Factors Influencing the Surface Modification of Mesenchymal Stem Cells with Fluorescein-Pegylated Lipids

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Artificial introduction of functional molecules on the cell surface may be a promising way to improve the therapeutic effects of cell therapy. Pegylated lipids are conventionally used in drug carriers. The lipid part of pegylated lipids noncovalently interacts with the cell surface. However, little information is available regarding conditions for cell-surface modification by using pegylated lipids. In this study, we synthesized fluorescein-labeled pegylated lipids and evaluated the factors that affect modification efficiency by using human mesenchymal stem cells (hMSCs). As the concentration of the pegylated lipid as well as the exposure time increased, the modification efficiency increased. The modification efficiency at 37°C was 20- and 3-fold higher than that at 4°C and 25°C, respectively. In addition, with an increase in the molecular weight of polyethylene glycol (PEG), more pegylated lipids were extracellularly distributed than those intracellularly distributed. At the optimal condition, pegylated lipids were observed mainly on the cell membrane by confocal microscopy. In contrast, the cell condition (adherent or nonadherent) had little or no effect on the cell-surface modification efficiency. The results of this study will be useful for constructing an optimal modification method for introducing functional molecules on the cell surface.

Key words pegylated lipid; mesenchymal stem cell; cell surface modification; cell therapy

Recently, the field of cell therapy has been rapidly expanding because of the considerable progress in cellular and molecular biology. The transplantation of dendritic cells,[1] fibroblasts,[2] corneal cells,[3] and myocardial cells[4] has shown good treatment outcomes in tumor immune therapy or regenerative therapy. Recently, multi- or pluripotent cells, including mesenchymal stem cells (MSCs), embryonic stem (ES) cells,[5] and induced pluripotent stem (iPS) cells,[6] have become attractive candidates for regenerative medicine. The dynamics of transplanted cells, involving adhesion, migration, and cell–cell interactions, are important factors that affect treatment outcomes of cell therapy.[7] Therefore, the artificial introduction of functional molecules on the cell surface to control cell dynamics is a promising way to improve the therapeutic effects of cell therapy.

Genetic and chemical methods are the main approaches in cell surface engineering.[8] For the genetic approach, transfection/transduction with plasmid DNA[9] or a viral vector[10] is generally used to induce the expression of functional molecules on the cell surface. However, it takes a long time to induce the expression of target molecules. Moreover, transfection efficiency when using nonviral techniques is extremely low, especially in primary and immature cells. In clinical applications, a rapid and effective method for cell surface engineering is necessary. For the chemical approach, it is difficult to reverse or prevent covalent conjugation with nontargeted proteins. In contrast, noncovalent modification with a lipid or polymer is a simpler and more biocompatible method.[11,12] Polyethylene glycol (PEG) is often used for the retention of drug carriers in blood or controlled release in drug delivery systems in clinical use.[13] Recently, it was reported that by simply culturing cells in a medium containing pegylated lipids, the lipid part of the pegylated lipids could noncovalently interact with the cell surface.[11,14] In fact, pegylated lipid could encapsulate islets with thin membrane.[14] And antibodies modified on tumor cells through pegylated lipid could enhance dendritic phogocytosis.[15] Therefore, pegylated lipids would be useful for adding functional molecules to the cell surface. Using this method, cells can be more quickly modified using functional molecules than when using the genetic method. Moreover, most of the cells in culture could be modified using functional molecules. Considering these factors, the cell-surface modification with pegylated lipid method may be useful for clinical applications because of its operability, high efficiency, and biocompatibility. In addition, cell-surface modification with pegylated lipids is applicable to all cell types. However, little information is available regarding the modification conditions. Such information, which determines the modification efficiency of functional molecules, should be determined because the number of functional molecules on the cell surface directly influences the cell's fate or function. To improve the therapeutic effect of cell transplantation by cell modification and to optimize the modification method, it is essential to gather and organize fundamental information regarding the effects of these factors on the modification efficiency.

MSCs, which can be isolated from the bone marrow, cord blood or adipose,[16,17] can be used for autologous transplantation; therefore, MSC transplantation generally does not have ethical concerns. In fact, MSCs have been used in clinical trials for severe diabetes,[18] acute myocardial infarction,[19] and neurological deficits.[20] In this study, we used a human MSC (hMSC) line to evaluate the effect of each of the following factors on the modification efficiency: concentration

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of pegylated lipids, exposure time, cell condition (adherent or nonadherent), temperature, and the molecular weight of the PEG chain. Cytotoxicity was also evaluated under several conditions for cellular modification.

MATERIALS AND METHODS

Fluorescent Labeling of Pegylated Lipids NH_2-PEG-distearoyl phosphatidyl ethanolamine (DSPE; molecular weight of the PEG part: 2000, 5000, and 10000; NOF Corporation, Tokyo, Japan) and fluorescein-*N*-hidroxysuccinimide (NHS) were dissolved in acetone (molar ratio, 1 : 2) and incubated overnight at room temperature. Through gel filtration using a PD10 column (GE Healthcare, Buckinghamshire, U.K.), the solvent was exchanged with water, and unreacted fluorescein-NHS was excluded. After freeze-drying, fluorescein-PEG-DSPE (Flu-PEG-DSPE) was obtained.

