Gene delivery to dendritic cells mediated by complexes of lipoplexes and pH-sensitive fusogenic polymer-modified liposomes

Gene Delivery to Dendritic Cells Mediated by Complexes of Lipoplexes and pH-Sensitive Fusogenic Polymer-Modified Liposomes

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Abstract

Dendritic cells (DCs) are potent professional antigen presenting cells that are useful for cancer immunotherapy. We previously reported the preparation and characterization of complexes of lipoplexes with pH-sensitive fusogenic liposomes, which comprise polymers based on poly(glycidol) with carboxyl groups, to transfect various malignant cell lines. The present study applied this kind of vectors to transfection of a murine DC line DC2.4. We first optimized the ratios of their components for efficient transfection. We subsequently investigated the effects of ligands and pH-sensitive polymers to improve transfection activities. Our results indicate that the anionic surface derived from pH-sensitive polymers might be recognized by scavenger receptors on DC2.4 cells. In addition, no effects on transfection or cell association were observed by attaching ligands such as transferrin and mannan. We found that more sensitive pH-responding polymers led to higher transfection activities into DC2.4 cells, which suggests that endosomal escape is important for transfection into DC2.4 cells. These complexes with

pH-sensitive fusogenic polymers exhibited higher activity than commercial reagents. For those reasons, they are promising as gene vectors for DCs.

Keywords: dendritic cell / gene delivery / pH-sensitive polymer / lipoplex / fusogenic liposome

Introduction

Dendritic cells (DCs) are potent professional antigen presenting cells. They play a crucial role in innate and adaptive immune responses [1–3]. In fact, DCs recognize, acquire, process, and present antigens to native and resting T cells for induction of an antigen-specific immune response. Because DCs presenting tumor-associated antigens (TAAs) can activate TAA-specific immune response, many researchers have been attracted to TAA-presenting DCs as a vaccine for cancer immunotherapy [4, 5]. A key for vaccine production is delivery of TAA into DCs. The most extensively studied approach for TAA-loading of DCs is to expose them to defined antigenic proteins or the entire contents of tumor cells. Antigenic proteins are internalized *via* endocytosis and degraded to peptide fragments. These peptides are presented by binding to major histocompatibility complex (MHC) class II molecules, which mainly activate CD4+ T cells, thereby inducing humoral immunity. Nevertheless, these vaccines have not been useful for cancer immunotherapy to date, because fragmented antigen peptides do not necessarily bind to MHC class II molecules efficiently because of the MHC molecules'

diversity [6].

Another promising strategy for supplying DCs with TAA is the delivery of TAA-coded DNA or mRNA to express TAA in DCs as an endogenous antigen. The TAAs expressed in DCs are degraded by proteasomes after ubiquitination. These fragmented peptides are presented by binding to MHC class I molecules, which mainly activate CD8+ T cells to induce cellular immunity. Antigen peptides processed from endogenous proteins bind efficiently to MHC molecules. Reportedly, some cancer cells present their own TAA on the surface *via* MHC class I molecules for recognition by CD8+ T cells. Therefore, the preparation of TAA-loading DCs by gene delivery can induce antitumor immune responses in a practical manner, constituting a powerful tool for cancer immunotherapy [5, 7].

Attempts have been made to develop efficient vectors for DCs from adenovirus [8, 9]. Although the transfection efficiency of a conventional adenovirus is not high, toward DCs, Okada et al. achieved efficient transfection of 90% DC2.4 cells using the fiber-mutant adenoviral vector [8], indicating that modification of adenovirus might engender production of efficient vectors for DCs. However, adenoviruses induced not

only gene transfer, but also dispensable immune responses [10]. Consequently, non-viral vectors have been attractive for gene delivery into DCs because of their low immunogenicity and lack of pathogenicity, even though they have lower transfection activity than virus-based vectors.

Recent progress in the area of non-viral vector-mediated gene delivery has revealed various cellular processes that are involved in the vector-mediated transfection of cells. They include cellular binding and subsequent internalization, transfer from endosome into cytosol (endosomal escape), nuclear entry, and gene transcription [11, 12]. Therefore, to achieve efficient transfection of DCs, vectors must be rationally designed specifically for DC to pass through these cellular processes efficiently. Among these cellular processes, efficient cellular binding and endosomal escape greatly affect the efficiency of non-viral vector-mediated transfection [11–13].

Previously, we prepared hybrid complexes comprising lipoplexes and liposomes modified with pH-sensitive fusogenic polymers, such as succinylated poly(glycidol) (SucPG) and 3-methylglutarylated poly(glycidol) (MGluPG) [14–17]. These complexes, which are designated respectively as SucPG complex and MGluPG complex, generate

fusion capability under mildly acidic conditions. In addition, these complexes have a structure in which the positively charged lipoplexes are covered with negatively charged polymer-modified liposomes. Consequently, once an appropriate ligand, such as transferrin, was conjugated, they achieved efficient transfection of various cancer-derived cell lines, such as HeLa and K562 cells, through efficient cellular association via receptor-mediated endocytosis and through endosomal escape via membrane fusion with endosome (Fig. 1) [14–17].

To advance the development of potent vectors that are designed specifically for DCs based on the hybrid complexes consisting of pH-sensitive fusogenic liposomes and lipoplexes, we attempted to optimize their structure from the viewpoints of ligand and pH-sensitive properties, which would contribute to high cellular uptake and efficient endosomal escape, respectively, using DC2.4 cells, a murine DC line, as a model of DCs. Our results demonstrated that the structural optimization of the complexes was able to produce an efficient non-viral vector for DCs.

Materials & Methods

Materials

SucPG and MGluPG were synthesized according to our reports [18, 19]. SucPG and MGluPG with the composition (x:y:z, Fig. 1) of 18:74:8 and 9:81:10 (mol/mol/mol), respectively. TRX-20 (Fig. 2a) [20] and L-dioleoyl phosphatidyl-ethanolamine (DOPE) were provided from Terumo corp. and NOF corp., respectively. Dilauroyl phosphatidyl choline (DLPC) and transferrin were purchased by Aldrich. Aminoethylcarbamylmethyl mannan (Fig. 2b) was synthesized as follows [21]. 500 mg of Mannan (Sigma) was reacted with 483 mg of sodium monochloroacetate in 1 N NaOH solution at 55 ^{o}C for 5 h. The solution adjusted to pH 4.7 was followed by the reaction with ethylenediamine dihydrochloride *via* 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Dojindo) on ice for 1 day. The reaction mixture was purified by dialysis, and then lyophilized. The amounts of amino groups were detected by fluorescamine assay [22], by which an amino group per 22 units was estimated (Fig. 2b).

