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Target-cell specificity of fusogenic liposomes: Membrane fusion-mediated macromolecule delivery into human blood mononuclear cells

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Abstract

Fusogenic liposome, a unique vector prepared by fusing ultraviolet-inactivated Sendai virus and liposome, is known to efficiently deliver content into various animal cells through membrane fusion. In this study, we examined the target-cell specificity of fusogenic liposome (FL)-mediated macromolecule delivery into human blood cells using diphtheria toxin fragment A (DTA) as a probe. Among the peripheral blood mononuclear cells (PBMC), FL was able to deliver its encapsulates into $CD14^+$ monocytes and $CD4^-/CD8^-$ T-cells, but not into $CD19^+$ B-lymphocytes, $CD4^+$ T-cells or $CD8^+$ T-cells. The susceptibility of human leukemia cell lines to FL was similar to that of PBMC; the order of the reactivity was U937 (monoblastic leukemia) > MOLT4, Jurkat (T-lymphoma) > Daudi, BALL1 (B-lymphoma) > K562 (erythroblastic leukemia). Interestingly, FL showed similar binding activity to all of these leukemia cell lines. These findings indicate that, among blood cells, monocytes, monoblastic leukemia cells, $CD4^-/CD8^-$ T-cells and T-lymphoma cells are preferable targets for FL-mediated macromolecule delivery. This is the first demonstration of the existence of non-permissive cells against FL. Our results also suggest that some molecules on target-cells other than the binding targets of SV-derived protein may participate in fusion between FL and cells.

Keywords: Fusogenic liposome; Fusion; Sendai virus; Peripheral blood mononuclear cell; Human leukemia cell

1. Introduction

Efficient delivery of biological materials into living cells is important in biological research. It is also of primary importance in vector development for gene therapy. Various methods and carriers have been developed, e.g. microinjection, electroporation, liposomes, DEAE-dextran, and viral vectors, although

Abbreviations: FL, fusogenic liposome; OD, optical density; PBMC, peripheral blood mononuclear cells; DTA, diphtheria toxin fragment A; SV, Sendai virus

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each of these has both advantages and disadvantages. Liposome introduction is the simplest among these methods, and for this reason, liposomes have been used as carriers for drugs and macromolecules [1], and for DNA transfection of cells in vitro [2] and in vivo in gene therapy [3,4]. However, using liposomes, biological materials are introduced into cells via endocytosis, and they are often digested within endosomes by lysosomal enzymes, which results in complete disappearance of their biological activity [5].

Nakanishi and his colleagues developed the fusogenic liposome (FL) as a macromolecule delivery system [6,7]. The FL is prepared by fusing a liposome with a Sendai virus particle. The liposome is modified by the envelope proteins of Sendai virus. This liposome can deliver its content into the cytoplasm directly and efficiently because it can fuse with cell membrane with the aid of Sendai virus envelope glycoproteins. To date, liposomes modified by Sendai virus-derived proteins have been used to successfully introduce biologically active materials, e.g. proteins, plasmid DNA, oligonucleotides, and ribozymes, into animal cells [8–11]. In murine fibroblast L-cells, it has been clearly demonstrated that FL can transfer DNA with higher efficiency and less cytotoxicity than lipofectin, a typical liposome reagent used for DNA delivery [12]. The high efficiency of this system is attributable to the fact that materials introduced by membrane fusion can escape lysosomal degradation and reach the cytoplasm in an intact form [13]. At present, FL is expected to become a highly useful tool for use in gene therapy.

When using FL as a carrier, knowledge of FL target-cell specificity is highly important. Based on our previous study, FL has a wide range of target cells. However, little is known about its reactivity with blood cells (Mizuguchi and Nakanishi, manuscript in preparation). Since these cells are an important target of gene therapy and molecular biology, we here examined the target-cell specificity of FL-mediated macromolecule delivery into blood cells. In order to examine FL-mediated macromolecule transfer in a manner that precisely reflected the specificity of membrane fusion, diphtheria toxin fragment A (DTA) was used as an encapsulated molecule. DTA is a useful probe for detecting membrane-fusion mediated macromolecule delivery, because one molecule of DTA can kill a target cell, and DTA-containing FL (DTA-FL) has been shown to provide data on direct fusion between FL and cytoplasmic membranes [5,14,15]. In this report, our findings demonstrate that susceptibility of blood cells to FL differs among cell types and that binding of FL with cells through Sendai virus-derived protein is not enough to guarantee membrane fusion.