Cell Culture hMSCs (UE7T-13 cells; RIKEN Cell Bank, Japan, RCB2160) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; GIBCO Invitrogen, Tokyo, Japan), 1000 U/mL penicillin G, and 100 mg/L streptomycin. Cells were dissociated using trypsin and suspended in phosphate buffered saline (PBS).

Cytotoxicity of Flu-PEG-DSPE The cell viability upon exposure to Flu-PEG-DSPE was evaluated using the CCK-8 assay (Dojindo, Kumamoto, Japan). In the evaluation of cytotoxicity on adherent hMSCs, cells were seeded on a 96-well plate and incubated in the culture medium for 24 h. Then, cells were incubated in the medium containing Flu-PEG-DSPE. On the other hand, in the evaluation of cytotoxicity on nonadherent hMSCs, cells were dispersed in the medium containg Flu-PEG-DSPE in a container that was inverted at 3-s intervals. After treatment with Flu-PEG-DSPE and washing with culture medium, new medium containing CCK-8 solution was added to hMSCs. After incubation for 0.5 h, UV-Vis absorption was measured at 450 nm with 655 nm as the reference wavelength by using a Bio-Rad Model 550 microplate reader (Bio-Rad, Irvine, CA, U.S.A.). As a negative control, cell viability of hMSCs treated with 0.1% Triton-X for 4 h at 37°C was measured.

Evaluation of the Fluorescence Intensity of Modified MSCs by Flow Cytometry Flu-PEG-DSPE was dissolved in DMEM and applied to hMSCs. hMSCs were incubated for a predetermined time and washed in fresh DMEM for 10 min. After incubation, modified hMSCs were divided into 2 groups: a trypan blue-negative [TB (−)] group and a trypan blue-positive [TB (+)] group. Trypan blue solution (20 μL) was applied to a pellet of hMSCs of the TB (+) group, which was then kept for several tens of seconds on ice. Then, PBS was added to adjust the volume to 200 μL. Thus, the fluorescence on the cell surface of hMSCs in the TB (+) group was quenched.[22,23] The median fluorescence intensity of each group was measured by flow cytometer (BD FACS Canto II, BD Biosciences, San Jose, CA, U.S.A.). The fluorescence intensity of the cell surface was calculated using the following expression:

$$\begin{aligned}&\text{intensity of cell surface}\\&\quad=(\text{intensity of TB }(-)\text{ group})\\&\qquad-(\text{intensity of TB }(+)\text{ group})\end{aligned}$$

The ratio of the fluorescence intensity of the cell surface to the intracellular fluorescence intensity (S/I ratio) was calculated using the following expression:

$$\text{S/I ratio}=\frac{\text{intensity of cell surface}}{\text{intensity of TB (+) sample}}$$

To evaluate the stability of the Flu-PEG-DSPE modification, modified hMSCs were incubated in fresh DMEM (without Flu-PEG-DSPE) containing 10% fetal bovine serum (FBS) for the indicated period at 37°C in a container that was inverted at 3-s intervals. The fluorescence intensity of hMSCs was measured by flow cytometry. hMSCs modified using 1 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C were used to evaluate stability.

Observation of Modified MSCs by Confocal Laser Scanning Microscopy After hMSCs were modified using 0.1, 0.25, 1, 3, and 5 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C, they were fixed with 4% paraformaldehyde and then observed using a FLUOVIEW FV10i confocal laser scanning microscope (Olympus, Tokyo, Japan). To confirm the quenching of fluorescence by trypan-blue staining, hMSCs modified with 0.1 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C were stained with Hoechst 33342 (Life Technologies, Carlsbad, CA, U.S.A.) and dispersed in 200 μL of PBS with or without 20 μL of trypan blue. The cells were transferred to a chamber slide and observed using a Nikon A1R MP confocal imaging system (Nikon, Tokyo, Japan).

Measurement of Fluorescence Intensity of the Lysate of Modified hMSCs hMSCs modified using 0.1 mM Flu-PEG_{2000}-DSPE were lysed with 5% Triton-X. The fluorescent intensity of the hMSC lysate was measured by fluorometer (Fluoromax-4; HORIBA, Japan).

Calculation of the Number and the Density of Flu-PEG-DSPE on hMSC Surface The number of Flu-PEG_{2000}-DSPE on cell surface was calculated using S/I ratio and fluorescent intensity of cell lysate measured by fluorometer. The density (the area of one Flu-PEG_{2000}-DSPE molecule introduced on cell surface) was calculated from the number of introduced Flu-PEG_{2000}-DSPE and the surface area of hMSC. The cell surface area was calculated by the estimation that hMSC is sphere form whose diameter is 15 μm, which was following to a previous report.[14]

RESULTS

Cytotoxicity of Flu-PEG-DSPE hMSCs cultured under adherent or nonadherent condition were treated with 5 mM Flu-PEG_{2000}-DSPE or Flu-PEG_{5000}-DSPE, 3 mM Flu-PEG_{10000}-DSPE, or 0.1% Triton X (negative control) for 4 h at 37°C. The absorbance measured at 450 nm, which indicated cell viability, was approximately equal in the hMSC group modified with Flu-PEG-DSPE and was independent of the molecular weight of PEG. Remarkable cytotoxicity in hMSCs was not detected after treatment with 5 mM Flu-PEG_{2000}-DSPE or Flu-PEG_{5000}-DSPE, or with 3 mM Flu-PEG_{10000}-DSPE under adherent (Fig. 1A) or nonadherent conditions (Fig. 1).