Cell culture

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI 1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100 μM nonessential amino acid, 50 μM 2-mercaptoethanol and antibiotics at 37 ^{o}C [23].

Preparation of pH-sensitive polymer-modified liposome-lipoplex complexes

Lipoplexes were prepared as reported previously [16]. Plasmid DNA, pCMV-Luc or pEGFP-C1, in 5% glucose solution was added to lipid film including TRX-20, DLPC and DOPE at the ratio of 1/1/2 (mol/mol/mol), and they were incubated for 10 min on ice. SucPG or MGluPG-modified liposomes were prepared by suspending a mixture of SucPG or MGluPG and EYPC in 5 mM Hepes solution containing 5% glucose (pH 7.4) and subsequent extrusion through a polycarbonate membrane with a pore size of 50 nm. Transferrin or aminoethylcarbamylmethyl mannan was conjugated to SucPG or MGluPG using EDC as previously reported [14]. Briefly, to the liposome suspension was added EDC (0.7 mg) at pH 6.0 and stirred for 2 h at 4 °C. Then, transferrin (3 mg)

and 0.5 M ferric citrate (5 μl) or aminoethylcarbamylmethyl mannan (3 mg) was added to the liposome suspension and the suspension was kept at 4 °C overnight. After the liposome suspension was adjusted to pH 7.4, the transferrin or aminoethylcarbamylmethyl mannan- conjugated SucPG or MGluPG-modified liposome was purified using a Sepharose 4B column at 4 °C with 5 mM Hepes solution containing 5% glucose (pH 7.4). A suspension of SucPG or MGluPG-modified liposome either bearing or not bearing ligand (0.2 mM) was added to the lipoplex suspension and incubated for 10 min in an ice bath.

Dynamic light scattering and zeta potential

Diameters and zeta potentials of the lipoplexes and the SucPG and MGluPG complexes were measured using a Nicomp 370 ZLS dynamic light scattering instrument (Particle Sizing Systems, Santa Barbara, CA) equipped with a 35 mW laser (632.8 nm wavelength). Zeta potentials were measured by equipped an Avalanche photodiode detector, and were detected at an 18.9 angle treated with 9.75 mV. Data was obtained as an average of more than three measurements on different samples.

Transfection

Transfection to DC2.4 cells was done according to the following procedures. For luciferase assay, DC2.4 cells (7.5×10^4 cells) cultured for 2 days in a 24-well plate were washed with Hank's balanced salt solution (HBBS, Sigma) and then incubated in culture medium. The complexes or the lipoplexes containing pCMV-Luc (0.75 μg) were added gently to the cells and incubated for 4 h at 37 °C. The cells were washed with HBBS three times, followed by the incubation in culture medium at 37 °C for 40 h. Then, transfected cells were evaluated by luciferase assay [14].

For GFP expression, the complexes or the lipoplexes containing pEGFP-C1 (0.75 μg) were added gently to the DC2.4 cells (1.5×10^5 cells) incubated for 2 days in a 12-well plate. After 4 h-incubation at 37 °C, the cells were washed with HBBS three times, followed by the incubation in culture medium at 37 °C for 24 h. Then, GFP-transfected cells were evaluated using flowcytometer [16]. SuperFect (QIAGEN) and Lipofetamine2000 (Invitrogen) were also used according to the manufacturer's instructions.

Cellular uptake

The DC2.4 cells (1×10^5 cells) cultured for 2 days in a 12-well plate were washed with HBBS and then incubated in culture medium. The complexes or the lipoplexes containing plasmid DNA (1 μg), in which DOPE was substituted by NBD-PE (3 mol%), were added gently to the cells and incubated for 4 h at 37 °C. The cells were washed with HBBS three times, and then flowcytometerical analysis of detached cells using trypsin was performed [16]. For inhibition assay, free transferrin, mannan or dextran sulfate at different concentrations was preincubated to cell for an hour before the incubation of these complexes labeled with NBD-PE.

Microscopic analysis

The DC2.4 cells (1.5×10^5 cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBBS, and then incubated in culture medium. The Lipoplexes or the SucPG or MGluPG complexes containing FITC-labeled plasmid DNA (0.5 μg) were added gently to the cells and incubated for 4 h at 37 ^{o}C. After the incubation, the

cells were washed with HBBS three times, and then replaced by serum-free medium. LysoTracker Red DND-99 (Molecular Probes) was used by the staining of intracellular acidic compartments according to the manufacturer's instructions. Briefly, LysoTracker Red was added to cells at the final concentration of 75 nM. After the 5 min-incubation, the cells were washed with HBBS there times. Confocal laser scanning microscopic analysis of these cells was performed using LSM 5 EXCITER (Carl Zeiss Co. Ltd.).

Cytotoxicity

The cytotoxicity of the lipoplexes and the SucPG and MGluPG complexes was assessed by WST-8 assay [24]. The cells were treated with the complexes for 4 h and incubated for 40 h according to the transfection procedures. Then, the culture medium was carefully replaced with 0.11 ml of fresh RPMI containing 10% FCS and 10 μl of WST-8 (5 mg/ml) was added to each well. After 2 h-incubation at 37 °C, the survived cells was determined by absorbance at 450 nm using Wallac 1420 ARVO SX multilabel counter (Perkin Elmer Life Sciences).