2. Materials and methods

2.1. Preparation of FL

Unilamellar liposomes were prepared by the reverse-phase evaporation method with some modifications [16]. Briefly, 46 μmol of lipids (egg phosphatidylcholine/L-α-dimyristoyl phosphatidic acid/cholesterol, 5:1:4, molar ratio) in 1 ml of a mixture of dichloromethane and diethyl ether (53:47 (w/w)) were added to 300 μl of an aqueous solution containing 100 μg of diphtheria toxin fragment A (DTA) or 1 mM calcein in BSS(−) (150 mM NaCl/10 mM Tris HCl, pH 7.6). After sizing by extrusion through a 0.4-μm polycarbonate membrane (Nucleopore, Costar, Cambridge, MA), liposomes were separated from unencapsulated molecules by centrifugation. Sendai virus, grown in 10-day-old embryonated chicken eggs at 35.5°C, was purified by differential centrifugation and inactivated by ultraviolet (2000 J/cm^2) irradiation. The liposomes were then mixed with inactivated Sendai virus and incubated for 2 h at 37°C with shaking. FL were separated from free liposomes and free Sendai virus by stepwise sucrose gradient centrifugation (15–10%). FL was quantified by an optical density (OD) of 540 nm.

2.2. Preparation of diphtheria toxin fragment A

Diphtheria toxin fragment A (DTA) was prepared according to the method of Uchida [15] with some modifications. Briefly, DTA was produced by C7hm7210, a mutant bacteria strain of *Corynebacterium diphtheriae* C7(β), and then purified by ammonium sulfate fractionation and column chromatography. Activity of DTA was confirmed by the ADP-ribosylation of elongation factor-2 as described previously [17].

2.3. Cell culture

HeLa (JCRB9004) cells were obtained from the Japan Cancer Research Resources Bank (JCRB) and maintained in MEM supplemented with 10% fetal calf serum (FCS). K562 (JCRB0019), Daudi (JCRB9071), BALL-1 (JCRB0071), MOLT4 (JCRB9031) and U937 (JCRB9021) cells obtained from the JCRB and Jurkat (RCB0806) cells from the RIKEN Cell Bank were maintained in RPMI 1640 medium supplemented with 10% FCS. Cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Before each experiment, cells were washed and resuspended in serum-free medium.

2.4. Preparation and separation of human peripheral blood mononuclear cells (PBMC)

Peripheral blood was drawn from healthy volunteers using heparin as an anti-coagulant. The blood was diluted by an equal volume of buffer (0.01% glucose, 5 μM $CaCl_2$, 98 μM $MgCl_2$, 540 μM KCl, 14.5 mM Tris, and 126 mM NaCl) and layered on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). After centrifugation for 40 min at $400\times g$, cells in the interface of plasma and Ficoll-Paque were collected as PBMC, washed twice with RPMI medium and subjected to experiments.

Separation of PBMC was performed using a magnetic cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) [18]. To obtain monocytes, PBMC were labeled by incubating with CD14 MicroBeads (anti human CD14 antibody-conjugated microbeads, 20 μl for 1×10^7 cells; Daiichikagaku, Tokyo, Japan) for 15 min at 4°C in PBS(−) containing 2 mM EDTA and 0.5% BSA. Labeled cells were applied and bound to a steel wool column in a high magnetic field. After washing with PBS(−) containing 2 mM EDTA and 0.5% BSA, the column was removed from the magnetic field and $CD14^+$ cells were eluted. B-cells were obtained using CD19 MicroBeads in the same manner as monocytes. After removal of $CD14^+$ cells and $CD19^+$ cells by means of the MACS system, T-cells were collected by the panning method [19] as follows. The cells were poured into a cell culture plate coated with anti-human IgG antibody (Rockland, Gilbertsville, PA) and incubated for 30 min at 37°C. Non-adherent cells were then collected as purified T-cells and washed. Further separation of T-cells into $CD4^+$, $CD8^+$, and $CD4^-/CD8^-$ cells was performed using the MACS system. $CD4^+$ T-cells were labeled with CD4 MicroBeads and separated by binding to a magnetic column. $CD8^+$ T-cells were also labeled with CD8 MicroBeads. After separation of $CD4^+$ T-cells and $CD8^+$ T-cells, the remaining cells were once labeled with a mixture of CD4 and CD8 MicroBeads, and then $CD4^-/CD8^-$ T-cells were obtained as a passed through fraction from the magnetic column.

The population of the separated cells was confirmed by flow cytometry with a Cyto ACE-150 Auto Cell Screener (JASCO, Tokyo, Japan). Briefly, 5×10^5 cells were labeled with 20 μl of dye-conjugated antibodies for 30 min at room temperature. After washing with PBS(−) containing 0.2% BSA and 0.1% NaN_3, cells were subjected to flow cytometry. The dye-conjugated antibodies used were as follows: fluorescein-labeled anti-CD14 antibody (Leinco Technologies, Ballwin, MO) for monocytes, FITC-labeled anti-CD3 (Immunotech, Marseille, France) for T-cells, phycoerythrin-labeled anti-CD19 (Immunotech) for B-cells, FITC-labeled anti-CD4 (Immunotech) for $CD4^+$ T-cells and phycoerythrin-labeled anti-CD8 (Immunotech) for $CD8^+$ T-cells.