Influence of the Cell Condition (Adherent or Nonadherent) on Modification Efficiency The cell-surface modification efficiency was compared between adherent and nonadherent hMSCs modified with Flu-PEG-DSPE. hMSCs were incubated under both conditions with 0.1 mM Flu-PEG_{2000}-DSPE

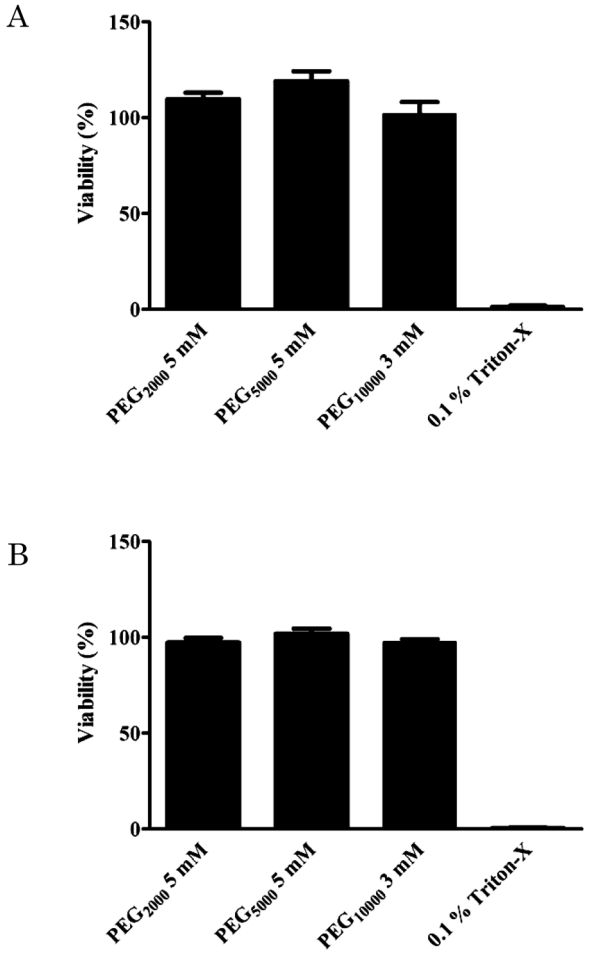

Fig. 1. Cell Viability of hMSCs Exposed to Flu-PEG-DSPE

hMSCs were treated with 5 mM Flu-PEG_{2000}-DSPE or Flu-PEG_{5000}-DSPE, 3 mM Flu-PEG_{10000}-DSPE, or 0.1% Triton X as a negative control for 4 h at 37°C under adherent (A) or nonadherent (B) condition. Each result represents the mean±S.D. (n=4).

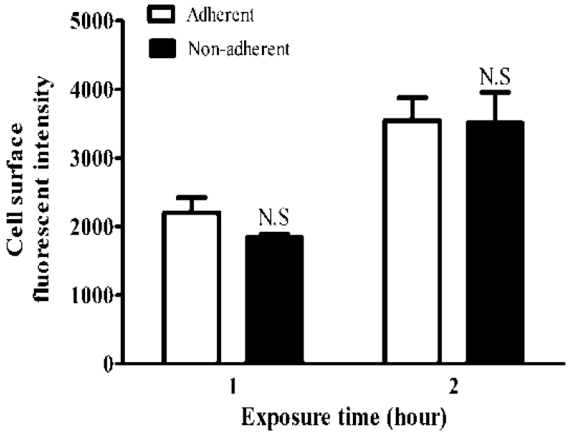

Fig. 2. Influence of Cell Conditions on Cell-Surface Modification Efficiency

hMSCs were modified under 2 cell conditions [adherent (□) or nonadherent (■)] using 0.1 mM Flu-PEG_{2000}-DSPE for 1 or 2 h at 37°C. In the nonadherent condition, hMSCs were dispersed in the medium containing Flu-PEG-DSPE and rotated at 3-s intervals in an incubator. Each result represents the mean±S.D. (n=3). Statistical significance was analyzed by Student's t-test *versus* the adherent group at each exposure time (N.S., not significant).

for 1 or 2 h at 37°C in DMEM containing 10% FBS. In the nonadherent condition, hMSCs were dispersed in the medium containing Flu-PEG-DSPE and were inverted. Thus, the cell condition does not affect cell-surface modification efficiency (Fig. 2).

Confirmation and Measurement of Fluorescein Introduced on the Cell Surface Fluorescein fluorescence was observed along the cell membrane after hMSCs were modified with Flu-PEG-DSPE (Fig. 3A). In contrast, after addition of trypan blue, the fluorescence along the cell membrane disappeared, which suggests that trypan blue quenched the fluorescence on the cell membrane. To prepare hMSCs modified using different concentrations of Flu-PEG-DSPE, adherent hMSCs were incubated with 1 mM Flu-PEG_{2000}-DSPE for 1, 3, 6, 12, or 24 h at 37°C in DMEM containing 10% FBS. After incubation, modified hMSCs were divided into 2 groups. The fluorescence intensity of the cell lysate in one group was measured by fluorometry (Fig. 3B). The fluorescence intensity of the whole cell was measured in the other group by flow cytometry (FACS; Fig. 3C). The fluorescence intensity of the cell lysate was highly correlated with the fluorescence intensity of whole cells (Fig. 3D).