MHC class I presentation

Surface marker expression was analyzed according to the previous report [8]. DC2.4 cells were treated under the transfection condition. DC2.4 cells treated with 10 mg/ml Lipopolysaccharide (LPS; Nacalai Tesque, Inc., Kyoto, Japan) and 100 units/ml recombinant murine interferon-γ (IFN-γ; Pepro Tech EC LTD., London, England) for 4 h were used as positive controls for DC maturation. At 24 h after transfection, cells were analyzed by flowcytometry. The cells were scraped by a cell scraper (Sumitomo Bakelite Co, Ltd, Japan) and 1×10^4 cells in 100 μl of staining buffer (phosphate buffered saline (PBS) containing 0.1 % BSA and 0.01 % NaN_3) were incubated with the anti-mouse CD16/32-block Fc binding (eBioscience, CA, USA) for 30 min on ice to block nonspecific binding of the antibody. After three times wash with the staining buffer, the cells were incubated with biotin-conjugated mouse anti-mouse H-$2K^b$/H-$2D^b$ (MHC class I) monoclonal antibody (BD Bioscience, NJ, USA) in the staining buffer for 30 min on ice according to the manufacturer's instruction. After three times wash with the staining buffer, MHC class I expressed at the surface was detected by the 30 min-incubation of PE-conjugated streptavidin (SIGMA, Missouri, USA) at a 1:200

dilution.

Results and Discussion

Transfection of DC2.4 cells

We performed luciferase assay to examine the transfection activity of the SucPG complexes to DC2.4 cells. Our previous reports described that the most effective SucPG complexes toward the transfection of HeLa cells were composed of the lipoplexes of TRX-20, DLPC, and DOPE at the molar ratio of 1/1/2 and transferrin-conjugated SucPG liposomes [16]. Accordingly, we first performed transfection of DC2.4 using SucPG complexes consisting of the same components. Then we optimized their composition for maximum transfection activity. Figure 3 shows that transfection activity of SucPG complexes is affected by both the cationic lipid/nucleotide unit ratio (N/P ratio) of the lipoplex and the SucPG-modified liposomes/lipoplex ratio, which is defined as the ratio of the SucPG carboxylated unit to DNA nucleotide unit of the complexes. The SucPG complexes achieved the highest transfection activities when the lipoplex with the N/P ratio of 4 was used for their preparation, indicating that greater

amounts of lipid components to DNA were necessary for efficient transfection of DC2.4 cells. The decreasing activities at higher N/P ratio might result from higher toxicity of the complex. This figure also shows that the SucPG complexes at the N/P ratio of 4 exhibited more efficient transfection than the parent lipoplex, indicating that the complexation of SucPG-modified liposomes was as effective for transfection of DC2.4 cells as for cancer cells [14-17]. The following assay was performed using lipoplexes and SucPG complexes at the N/P ratio of 4.

Ligand effect on transfection of DC2.4 cells

The SucPG complexes with transferrin exhibited higher transfection activities than the intact lipoplexes (Fig. 3). Although DCs have transferrin receptors, mannose receptors are known to be largely expressed in DCs. Mannan, as a ligand for mannose receptors, was used for delivery to DCs [10, 25–27]. Therefore, mannan was incorporated to the SucPG complexes to increase their transfection activity. Mannan derivatives with amino groups were synthesized [21] and bound to carboxyl groups of SucPG using a condensation reagent, as in the method of attachment of transferrin to the

complexes [14–17].

Figure 4 shows that the transfection activities of both the complexes bearing transferrin and mannan were unexpectedly almost equivalent over a wide range of carboxylated unit/nucleotide unit ratios. In addition, the complex without ligands also exhibited activity at the same level. This figure indicates that ligands such as transferrin and mannan were not effective to improve transfection activity of SucPG complexes to DC2.4 cells.

Complexation of plain SucPG-modified liposomes with the lipoplex also tends to increase the transfection activity (Fig. 4). Our previous study showed that their complexation resulted in significant reduction of the transfection activity toward HeLa cells because of reduction of affinity to cell [14, 16]. These facts imply that the negatively charged surface derived from the SucPG-modified liposomes might not suppress interaction between the complex and DCs.

We next investigated the cellular association of SucPG complexes to elucidate the ligand effect. Figure 5a shows that the amount of plain SucPG complex associated to DCs was almost equal to those of ligand-bearing complexes and was equal to that of the

parent lipoplex. Our previous studies showed that the SucPG complexes without transferrin to HeLa cells exhibited a much lower level of association to HeLa cells than the parent lipoplex because the positively charged surface of the lipoplex was shielded by negatively charged polymer-modified liposomes [14, 16]. Indeed, the SucPG complexes without ligand showed lower association to the cells than those with ligand. Therefore, Figure 5a suggests that the negatively charged liposomes of the complex might be involved in its association to DC2.4 cells.

Inhibition assay was also performed using free transferrin and free mannan (Fig. 5b), which indicates that neither free transferrin nor free mannan inhibited the association of these complexes. This result further confirms that transferrin and mannan in the complexes only slightly affected association of the complexes to DC2.4 cells.

It is known that DCs engulf microorganisms or apoptotic cells with an anionic component *via* scavenger receptors [28]. Considering that SucPG complexes contain negatively charged moieties derived from SucPG-modified liposomes, involvement of scavenger receptors is possible. Therefore, we also examined inhibitory effects of dextran sulfate, which has been used as an inhibitor of interaction between negatively

charged compounds and scavenger receptors [29]. Figure 5b shows that the addition of dextran sulfate strongly suppressed cellular association of the SucPG complexes, depending on the concentration. Consequently, the SucPG complexes were likely to have been internalized mainly *via* scavenger receptors. In fact, enhanced uptake by macrophages through scavenger receptors was also reported for poly(acrylic acid)-coated liposomes [29].

Although we conjugated mannan and transferrin to the complexes, they only slightly affected their association to the cells. Instead, the complexes were taken up efficiently by DC2.4 cells through interaction with scavenger receptors, which bind to anionic molecules [30]. In fact, DCs are known to have many kinds of scavenger receptors [30]. Therefore, multivalent binding between these receptors and many carboxylate anions of the polymer chains fixed on the complexes might cause their strong interaction, resulting in the highly efficient association of the complexes to DCs.

Effect of pH-sensitive fusogenic polymers on transfection activity of complexes

We next examined the endosomal escape to improve transfection activities.

Recently, another type of pH-sensitive complex, MGluPG complexes, in which SucPG is substituted by MGluPG (Fig. 1), was prepared. The MGluPG was synthesized by the reaction of poly(glycidol) with 3-methyl glutaric anhydride [19]. This polymer has more hydrophobic side groups than SucPG and was therefore more fusogenic than SucPG [19]. In addition, the MGluPG complexes exhibited greater fusion ability under mildly acidic conditions than the SucPG complexes because of the stronger fusion activity of MGluPG [17].

