α-CA*, were expressed in hADMSCs, but no expression of *MLC* or *MHC* was detected. After induction with DMSO for 48 h, the hADMSCs also expressed *MLC* and *MHC*, indicating a phenotypic change into CLCs. (**B**) Preparation of hADMSC-derived CLC patches. The hADMSCs were cultured on temperature-responsive dishes for 48 h with 0.1% of DMSO for differentiation into CLCs. As the culture temperature was decreased from 37°C to 20°C, the CLCs detached spontaneously as sheets and floated up within 30 min into the culture media as a layered CLC patch. (**C**) Transplantation of hADMSC-derived CLC patches. Detached patches were transplanted onto the left ventricular wall scar in the myocardial infarction (MI) model Nude rats. (**D**) Protocol used for assessment of cardiac function. The left anterior descending artery was ligated in Nude rats at 4 weeks before graft implantation. Cardiac function was assessed by echocardiography at preligation, pretransplantation, and every 2 weeks following transplantation. The treated animals were sacrificed 16 weeks after transplantation and prepared for histological examination.

fractions improved in both the implanted groups until 8 weeks, after which time it worsened only in the group transplanted with noncommitted hADMSC patches (Fig. 2B).

ANP was then measured to confirm that chronic heart failure due to MI could be treated by CLC patch transplantation (Fig. 2C). The ANP levels were significantly increased after MI in all groups (Fig. 2C). The sham-operated MI control group showed incremental increases in plasma ANP over the time course of examination, whereas both CLC patch- and hADMSC patch-transplanted animals had low ANP levels until 8 weeks after treatment. However, ANP levels increased subsequently in the hADMSC patch-transplanted group, whereas the CLC patch-transplanted group maintained the improvement in ANP levels.

The Kaplan–Meier survival curve showed higher long-term survival rates in cell patch-transplanted groups than in sham-operated MI controls (Fig. 2D). Notably, no rat died after transplantation of an hADMSC-derived CLC patch. Survival at 16 weeks after surgery was 100% for the CLC group, 80% for the hADMSC group, and 16% for the sham-operated group, with a significant difference between the two transplanted groups. These results suggest that transplantation of hADMSC-derived CLCs has beneficial effect in rats with heart failure induced by MI.

Effects of CLC transplantation on cardiac structure

Cardiac structure was next examined histologically to analyze further the difference between CLC patch- and noncommitted hADMSC patch-transplanted animals in the longer term (Fig. 3). On hematoxylin and eosin and Masson trichrome staining, the sham-transplanted MI control rats