The number and density of Flu-PEG_{2000}-DSPE molecules introduced to the cell surface was calculated from the fluorescence intensity of the hMSC lysate and the S/I ratio (Table 1). The area of a single Flu-PEG_{2000}-DSPE molecule introduced to the cell surface was approximately 2 nm^2 when hMSCs was modified with high density.

Influence of Flu-PEG-DSPE Concentration, Exposure Time, and Temperature on Modification Efficiency The effect of Flu-PEG-DSPE concentration on the efficiency of cell surface modification with Flu-PEG-DSPE was evaluated. Nonadherent hMSCs were incubated with 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C in DMEM containing 10% FBS. As the concentration of Flu-PEG_{2000}-DSPE increased, the fluorescence intensity of the cell surface of the hMSCs increased (Fig. 4A). Images of modified hMSCs show that the fluorescence was localized mainly at the cell surface (Fig. 4B).

Next, the effect of Flu-PEG-DSPE exposure time on cell-surface modification efficiency was evaluated. Adherent hMSCs were incubated in 0.1 or 1 mM Flu-PEG_{2000}-DSPE at 37°C in DMEM containing 10% FBS. As the exposure time was prolonged, the surface fluorescence intensity of the hMSCs increased (Fig. 4C).

Then, the influence of temperature on cell-surface modification efficiency with Flu-PEG-DSPE was investigated. Adherent hMSCs were incubated in 0.1 mM Flu-PEG_{2000}-DSPE for 2 h in DMEM containing 10% FBS. The increase in temperature enhanced the cell surface fluorescence intensity. hMSCs modified at low temperature (4°C or 25°C) showed remarkably low cell-surface fluorescence intensity relative to hMSCs modified at 37°C or 42°C (Fig. 4D).

Influence of Serum on Modification Efficiency To elucidate the influence of serum on the efficiency of cell-surface modification with Flu-PEG-DSPE, nonadherent hMSCs were incubated with 0.1 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C in DMEM containing 0%, 2.5%, 5%, or 10% FBS. As the FBS concentration was increased, the surface fluorescent intensity of hMSCs decreased (Fig. 5).

Stability of Flu-PEG-DSPE Modification of hMSCs in Medium with Serum The amount of Flu-PEG-DSPE on the