We transfected DC2.4 cells with MGluPG complexes containing EGFP gene. First, the transfection condition of the complexes was optimized using MGluPG liposomes. The SucPG complexes at N/P ratios of 4 exhibited the most efficient transfection (Fig. 3). Therefore, we prepared the MGluPG complexes at the same N/P ratio with varying ratios of carboxylated unit of MGluPG liposomes to the DNA nucleotide unit of the lipoplex. Figure 6a shows that the complexation of MGluPG liposomes improved the transfection activity of the parent lipoplex. In addition, the MGluPG complex with the carboxylated unit/nucleotide unit ratio of 2 exhibited the highest transfection activity. At that ratio, 25% of GFP-expressed cells were observed.

Transfection activities of the complexes with optimal composition were compared to those of commercially available reagents, such as Lipofectamine2000 and SuperFect. Fluorescence intensity of EGFP for the transfected cells was detected using flowcytometry (Fig. 6b). A larger population of the cells displayed more intensive fluorescence for the cells treated with MGluPG complex than cells treated with other complexes or reagents. Percentages of EGFP-positive cells and mean fluorescence intensity of the treated cells were evaluated using flowcytometry and are shown in Fig. 6c. The MGluPG complex induced a much higher percentage and a much higher mean fluorescence intensity to DC2.4 cells, that those of other reagents and complexes.

To confirm the high transfection activity of MGluPG complex, we further compared the transfection activity of MGluPG complex with SucPG complex using the luciferase gene. Results showed that luciferase activities of DC2.4 cells treated with SucPG complex and those treated with MGluPG complex were $1.16 \pm 0.14 \times 10^7$ RLU/mg protein (SucPG complex) and $2.02 \pm 0.17 \times 10^7$ RLU/mg protein (MGluPG complex), respectively, indicating that MGluPG complex induced twice-higher luciferase activity than the SucPG complex.

The cytotoxicity of the complexes was also examined using WST-8 assay [24] and compared to that of the parent lipoplex. The survived cells after transfection with the SucPG and MGluPG complexes and the lipoplex were estimated respectively as 50%, 54%, and 39%. Although these complexes exhibited some toxicity to DC2.4 cells during the cellular treatment, their cytotoxicity was still lower than that of the parent lipoplex. It is likely that, for the complexes, the negatively charged liposomes shielded positively charged surface of the lipoplex, resulting in the lower toxicity for these complexes than for the lipoplex.

Mechanism of the efficient transfection activity by the MGluPG complexes

We examined the cell association and the intracellular localization of this complex to elucidate the cause of the high activity of the MGluPG complex. Figure 7A shows mean fluorescence intensities of DC2.4 cells treated with the fluorescent-lipid-labeled lipoplex or the complexes, as estimated using flow cytometry. The mean fluorescence intensities of these treated cells were almost the same, indicating that approximately equal amounts of the SucPG and MGluPG complex and lipoplex

were associated with DC2.4 cells. Mean diameters and zeta potentials of these complexes were estimated as 258 ± 13 nm and 19.6 ± 1.0 mV (SucPG complex) and 258 ± 15 nm and 19.8 ± 6.0 mV (MGluPG complex), respectively, indicating that these complexes have similar particle size and surface charge. Consequently, it is highly likely that these complexes are taken up through interaction with a scavenger receptor at similar efficiencies.

We also examined the subcellular distribution of these complexes containing FITC-labeled DNA in DC2.4 cells, in which the acidic compartments were stained using LysoTracker. Figure 7B shows that many yellow dots were observed by treatment with lipoplexes, indicating that the lipoplexes were localized dominantly at acidic compartments such as endosomes/lysosomes. In cells treated with the SucPG or MGluPG complex, green fluorescence was also observed at different locations from those of the red dots, which suggests that the SucPG and MGluPG complexes were able to escape from endosomal compartments. No large difference was found in the intracellular localization of the complexes. Indeed, it might be difficult to identify small differences in amounts of DNA existing in the cytosol using this technique. We

observed that the MGluPG complex had about three-times-higher ability to induce membrane fusion than the SucPG complex at around pH 5–4.5, as judged by fluorescence resonance energy transfer assay using fluorescent lipids [17]. Such a high fusion ability of MGluPG complexes might strongly promote the transfer of plasmid DNA from endosomes to cytosol, resulting in the high transfection activities to DC2.4 cells.

Toward application to immunotherapy

We showed that SucPG and MGluPG complexes efficiently induced expression of EGFP and luciferase in DC2.4 cells. Considering their use as a vector for DC-mediated immunotherapy, MHC class I presentation for transgenes is important. Therefore, we finally evaluated expression of MHC class I on the DC2.4 cell surface after transfection with the complexes or the parent lipoplex containing luciferase gene. Figure 8 shows that the expression level of MHC class I was up-regulated by transfection with the lipoplex, SucPG complex or MGluPG complex, suggesting the MHC class I presentation responded to the transgene expression. Indeed, it is necessary to examine

whether the MHC class I is specific for transgene. We will examine that issue using more appropriate genes, such as ovalbumin gene, as well as their ability to induce MHC class I, which has target-specificity.

Conclusion

In this study, we prepared hybrid complexes of lipoplex and pH-sensitive polymer-modified liposomes designed for transfection of DCs from the viewpoints of ligand and pH-sensitive fusogenic properties. Results showed that, irrespective of possession of ligands, such as mannan and transferrin, the complexes were taken up efficiently by DC2.4 cells, probably because carboxylate anions of the polymers on the complexes were recognized by scavenger receptors of the cells. In addition, the complexes with polymers showing higher fusion ability, MGluPG, exhibited more efficient transfection of DC2.4 cells, which might be attributable to their efficient escape from endosomes. The complexes with the optimized structure achieved efficient transfection, producing 25% of GFP-expressing DC2.4 cells, which was much higher efficiency than some widely used commercially available reagents. Indeed, a wide

disparity remains between viral vectors and the complexes. However, further functionalization, which might enhance passage through intracellular barriers, such as entry into the nucleus, can improve their transfection activity. For those reasons, the MGluPG complexes and their relevant complexes are promising nonviral vectors for DCs, which are useful for cancer immunotherapy.

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Figure legends

Figure 1, SucPG (a) and MGluPG (b) complexes.

Figure 2, Structures of TRX-20 (a) and aminoethylcarbamylmethyl mannan (b).