2.5. FL-mediated DTA transfer into PBMC

Cells (1×10^5) were seeded in 96-well plates and incubated with various concentrations of DTA-FL in 100 μl of RPMI(−) media for 30 min at 37°C. Then, 100 μl of RPMI containing 20% FCS, which was not heat-inactivated, was added to each well, and cells were cultured for 24 h. Cell viability was evaluated by Alamar blue assay [20]. Briefly, 20 μl of Alamar blue dye (Iwaki Glass, Chiba, Japan) was added to each well, and the cells were further cultured for 12 h. The optical density (OD) of each well was then measured with a microplate reader (Bio-Tek Instruments, Winooski, VT), at test and reference wave length of 570 and 595 nm, respectively. Viability was calculated as [($OD_{570}-OD_{595}$ for DTA-FL treated cells)/($OD_{570}-OD_{595}$ for untreated cells)$\times100$] and expressed as a percentage of each untreated cell. When evaluating the viability of

$CD4^-/CD8^-$ T-cells, the change in OD of Alamar blue dye by an equal number of platelets in the fraction was also measured as a control and subtracted. Because $CD4^-/CD8^-$ T-cells were obtained as a fraction which passed through the magnetic column, platelets were co-eluated with the cells.

2.6. Binding assay

Cultured human leukemia cells were washed twice with BSS(+) (150 mM NaCl, 2 mM $CaCl_2$, and 10 mM Tris HCl; pH 7.6). 2.5×10^5 cells were incubated with calcein-containing FL ($OD_{540}=0.1$) in 250 μl of BSS(+) for 30 min at 37°C in a microtube. Cells were washed twice with PBS(−) and resuspended in PBS(−). Fluorescence intensity of the cells was measured by flow cytometry. The binding of calcein-containing liposomes not containing Sendai virus-derived proteins (calcein-L) was also measured to detect non-specific binding.

3. Results

3.1. Fusion between DTA-containing FL and PBMC

To confirm whether membrane fusion-mediated macromolecule delivery can be detected using DTA as a probe in our system, reactivity of three kinds of liposomes, DTA-FL, DTA-liposome (DTA-L; does not contain Sendai virus-derived proteins), and vehicle-FL, was examined using HeLa cells. HeLa cells were chosen as a representative of human cell lines. Each kind of liposome was added to HeLa cells and incubated for 30 min at 37°C then cultured for 24 h. Viability of the cells was then examined by measuring the change of OD due to the reduction of Alamar blue dye (Fig. 1A) [20]. DTA-FL decreased the viability of HeLa cells in a dose-dependent manner. In contrast, neither DTA-L nor vehicle-FL showed any effect, indicating that the decrease in viability is specific to membrane fusion-mediated delivery of DTA by FL. Having verified the assay method, we next examined the effects of DTA-FL on PBMC. Interestingly, the viability of PBMC treated with DTA-FL was maintained at about 60% that of untreated cells, even at a DTA-FL dosage sufficient to kill HeLa cells (Fig. 1B). These results suggest that PBMC con-