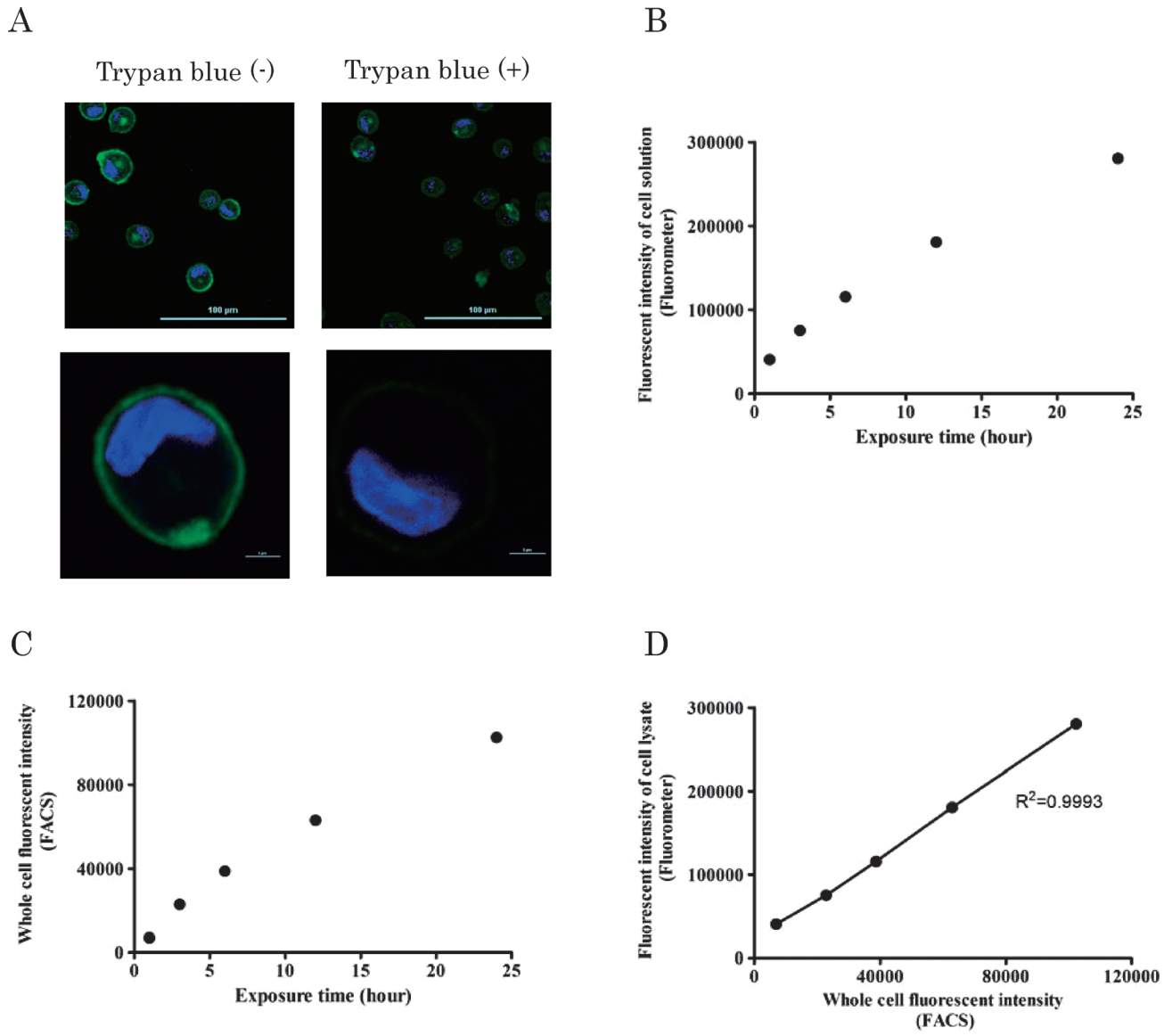

Fig. 3. Confirmation and Measurement of Fluorescein Modified on the Cell Surface

Fluorescent images of hMSCs modified by Flu-PEG$_{2000}$-DSPE with or without trypan-blue staining were observed by confocal microscopy. Scale bars, 100 μm (upper images) and 5 μm (lower images) (A). hMSCs were incubated with 1 mM Flu-PEG$_{2000}$-DSPE for 1, 3, 6, 12, and 24 h at 37°C in DMEM containing 10% FBS. The fluorescence intensity of the cell lysate was measured by fluorometry (B). Whole-cell fluorescence intensity was measured by flow cytometry (FACS; C). Correlation between fluorescence intensity of the cell lysate measured by fluorometry, and whole-cell fluorescence intensity measured by flow cytometry (D).

Table 1. The Number and Density of Flu-PEG$_{2000}$-DSPE Molecules Introduced to hMSC Surface

Incubation time (h)	Fluorescent intensity of lysate	Whole cell fluorescent intensity		S/I ratio	The number of Flu-PEG-DSPE molecule introduced on cell surface (per cell)	The area of one Flu-PEG-DSPE molecule introduced on cell surface (nm^2)
		Without TB	With TB			
1	40273	6920	4290	0.61	2.68E+07	26.35
3	75060	22849	12938	0.77	6.08E+07	11.61
6	115546	38729	20733	0.87	1.03E+08	6.87
12	180500	62908	26730	1.35	2.02E+08	3.49
24	280416	102358	38964	1.63	3.42E+08	2.07

S/I ratio, ratio of the fluorescence intensity of the cell surface to that of the inside of cell.

surface of hMSCs decreased slightly as the length of incubation of the cells in DMEM without Flu-PEG-DSPE increased. After incubation for 8 h, 50–60% of the initial fluorescence intensity was sustained on cell surfaces modified with Flu-DSPE$_{2000}$-DSPE or Flu-DSPE$_{5000}$-DSPE (Fig. 6).

Influence of Molecular Weight of PEG Chain on Modification Efficiency The cell-surface modification efficiency was compared for hMSCs modified with Flu-PEG-DSPE