Figure 3, Expression of luciferase in DC2.4 cells treated with SucPG complexes with various compositions prepared using TRX-20 lipoplexes of varying N/P ratios. Compositions of SucPG complexes are expressed as molar ratios of succinylated units (carboxylated units) of SucPG-modified liposomes and DNA nucleotide units of TRX-20 lipoplexes.

Figure 4, Comparison of transfection activities of SucPG complexes modified with various ligands, transferrin (square) and mannan (triangle). Plain SucPG complexes without ligands were also shown as a control (diamond).

Figure 5, Cell association of the lipoplexes and SucPG complexes modified with various ligands. (a) The amount of cell association of the lipoplexes and SucPG complexes containing transferrin (Tf) and mannan (Man) at the carboxylated unit/nucleotide unit of 1.5. The complex without ligands (Plain) was also shown as a

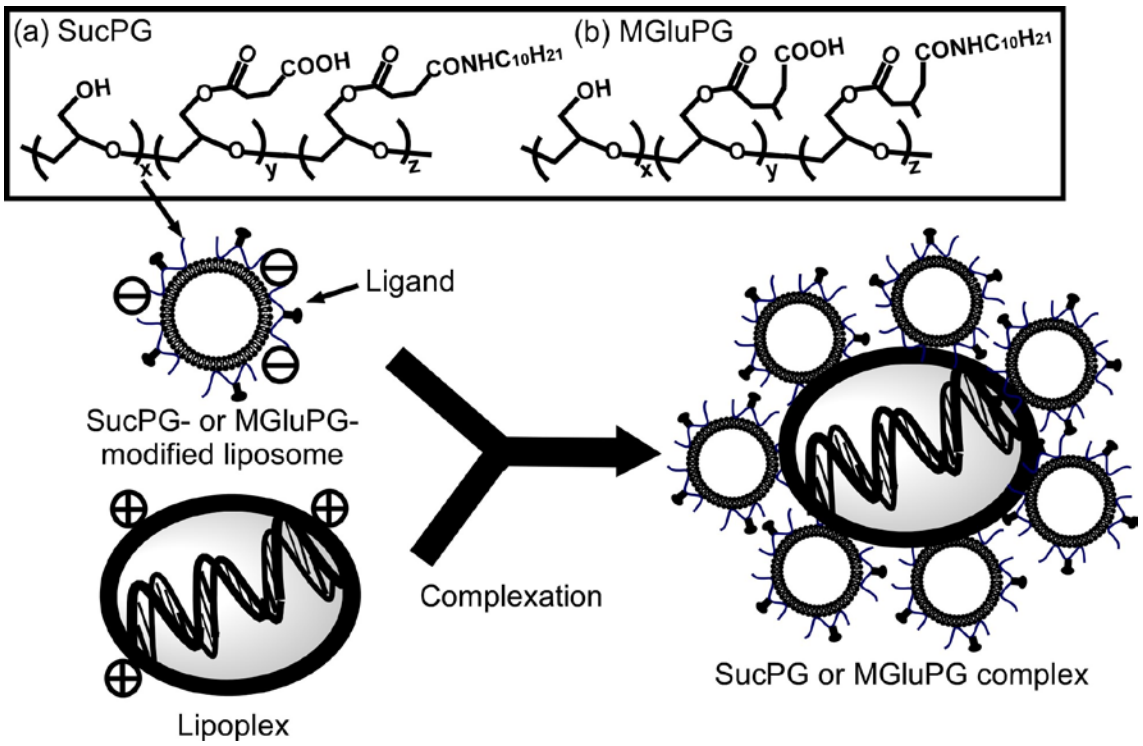
control. (b) Inhibition of the complexes with transferrin (square) and mannan (triangle) to the cell association. The complexes without ligands were also shown as a control (diamond). Free transferrin (solid lines), free mannan (dotted lines) and dextran sulfate (dash lines) were preincubated with DCs as an inhibitor. Relative fluorescence intensity was calculated as the ratios of the amount of association in the presence of ligands to that in the absence of ligands.

Figure 6, Transfection activities of the MGluPG complexes containing EGFP gene. (a) Expression of EGFP in DC2.4 cells treated with MGluPG complexes with various compositions prepared using TRX-20 lipoplexes with N/P ratio of 4. Compositions of MGluPG complexes are expressed as molar ratios of carboxylated units of MGluPG-modified liposomes and DNA nucleotide units of TRX-20 lipoplexes. (b) Expression levels of EGFP among various transfection reagents. (c) Comparison of transfection activities and gene expression levels among various transfection reagents.

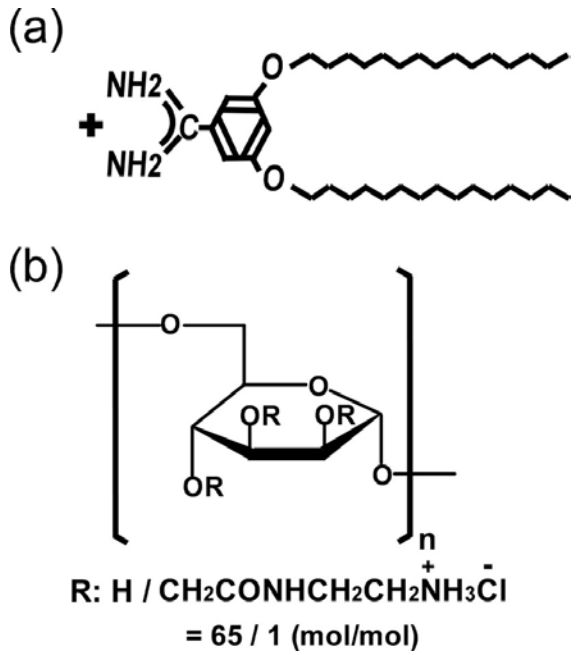
Figure 7, Comparison of cell association (A), and intracellular localization (B) between lipoplexes, the SucPG complexes and MGluPG complexes.