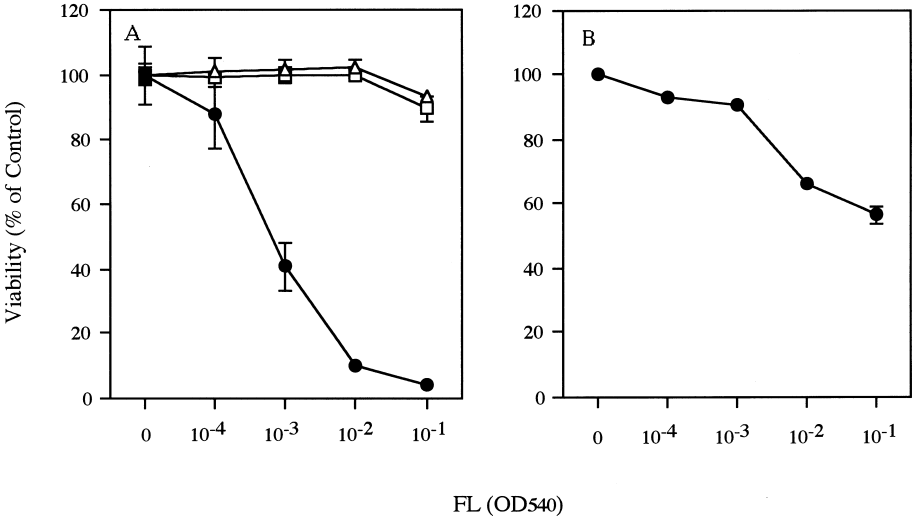

Fig. 1. Effect of DTA-FL on HeLa cells and PBMC. (A) HeLa cells were seeded in 96-well plates and incubated with various concentrations of DTA-FL (closed circle), DTA-liposome (open triangle), or vehicle-FL (open square) for 30 min at 37°C. After culturing for 24 h, Alamar blue dye was added to each well and the $OD_{570}-OD_{595}$ of the wells was measured. Viability was calculated as [($OD_{570}-OD_{595}$ for DTA-FL treated cells)/($OD_{570}-OD_{595}$ for untreated cells)×100] and expressed as a percentage of each untreated cell. (B) PBMC were prepared as described in Section 2 and incubated with DTA-FL for 30 min at 37°C, and then cell viability was measured by Alamar blue assay.