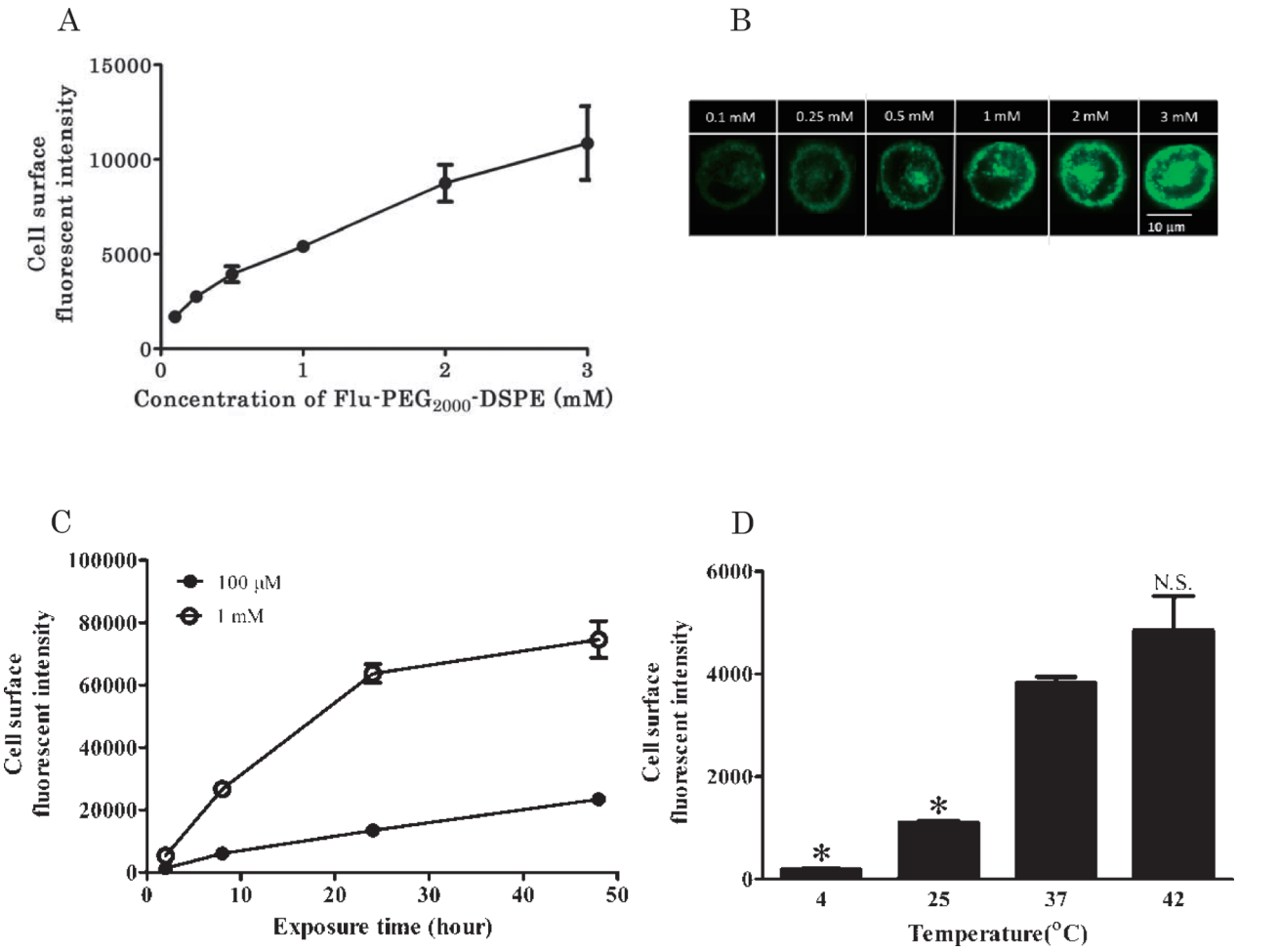

Fig. 4. Influence of Concentration, Exposure Time, and Temperature of Flu-PEG-DSPE on Cell-Surface Modification Efficiency

Nonadherent hMSCs were modified for 2 h at 37°C with each concentration of Flu-PEG_{2000}-DSPE. The cell surface fluorescent intensity of modified hMSCs was measured by flow cytometry. Each result represents the mean±S.D. (n=3; A). Fluorescent images of hMSCs modified using each concentration of Flu-PEG_{2000}-DSPE were obtained by confocal microscopy. Scale bar, 10 μm (B). Adherent hMSCs were modified with 0.1 or 1 mM of Flu-PEG_{2000}-DSPE at 37°C for the indicated times. Each result represents the mean±S.D. (n=3; C). Adherent hMSCs were modified with 0.1 mM Flu-PEG_{2000}-DSPE for 2 h at each temperature. Each result represents the mean±S.D. (n=3). Statistical significance was analyzed by Dunnett's test *versus* the group at 37°C (*p<0.05; N.S., not significant; D).

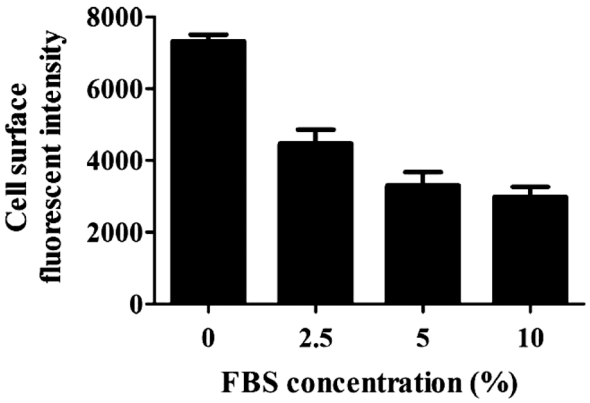

Fig. 5. Influence of FBS Concentration on Modification Efficiency

Nonadherent hMSCs were modified with 100 μM Flu-PEG_{2000}-DSPE for 2 h at 37°C in DMEM containing 0%, 2.5%, 5.0%, or 10% FBS. Each result represents the mean±S.D. (n=3).

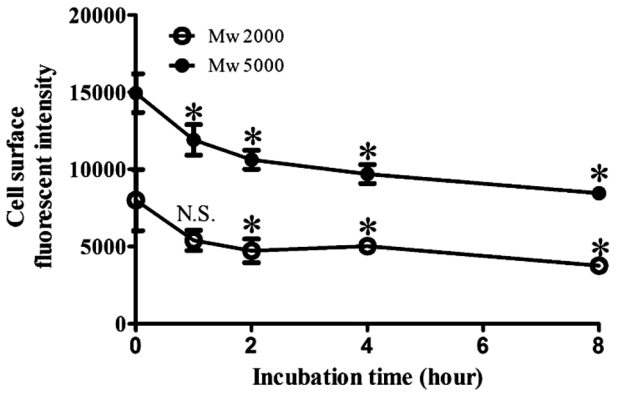

Fig. 6. Stability of Flu-PEG-DSPE Introduced to the Surface of hMSCs

hMSCs were modified with 1 mM Flu-PEG_{2000}-DSPE for 2 h at 37°C, and modified hMSCs were washed in the medium containing 10% FCS and rotated at 3-s intervals for the indicated times at 37°C in an incubator. Statistical significance was analyzed by Dunnett's test *versus* a no-wash group at each molecular weight (*p<0.05; N.S., not significant).

possessing PEG chains with different molecular weights. Nonadherent hMSCs were modified using 1 mM Flu-PEG-DSPE for 2 h at 37°C in DMEM containing 10% FBS. The fluorescence intensity of the whole cell was higher when the molecular weight of the PEG chain was lower (Fig. 7A). In contrast, the cell surface fluorescence intensity increased slightly as the molecular weight of the PEG chain increased (Fig. 7B).