Figure 8, Immunofluorescence analysis of DC2.4 cells treated with (a) no treatment, (b)

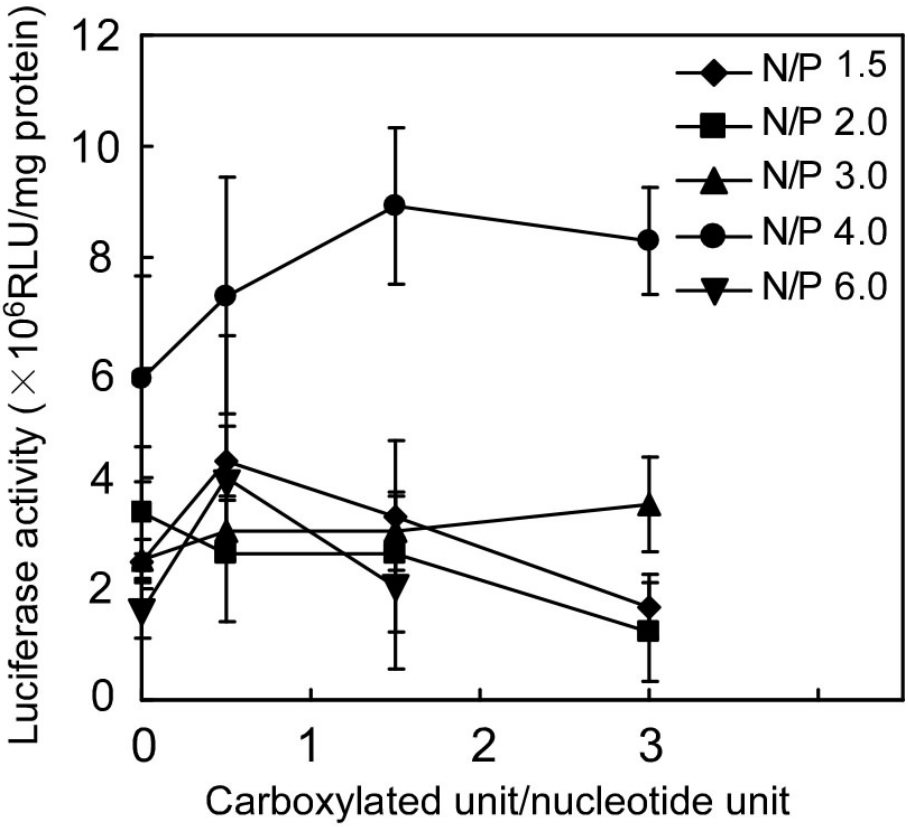
lipoplex, (c) SucPG complex and (d) MGluPG complex. DC2.4 cells treated with 10 mg/ml LPS plus 100 units/ml IFN-γ for 24 h were used as positive control for phenotypical DC maturation (e). Cells were stained by indirect immunofluorescence using biotinylated monoclonal antibodies of H-2K^b/H-2D^b (MHC class I) followed by PE-conjugated streptavidin. Value in the upper right-hand corner of each panel represents the mean fluorescence intensity in flow cytometry analysis in the presence of specific antibodies.



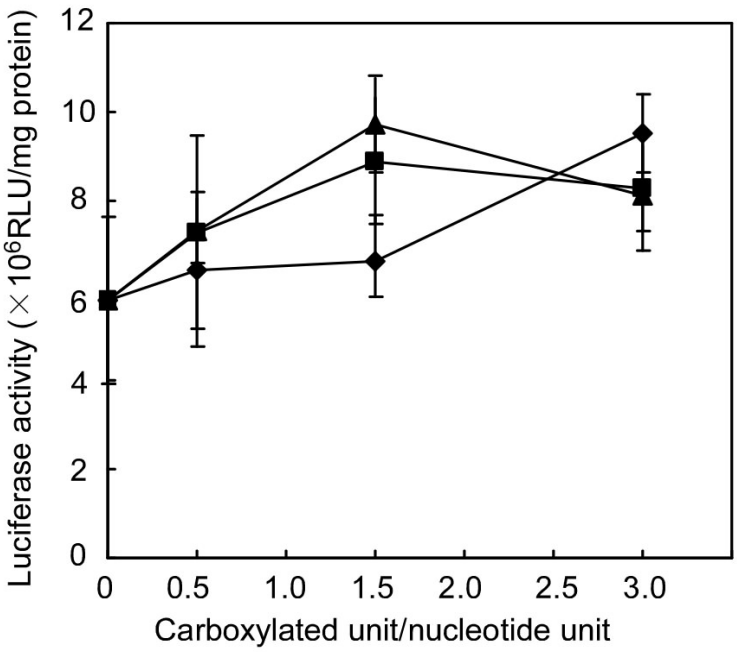
Yuba *et al*, Figure 1.



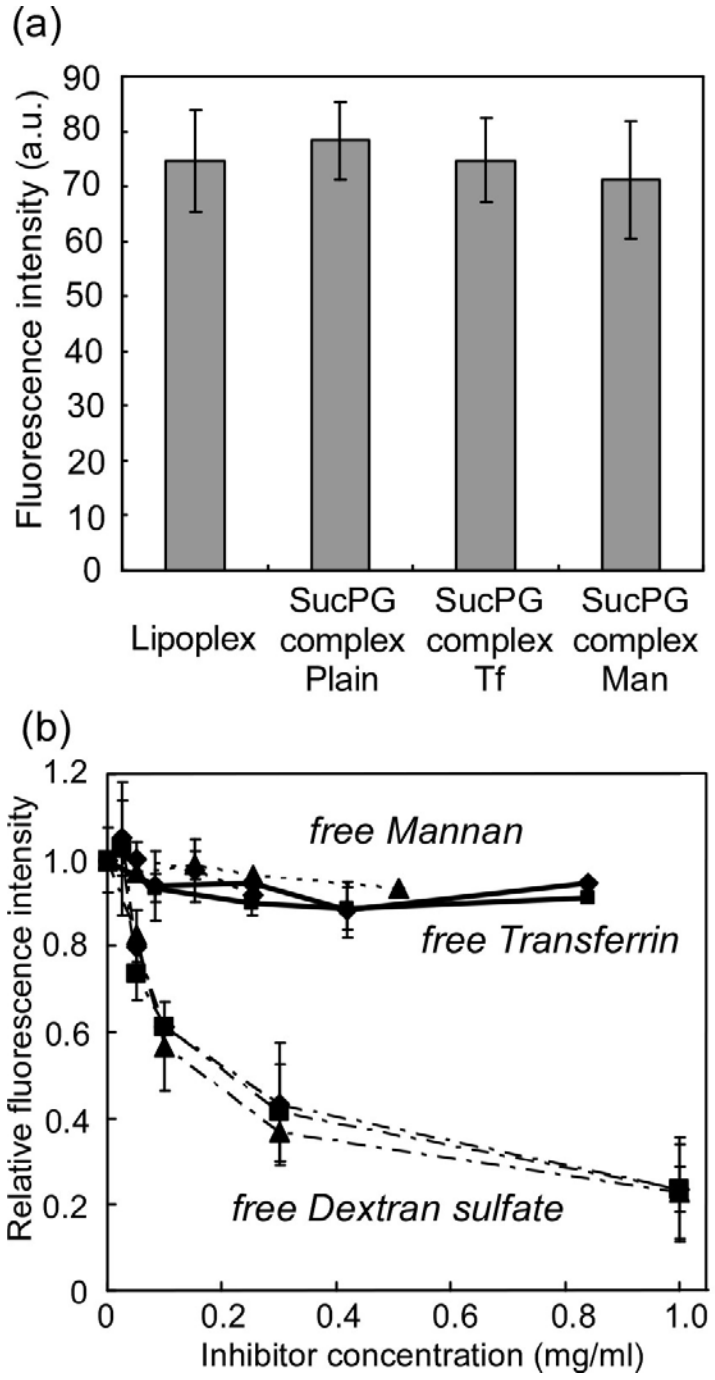
Yuba *et al*, Figure 2.