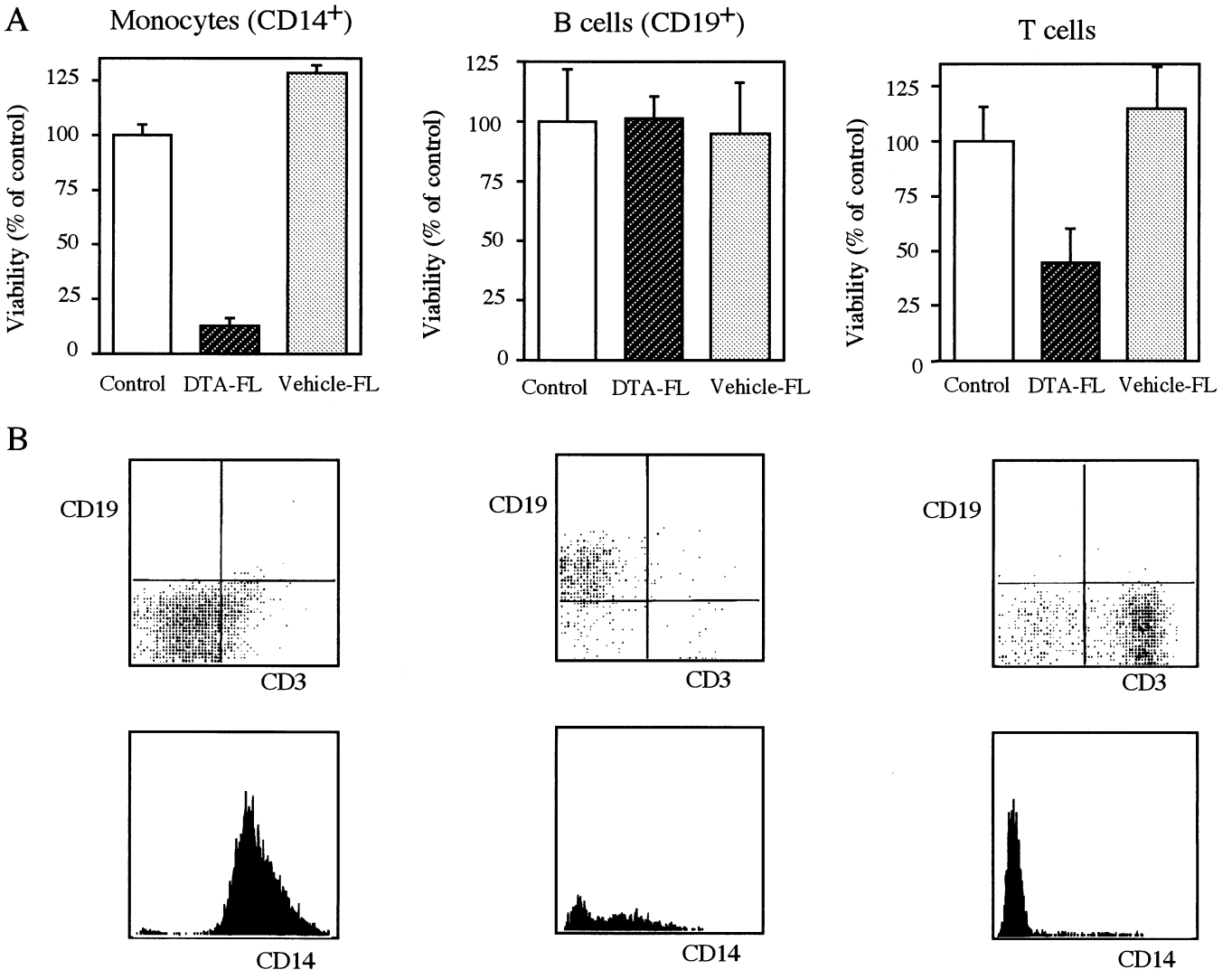

Fig. 2. Cell type specific fusion of FL among PBMC. Human PBMC were separated into monocytes, B-cells and T-cells as described in Section 2. Susceptibility to DTA-FL was examined (A) and the expression of cell surface markers was confirmed by flow cytometry (B). (A) Susceptibility to FL. Cells were incubated with FL containing DTA (DTA-FL) (hatched bar) or FL without DTA (Vehicle-FL) (dotted bar) for 30 min at 37°C. After culturing for 24 h, the viability of the cells was measured by Alamar blue assay as described in Section 2. Data represent the mean+S.D. for triplicate experiments. (B) Flow cytometric analysis of surface makers on separated cells. Cells were stained with anti-human CD14 antibody, anti-human CD19 antibody or anti-human CD3 antibody, then subjected to flow cytometry.

tain subpopulations which differ in their susceptibility to FL.

3.2. Fusion between FL and subpopulations of PBMC

Since human blood mononuclear cells are important targets not only for biochemical research, but also for therapy, we further examined the reactivity of FL with the respective cell populations of PBMC. PBMC were separated into subpopulations by antibody-conjugated magnetic beads as described in Section 2. After separating PBMC into monocytes, B-cells, and T-cells, each type of cell was incubated with DTA-FL (OD = 0.3) or vehicle-FL. This dose of DTA-FL was sufficient to kill HeLa cells, as shown in Fig. 1. DTA-FL caused a marked decrease in the viability of $CD14^+$ monocytes, but did not affect $CD19^+$ B-lymphocytes at all (Fig. 2A). On