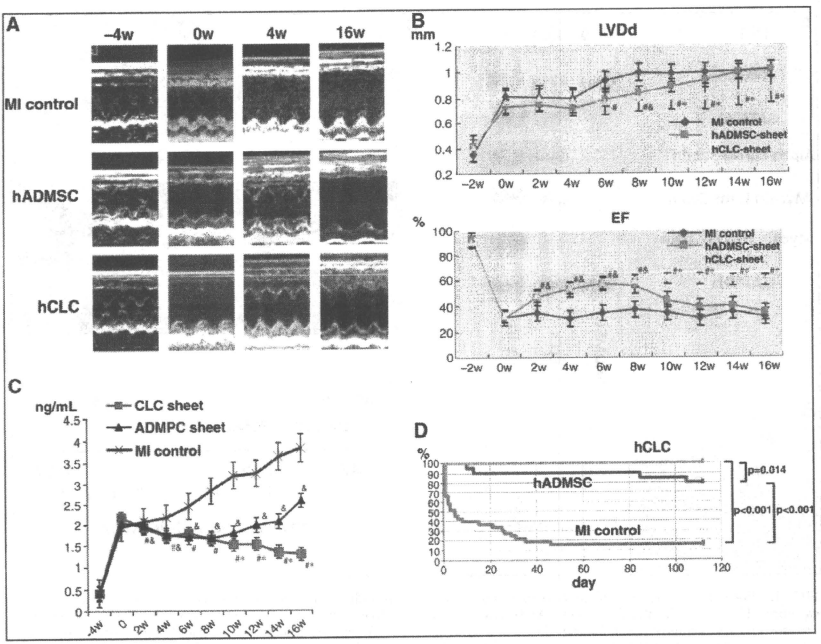

FIG. 2. Effects of CLC patch transplantation on cardiac function and long-term survival. (**A**) In both the patch-transplanted groups, echocardiography showed improved wall motion within 4 weeks of transplantation. However, at 16 weeks after transplantation, the wall motion of noncommitted hADMSC patch-transplanted rats worsened, whereas it was maintained in the CLC patch-transplanted animals. (**B**) Left ventricular diastolic dimension and ejection fractions improved significantly in both the patch-grafted groups in the early phase, as confirmed by echocardiography. However, these two parameters of cardiac function worsened at 8 weeks after implantation in the noncommitted hADMSC patch-transplanted groups, but not in the CLC patch-transplanted group. The numbers of all groups were five. Data are mean ± standard error of mean ($^{\#}p < 0.05$; MI control vs. the hCLC patch-transplanted animals; $^{\&}p < 0.05$; MI control vs. the noncommitted-hADMSC patch-transplanted rats; $^{*}p < 0.05$; hCLC patch-transplanted vs. the noncommitted hADMSC patch-transplanted rats, respectively). (**C**) Plasma ANP levels. Sham-operated MI control group showed increment of plasma ANP levels during the course of the experiment. Both the CLC patch- and hADMSC patch-transplanted groups showed suppression of ANP level increment till 8 weeks after treatment. ANP levels of the hADMSC patch-transplanted group increased from 8 weeks after transplantation, but no change in ANP levels was noted in the CLC patch-transplanted group. The numbers of all groups were four. (**D**) Long-term survival of rats with chronic heart failure that received the CLC patch ($n = 28$), noncommitted hADMSC patch ($n = 20$), or sham operation ($n = 37$). The Kaplan–Meier survival curve demonstrated that no rat died after transplantation of hADMSC-derived CLC patch. The survival rate at 16 weeks after surgery was 80% for the hADMSC group versus 16% for the sham-operated group. Log-rank test; p-values are indicated. LVDd, left ventricular diastolic dimension.

showed only a thin layer of cardiac muscle and fibrotic tissues in the scarred anterior left ventricular wall (Fig. 3A, B). Rats implanted with noncommitted hADMSCs showed small patches of cardiac muscles over that seen in the control MI rats (Fig. 3C, D). On the other hand, the rats transplanted with CLC patches showed significant reversal of the infracted myocardium and a full cardiac muscle layer overlaying the transplanted area (Fig. 3E, F, arrowheads).

CLCs differentiate into cardiac muscle in situ

The *in situ* differentiation capacity of the implanted cell sheets into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for α-CA and human troponin I (Fig. 4). Thin layers of α-CA-positive cells were observed on the scarred myocardium of sham-operated MI control rats (Fig. 4A). A similar but thicker

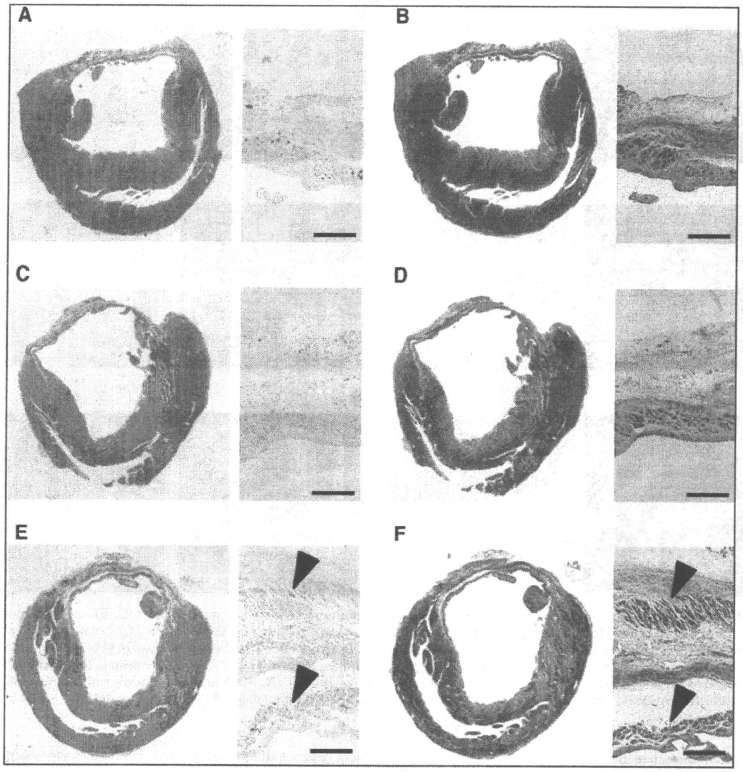

FIG. 3. Effects of CLC transplantation on cardiac structure. Photomicrographs showing representative myocardial sections stained with hematoxylin and eosin (**A, C, D**) and Masson trichrome (**B, D, F**) in the individual groups. The transplanted CLC patch reversed wall thinning of the infracted myocardium and another cardiac muscle was layered onto the transplanted area (arrowheads). (**A, B**) Sham-operated MI control group; (**C, D**) noncommitted hADMSC patch-transplanted group; and (**E, F**) hADMSC-derived CLC patch-transplanted group. Bars = 200 μm.