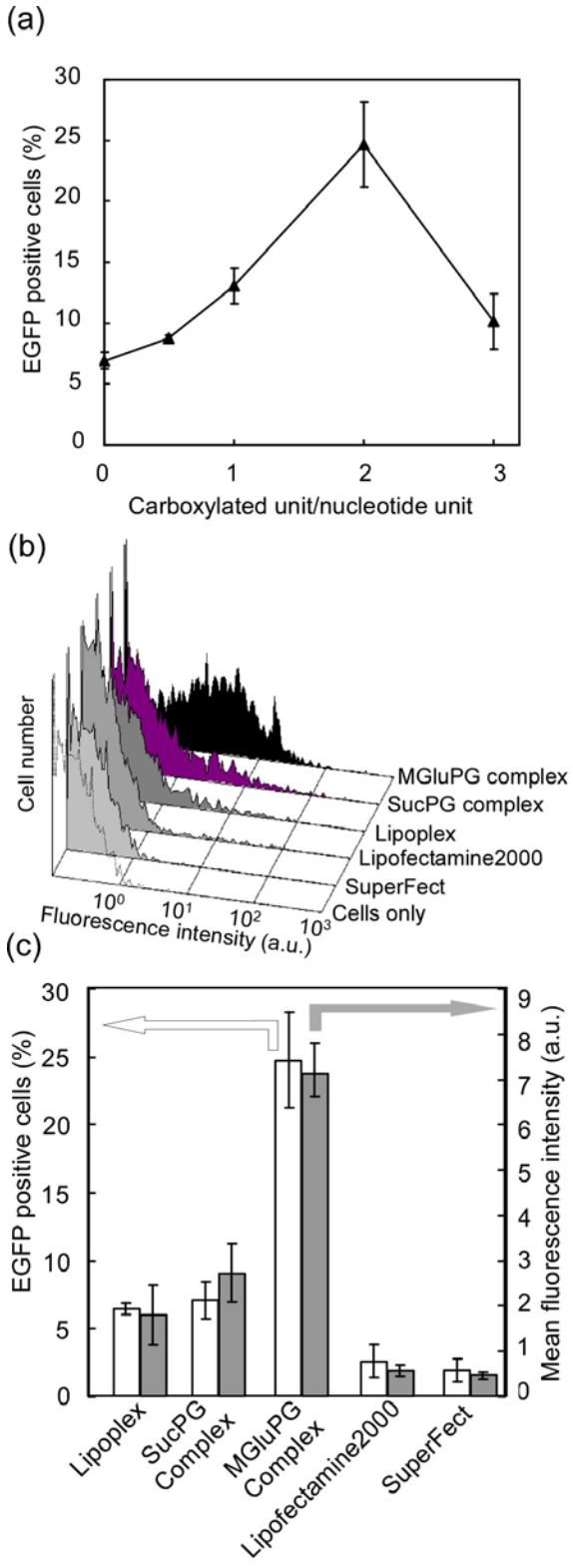
Yuba *et al*, Figure 3.



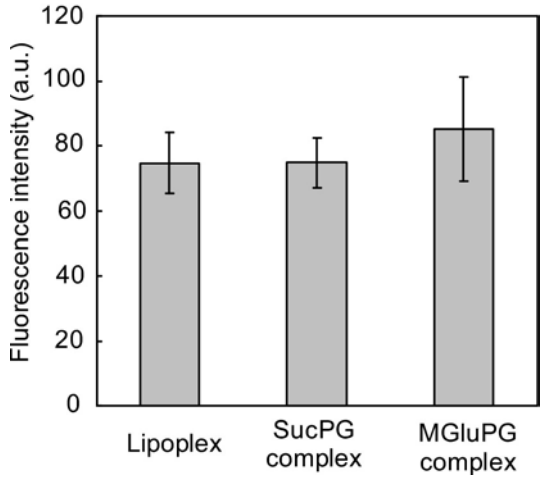
Yuba *et al*, Figure 4.