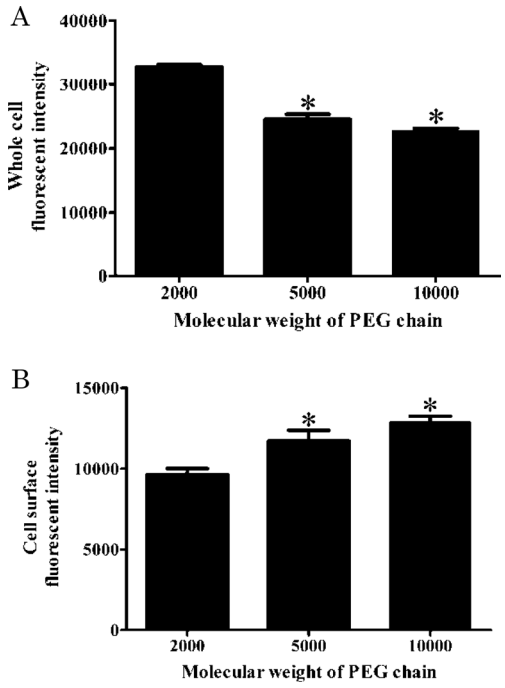

Fig. 7. Influence of the Molecular Weight of the PEG Chain on Cell-Surface Modification Efficiency

Nonadherent hMSCs were modified with 1 mM Flu-PEG-DSPE for 2 h at 37°C. Whole-cell fluorescence intensity of modified hMSCs (A). Cell surface fluorescence intensity of modified hMSCs (B). Each result represents the mean±S.D. (n=3). Statistical significance was analyzed by Dunnett's test *versus* the group with a molecular weight of 2000 (*p<0.05).

DISCUSSION

The purpose of this study was to investigate the factors which influence the surface modification of hMSCs with pegylated lipids, which is a promising tool to reinforce or add cell functions for cell therapy. We used pegylated lipids to modify the cell surface because this method is relatively simpler and quicker than genetic modification or chemical modification. Moreover, PEG-DSPE is clinically used as a liposomal carrier for drugs.[21] In this study, severe cytotoxicity of Flu-PEG-DSPE in hMSCs was not detected, even at the high concentration of 5 mM for Flu-PEG_{2000}-DSPE and Flu-PEG_{5000}-DSPE or 3 mM for Flu-PEG_{10000}-DSPE (Figs. 1A, B). In fact, Flu-PEG_{10000}-DSPE did not dissolve in DMEM containing 10% FCS at 5 mM, and 5 mM was nearly the maximal concentration for solubilization of Flu-PEG_{2000}-DSPE and Flu-PEG_{5000}-DSPE. Therefore, cell-surface modification using pegylated lipids has potential for clinical use.

The presence of pegylated lipids modified with functional molecules on the cell surface is desirable because only functional molecules presented outside of cells can have direct physiological effects in the host after transplantation. Therefore, it is important to confirm the localization of pegylated lipids, *i.e.*, whether they are inside or on the cell membrane, after modification. In this study, pegylated lipids were localized both intracellularly and on the cell surface, as determined from fluorescent images of modified hMSCs obtained using a confocal microscope (Figs. 3A, 4B). We then quantitatively evaluated the localization of the pegylated lipids in modified cells by flow cytometry. In the evaluation of cellular uptake by flow cytometry, trypan blue is generally added to cells for cell-surface fluorescence quenching.[22,23] Accordingly, we also added trypan blue for cell-surface quenching (Fig. 3A). We calculated the fluorescence intensity on the cell surface by subtracting the cellular fluorescence intensity with trypan blue from that without trypan blue, and thus, we separately evaluated the intracellular and extracellular fluorescence intensity. Therefore, cell-surface modification by pegylated lipids is valuable for the introduction of functional molecules outside of the cell membrane, which could thus be expected to have physiological effects in the host upon transplantation. Fluorescence intensity of fluorescein generally decreases in low pH.[24] In order to confirm the effect of pH on S/I ratio, we synthesized pegylated lipid conjugating with Alexa 488, which is less sensitive to low pH. Then, we compared S/I ratio evaluated by fluorescein with that evaluated by Alexa 488. As a result, there was no significant difference between S/I ratio of hMSCs modified with fluorescein-PEG-DSPE and cells modified with Alexa 488-PEG-DSPE (data not shown).