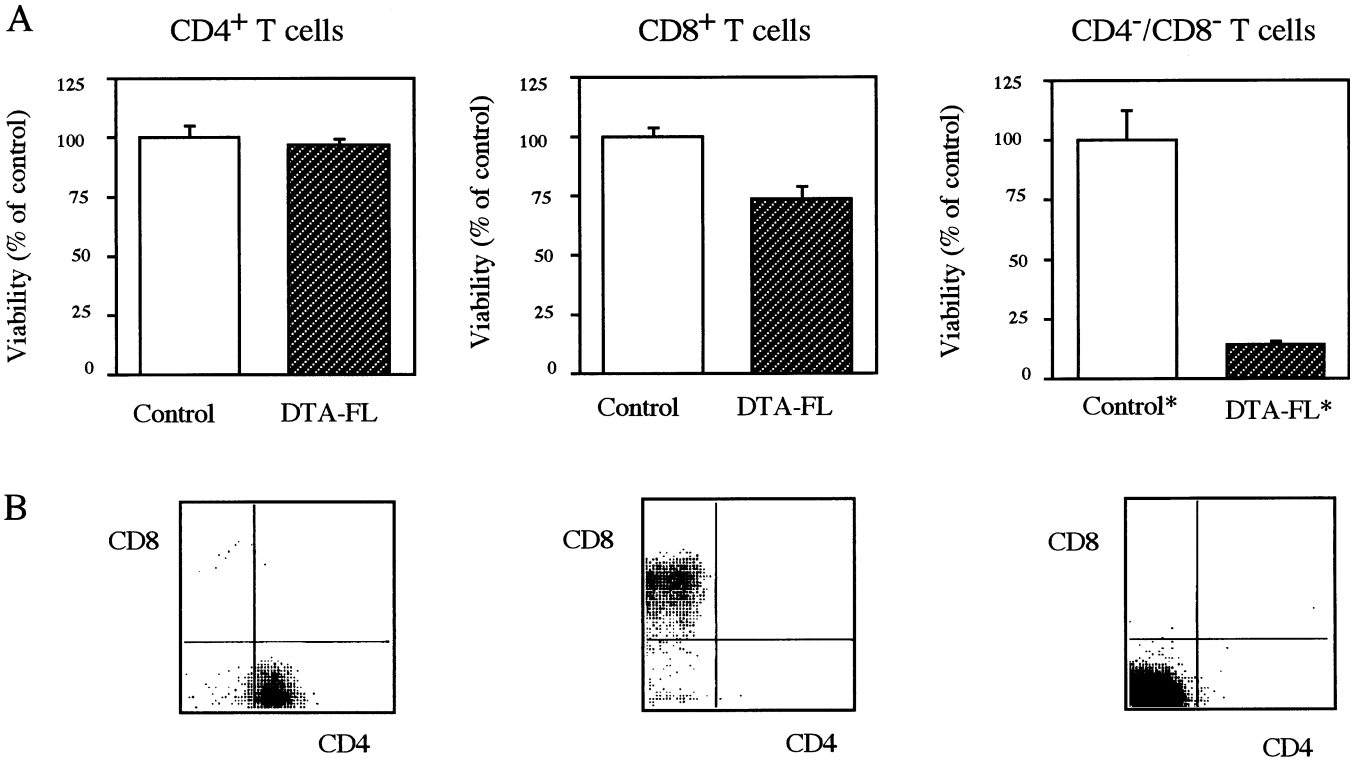

Fig. 3. Cell type specific fusion of FL among T-cells. Human T-cells were separated from PBMC and T-cell subpopulations were obtained as described in Section 2. Susceptibility to DTA-FL was examined (A) and the expression of cell surface markers was confirmed by flow cytometry (B). (A) Susceptibility to FL. Cells were incubated with FL containing DTA (DTA-FL) (hatched bar) for 30 min at 37°C. After culturing for 24 h, the viability of the cells was measured by Alamar blue assay as described in Section 2. Data represent the mean+S.D. for triplicate experiments. *For $CD4^-/CD8^-$ T-cells, the change in OD due to the co-existence of platelets in the well was subtracted. (B) Flow cytometric analysis of surface makers on separated cells. Cells were stained with anti-human CD4 antibody and anti-human CD8 antibody.

the other hand, DTA-FL caused a partial decrease in viability of T-cells. Vehicle-FL did not affect the viability of any kind of cell. The populations of the separated cells were confirmed by flow cytometry (Fig. 2B). These results indicate that FL can fuse with and deliver DTA into monocytes, whereas FL cannot fuse with B-cells. Since T-cells seem to include subpopulations with which FL cannot fuse, we then separated T-cells into $CD4^+$, $CD8^+$, and $CD4^-/CD8^-$ cells using anti-CD4 and CD8 antibody-conjugated microbeads and examined the reactivity of each with FL. As shown in Fig. 3, we found that DTA-FL did not reduce the viability of $CD4^+$ T-cells or $CD8^+$ T-cells; however, it killed $CD4^-/CD8^-$ T-cells. These results indicate that FL can fuse with and deliver DTA into $CD4^-/CD8^-$ T-cells, but can do neither with $CD4^+$ T-cells or $CD8^+$ T-cells.

3.3. Fusion between FL and human leukemia cells

Next, we examined the reactivity of DTA-FL with cultured human blood cells. Six types of typical cultured human leukemia cell lines were selected: U937 (monoblastic leukemia), Daudi and BALL1 (B-lymphoma), MOLT4 and Jurkat (T-lymphoma), and K562 (erythroblastic leukemia). DTA-FL was incubated with these cells and then cell viability was measured. As shown in Fig. 4, DTA-FL caused the most marked decrease in viability of U937 cells, whereas it did not affect K562 cells. The sensitivity to DTA-FL, in decreasing order, was as follows: U937 (monoblastic leukemia) > MOLT4, Jurkat (T-lymphoma) > Daudi, BALL1 (B-lymphoma) > K562 (erythroblastic leukemia). These results correlate well with the demonstrated susceptibility of PBMC against FL; i.e. both in PBMC and cultured blood

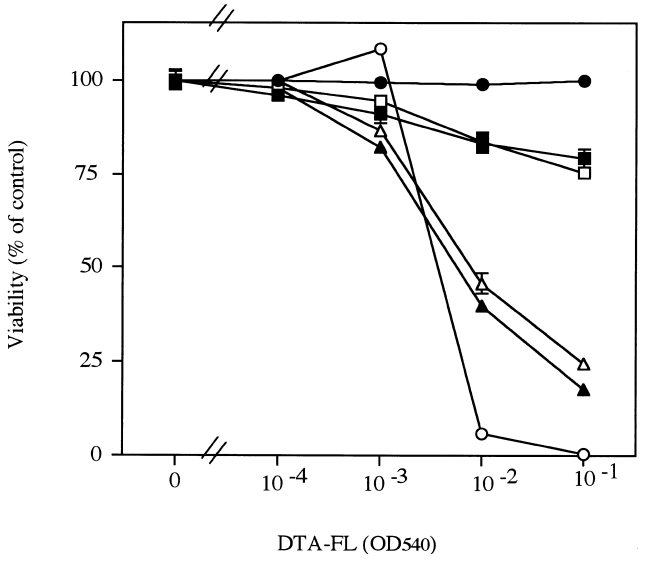

Fig. 4. Susceptibility of human leukemia cell lines to DTA-FL. Cells were incubated with DTA-FL for 30 min at 37°C. After culturing for 24 h, viability of the cells was measured by Alamar blue assay as described in Section 2. Data represent the mean ± S.D. for triplicate experiments. Closed circle, K562 (erythroblastic leukemia); closed square, Daudi (B-lymphoma); open square, BALL1 (B-lymphoma); closed triangle, MOLT4 (T-lymphoma); open triangle, Jurkat (T-lymphoma); open circle, U937 (monoblastic leukemia). The mean ± S.D. value of $OD_{570}-OD_{595}$ by reduction of Alamar blue dye for each untreated cell was as follows: 0.880 ± 0.007 for K562, 0.677 ± 0.019 for Daudi, 0.790 ± 0.014 for BALL-1, 0.818 ± 0.019 for Jurkat, 0.812 ± 0.007 for MOLT4 and 0.819 ± 0.014 for U937. These values suggested that the variation of proliferative activity among these cells is small.