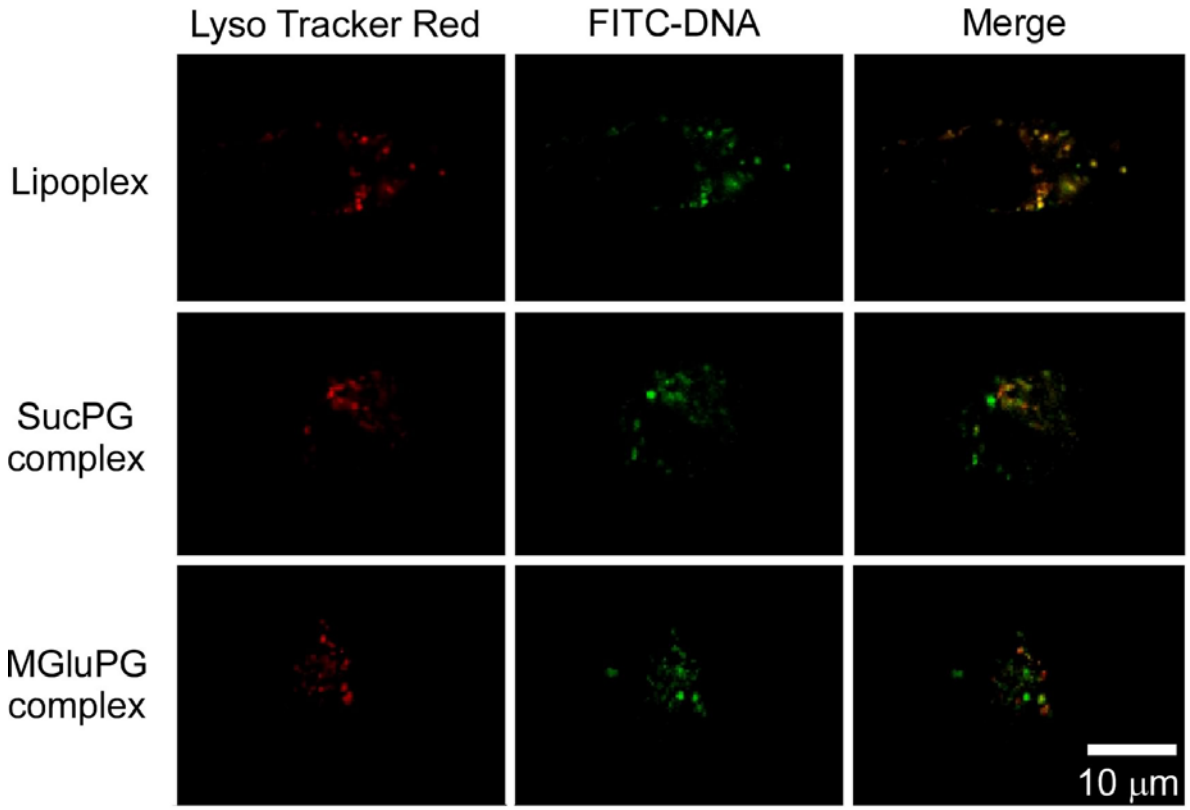
Yuba *et al*, Figure 5.



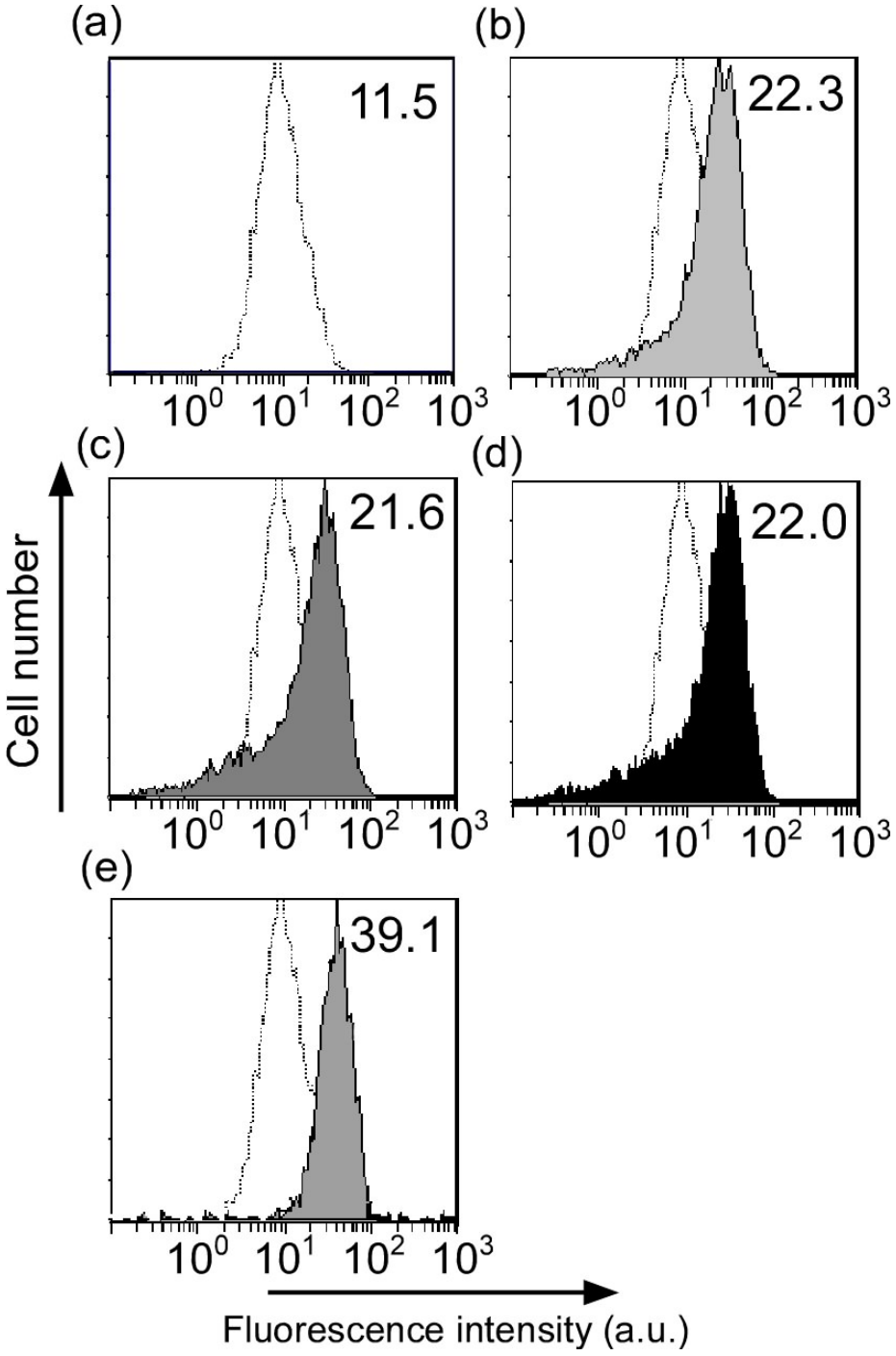
Yuba *et al*, Figure 6.



Yuba *et al*, Figure 7A



Yuba *et al*, Figure 7B.



Yuba *et al*, Figure 8.