To prepare cells modified with functional molecules to enhance the effects of cell therapy, it is essential to optimize the modification conditions. In this study, we systematically evaluated factors affecting modification efficiency, including temperature, exposure time, PEG length, and cell conditions, for the modification of cells by pegylated lipids. Increases in temperature (Fig. 4D), concentration of pegylated lipids (Figs. 4A, B), and exposure time (Fig. 4C) increased the amount of pegylated lipids introduced on the cell surface. Among the factors examined, temperature had the most significant effect on modification efficiency. Comparing the modification at 37°C to that at 4°C or 25°C, we found that the amount of modified pegylated lipid on the cells increased by 20- or 3-fold, respectively (Fig. 4D), probably because the decrease in temperature decreased the diffusional mobility of the raft in the cell membrane.[25] This significant decrease in the amount of modified pegylated lipid on the cell membrane at low temperatures may be explained by a reduction in the diffusional mobility of the cell membrane. Therefore, the use of a different technique to increase the diffusional mobility of the cell membrane may further enhance the cell-surface modification efficiency. Although exposure time and the concentration of pegylated lipid were both important for effective cell modification, prolongation of the exposure time would likely be limited in clinical use because a long operation time for the cell modification procedure could diminish cell viability and cell function. Therefore, temperature and concentration are likely the most useful parameters for cell-surface modification using pegylated lipids. In therapy using MSCs, it is sometimes necessary for the cells to adhere to a culture dish for cell processing, such as during proliferation or the induction of differentiation.[7,26] In such cases, there are 2 possible points where cell-surface modification can be applied: when cultured cells are on the dish or when dispersed cells are in the medium after detachment from the dish. Therefore, we compared the modification efficiency between these 2 conditions. The possible area for introduction of pegylated lipid would be small on the surface of adherent cell in the comparison with that of nonadherent cell. However, we did not observe any difference in the modification efficiency (Fig. 2). Previously, Lee et al demonstrated that the amount of liposome binding to adhered macrophage was approximately 2 fold larger than that biding to non-adhered macrophage, and discussed that expression of

receptors, that could bind to liposome, might be promoted in adherent cell.[27] Enhancement of the expression of such receptors might be a reason why there was no significant difference between the amount of pegylated lipids modified adherent hMSCs and nonadherent hMSCs in Fig. 2.

Although the pathway in the introduction of pegylated lipid to cell membrane is still unclear, there are 3 possible pathways. First, pegylated lipid assembly might be fuse with cell surface membrane. In fact, it was reported that DSPE-PEG form micelle in water.[28,29] Second, monomers of PEGyalted lipid might be directly inserted into cell membrane. Third, pegylated lipid transferred in the cells through endocytosis or phagocytosis might be transported on cell surface.

A decrease in the concentration of FBS in the DMEM medium used for modification enhanced the surface fluorescent intensity of the hMSCs (Fig. 5), which suggests that some component in the serum inhibits cell surface modification.

After the modified cells are injected, proteins or lipids in the blood may draw the pegylated lipids away from the cell membrane, resulting in a loss of function of the modified cells. Therefore, we measured the fluorescence intensity after cells modified by pegylated lipids were washed with the medium containing 10% FBS to remove the pegylated lipids that interact weakly with the cells. Furthermore, we evaluated the stability of the modification with pegylated lipids in the serum-containing medium. After incubation with 10% serum-containing medium for 1 h, the amount of pegylated lipids on the cell surface decreased to approximately 60% of the initial amount before washing, and it remained constant for 8 h (Fig. 6). This result suggests that a portion of the pegylated lipid strongly interacts with the cell membrane, and it could thus be expected to maintain its function after transplantation.

PEGylation of a drug can enhance the drug's stability and prolong its circulation in the blood because the presence of a modified PEG chain can prevent recognition from proteins, lipids, or cells through steric hindrance, and this effect increases according to an increase in the molecular weight of PEG.[19] Thus, we evaluated the effect of the molecular weight of PEG on cell-surface modification efficiency. As the molecular weight of the PEG chain increased, the amount of modified pegylated lipid on the cell surface also increased slightly (Fig. 7B). In liposomes modified by antibody-pegylated lipids, the recognition of the target antigen by the antibody decreased according to an increase in the molecular weight of PEG.[30] However, the length of the PEG chain is also important for recognition by the target molecule, as many proteins or lipids on the surface of the modified cells may disturb target recognition by the functional molecules. Therefore, the further optimization of the molecular weight of the PEG chain is important to modify functional molecules introduced to the cell surface by using pegylated lipids.

In conclusion, factors affecting the modification efficiency were systemically evaluated for cell-surface modification by using pegylated lipids. Increases in temperature, concentration, and exposure time increased the efficiency of cell-surface modification by pegylated lipids. Among these 3 factors, temperature and concentration are important for controlling cell-surface modification. Increasing the molecular weight of the PEG chain of the pegylated lipid enhanced the positioning of the pegylated lipid on the cellular membrane. The optimal molecular weight of PEG should be precisely determined to balance steric hindrance with the distance between the functional molecule and the cell membrane to protect against intermolecular recognition. Cell-surface modification with pegylated lipids can be applied to all types of cells. Thus, this method can be applied to all cell transplantation research. Furthermore, a portion of the pegylated lipid introduced to the cell surface strongly interacted with the cell membrane in the presence of serum. We also achieved introduction of much more pegylated lipids to the cell membrane by optimizing the modification conditions. Considering these findings, cells modified by pegylated lipids with functional molecules under optimized conditions will likely exert maximal function *in vivo*. Therefore, cell-surface modification is a promising tool that can be used to control the fate and function of cells in cell therapy.

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