layer of α-CA-positive cells was apparent in the tissues from noncommitted hADMSC-transplanted rats (Fig. 4A, C), whereas the CLC patch-transplanted group showed two cardiac muscle layers positive for α-CA (Fig. 4E, arrow and arrowhead). There were no human troponin I-positive cells in the sham-operated MI control group (Fig. 4B), but some were observed in the noncommitted hADMSC patch-transplanted group (Fig. 4D). As shown in Figure 4F, large amounts of human troponin I-positive myocardium was observed in the CLC-transplanted animals (arrow) in addition to some human troponin I-negative but α-CA-positive myocardium in the internal myocardial layer (Fig. 4E, F, arrowhead). These results indicated that CLCs can efficiently differentiate into cardiomyocytes *in situ*.

Discussion

There are several advantages to hADMSC-derived CLC patch transplantation for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and the cells can be obtained in large quantities, without serious ethical issues. Second, hADMSCs differentiate into CLCs by induction with DMSO, which is available in current good manufacturing practice grade. Third, hADMSC-derived

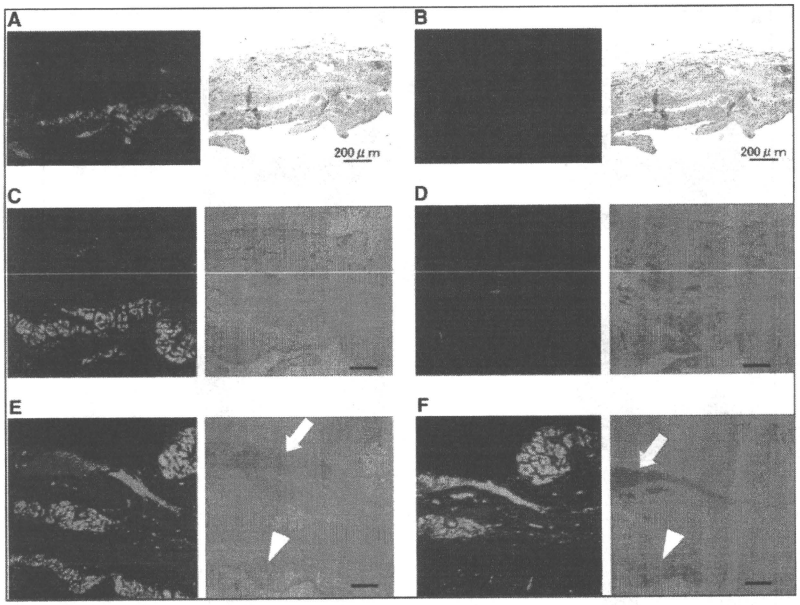

FIG. 4. CLCs differentiate into cardiac muscles *in situ*. Immunofluorescence with anti-α-CA (**A, C, E**) and anti-human-specific cardiac troponin I (**B, D, F**) antibodies, merged with phase contrast. In the hADMSC-derived CLC patch-transplanted groups, two cardiac muscle layers positive for α-CA are observed (**E**, arrow and arrowhead). A large mass of human troponin I-positive myocardium was observed in the CLC-transplanted tissues (**F**, arrows). In the same animals, the internal myocardium layer expressed α-CA but not human troponin I (**E, F**, arrowheads). (**A, B**) Sham-operated MI control group; (**C, D**) noncommitted hADMSC patch-transplanted group; and (**E, F**) CLC patch-transplanted group. Bars = 200 μm.

CLCs can differentiate into cardiomyocytes *in vivo* within the myocardial milieu, resulting in increment of myocardial muscle force. Finally, reconstruction of thick myocardial tissue rescued cardiac dysfunction after MI and improved long-term survival.