cell lines, FL showed same reactivity with cells belonging to the same population. For example, FL had high reactivity with both peripheral blood monocytes and monoblastic leukemia U937 cells, whereas FL had very little reactivity with both peripheral blood B-cells and cultured B-lymphoma cell lines such as Daudi and BALL cells (Figs. 2 and 4).

3.4. Binding between FL and human leukemia cells

Because binding of viral envelope proteins with target cells is thought to be a critical step in the fusion between the Sendai virus and cells [13], we next tried to determine whether FL can bind with cells which cannot fuse with FL (FL-resistant cells).

The binding ability of FL to both permissive and non-permissive human leukemia cells was examined using calcein-containing FL (calcein-FL). The cells were incubated with calcein-FL and the fluorescence intensity of cells was measured by flow cytometry (Fig. 5). In all cell lines examined, the fluorescence intensity of the cells increased by an almost equal degree after incubation with calcein-FL. On the other hand, incubation with calcein-liposomes not containing Sendai virus-derived proteins did not increase the fluorescence intensity of the cells, indicating that these liposomes do not bind with the cells. The data indicate that FL can bind with all these cell lines depending on the presence of Sendai virus-derived proteins. These results suggest that FL can bind with the cells, but binding is not sufficient to membrane fusion.

4. Discussion

We have revealed the target-cell specificity of membrane fusion-mediated macromolecule delivery by FL into human blood cells. The use of DTA as a model encapsulated molecule in FL provided good evidence of a direct fusion between FL and the cytoplasmic membrane, based on the following. DTA is known to inactivate elongation factor 2 and to stop protein synthesis in the cytosol of target cells. One molecule of DTA can kill a cell, if it is introduced into the cell through membrane fusion and not through endocytosis [6,14]. In addition, compared to the reporter gene assay, which is often used to examine the target-cell specificity of gene transfer vectors, this system has an advantage in that the evaluation of target-cell specificity is in no way affected by the gene expression activity of the cells. Using this excellent marker, we were here able to examine the target-cell specificity of FL in human blood cells and to discover the existence of FL-resistant cells.

From the standpoint of investigating the mechanisms of membrane fusion, the existence of FL-resistant cells (even though they can bind with FL) is interesting. Since fusion between FL and cell depends on the envelope proteins derived from the Sendai virus [7], these proteins may work similarly on FL and the Sendai virus. As to the membrane fusion between the Sendai virus and cells, two types of envelope proteins, F and HN protein, are known to be

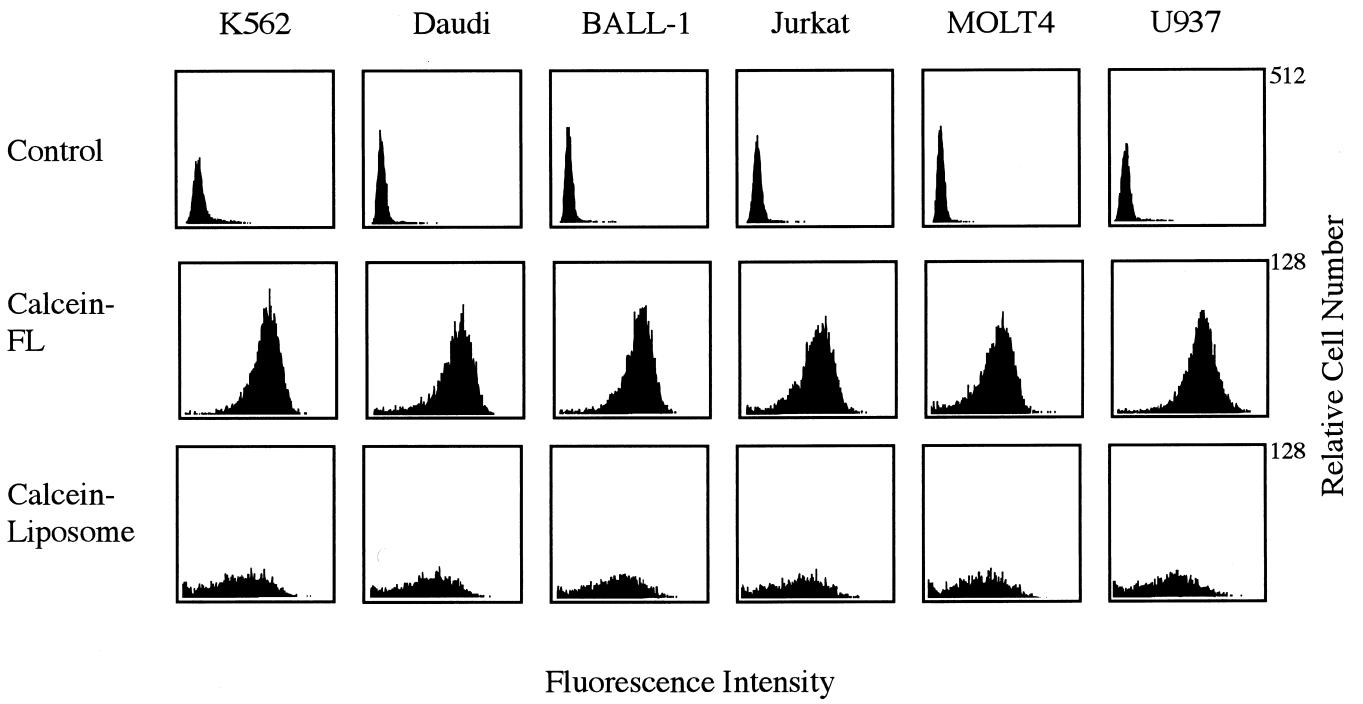

Fig. 5. Binding of calcein-containing FL with human leukemia cells. Cells were incubated with calcein-containing FL ($OD_{540} = 0.1$) (middle panels) or Sendai virus-free liposomes containing calcein (bottom panels) at 37°C for 30 min. After washing with PBS(−), the fluorescence intensity of the cells was measured by flow cytometry. The fluorescence intensity of untreated cells was measured as a negative control (top panels). The values of *y*-axes show relative cell number.

necessary. When the Sendai virus fuses with a cell, viral HN protein binds to the cell surface, and F protein then causes membrane fusion [21–24]. Sialic acid on the cell membrane is known to function as a binding target for HN protein [21,25], and the existence of a binding target for HN protein is believed to determine the susceptibility of fusion. In the present study, FL could bind to, but could not fuse with, FL-resistant cells. This means that binding to the cell surface is insufficient for FL to fuse with the cell membrane. In Fig. 5, binding of FL to Jurkat and MOLT4 cells was lower than to other cells including U937 cells. As shown in Fig. 4, DTA-FL showed higher reactivity with U937 cells than Jurkat and MOLT4 cells. If we consider only the results about U937, Jurkat, and MOLT4 cells, a possibility that the slight difference in binding leads to a more pronounced preferential cell killing by DTA-FL cannot be completely denied. However, FL showed the binding activity with all cell lines examined, depending on the existence of envelope proteins, despite these cells showed the large variation of the reactivity with DTA-FL. Therefore we concluded that the binding is not a critical step which determines the reactivity of FL with cells.

There are two possible explanations for this phenomenon. One is that not only sialic acid, but also other cell-surface molecules may be involved in the fusion between FL and cells, and FL-resistant cells may lack these molecules. Based on the report that HN protein has an unknown, but important, role in membrane fusion as well as receptor binding [22,23,26], it is speculated that HN protein may participate in membrane fusion by interacting with unknown molecules on the cell. The second possibility is that FL-resistant cells may possess an inhibitory factor for membrane fusion.

Research into these factors may provide important information not only for elucidating the mechanisms of membrane fusion, but also for improvement of the delivery by FL. In cases in which FL would be applied as a carrier to transfer biological materials into B-cells, $CD4^+$ T-cells, or $CD8^+$ T-cells, FL would have to be modified in order to fuse with these cells.

Information on the mechanisms of membrane fusion between FL and cells would be helpful in this regard.

In regard to FL as a therapeutic tool, the target-cell specificity we revealed here may be applicable for various purposes. For example, FL may be used for the treatment of genetic or infectious diseases in which monocytes are therapeutic targets, such as chronic granulomatous disease [27], Gaucher's disease [28] or AIDS [29]. We found that some T-lymphoma cell lines were also preferred targets of FL, although the majority of primary cultured T-lymphocytes were almost resistant. This characteristic prompts us to think that cancer cells derived from T-lymphocytes might obtain the reactivity with FL after their transformation. If FL can fuse with peripheral blood leukemia cells, it might be used to deliver anti-cancer materials specifically to tumor cells. In this way, FL might be useful in the treatment of leukemia. The reactivity of FL with peripheral blood leukemia cells will be an important subject for future research. Our findings make an important contribution to the basic characterization of FL. Further study of the mechanism of membrane fusion using FL-resistant cells will make it possible to modulate the target specificity of FL.

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