The choice of cell source is critical for realizing success in cellular therapy.[18] Liposuction surgeries yield from 100 mL to >3 L of lipoaspirate tissue.[19] The initial isolation of cells from adipose tissue was described by Bjorntorp *et al.*[14] This procedure was since modified to isolate cells from human adipose tissue specimens.[20–22] In this context, Zuk *et al.*[11] reported that the preadipocytes exhibited stem cell features as MSCs, currently known as ADMSCs. Because of the above-stated advantages of procuring cells for therapy from adipose tissues, hADMSCs present a potential and promising source for cellular therapy, even in patients with post-MI severe heart failure.

The *in vitro* differentiation of ADMSCs is now well reported, and experimental findings in recent years suggested considerable therapeutic potential for cellular replacement in the context of acute MI and chronic progressive cardiac disease.[23–27] Stem cells are differentiated into a cardiomyocyte lineage by treatment with 5-azacytidie, retinoic acid, oxytocin, and many other reagents.[28–32] We proposed that DMSO could differentiate hADMSCs into CLCs, based on the differentiation of P19 embryonic stems cells into cardiomyocytes with DMSO.[31–33] It was notable that DMSO is also available in current good manufacturing practice grade. Unfortunately, DMSO-treated hADMSCs did not show spontaneous beating as their terminal differentiation function, but the cells did express the mature markers α-CA, *myosin light chain*, and *myosin heavy chain* to a lesser extent. There are no reports of the use of DMSO to commit ADMSCs to a cardiomyocytic lineage. The mechanism by which DMSO elicits its effect on differentiation remains unclear. It is possible that DMSO increases intracellular calcium ion concentration, thereby elevating phosphatidylethanolamine levels in the cells and controlling the distribution of protein kinase C to commit the P19 stem cells.[33–36] These mechanisms should be investigated further in the near future.

The *in vitro* differentiation of ADMSCs has been well reported,[23–27] although only a few reports relate to the differentiation of these cells into cardiomyocytes *in vivo*. Recently,

Miyahara *et al.*[13] reported the use of monolayered ADMSCs for myocardial repair. In their study, rat ADMSCs were isolated and grown as intact monolayer sheets using temperature-responsive culture dishes. Placement of the ADMSC sheets onto a scarred myocardium in rats resulted in diminished scarring and enhanced cardiac structure and function. Histological analysis demonstrated that the engrafted ADMSC sheets grew to form a thickened layer over the infarcted muscle that included newly formed vessels and a few cardiomyocytes. In our study, hADMSC-derived CLCs differentiated into cardiomyocytes in a myocardial milieu, indicated by the immunohistological results in which transplanted cells expressed human troponin I *in vivo*. Newly developed myocardium might augment cardiac function, and thus hADMSC patch transplantation was performed as a control. Cardiac dysfunction was rescued in a short term, although the numbers of cardiomyocytes derived from transplanted cells were low. In this context, Gimble *et al.*[19] suggested that hADMSCs might secrete angiogenic factors and/or antiapoptotic factors.

Transplantation of the hADMSC-derived CLC regenerated the thick myocardial tissues, rescued cardiac dysfunction after MI, and improved long-term survival rate compared with the noncommitted hADMSCs and sham-operated MI controls. The existing literature suggests that ADMSCs can be engrafted and survive within an infarcted myocardial milieu, acquire phenotypic markers consistent with cardiomyocytic and vascular-related lineages, and have a positive impact on structural and functional endpoints.[19,23–27] These are desirable outcomes for cardiac function and survival. However, few reports have applied long-term observation of the transplanted animals. Our study therefore observed the three rat groups for 16 weeks after transplantation. Only CLC transplantation provided the desired outcome at the experimental endpoint. Despite these encouraging results, much progress is needed to realize the hope of cell therapies for myocardial damage. First, delivery of the cell sheets to patients should be optimized for each given disease. Second, the issue of vascularization should be considered in the infracted or affected tissues after transplantation, because many small CLC patches would be necessary for a clinical cure. Finally, the value and impact of CLC patch transplantation should be confirmed in large animal models before embarking on clinical applications.

In conclusion, we showed that the phenotype of hADMSCs could be changed to that of CLCs by induction with DMSO. These hADMSC-derived CLCs engrafted into a scarred myocardium and differentiated into cardiomyocytes. The CLC patch transplantation also resulted in recovery of cardiac function and improved survival rate. Thus, transplantation of hADMSC-derived CLC patches in heart patients might be a potentially effective therapeutic strategy for cardiac tissue regeneration in the near future.

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Disclosure Statement

No competing financial interests exist.

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

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

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