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Targeted Delivery using Immunoliposomes with a Lipid-Modified Antibody-Binding Protein

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Abstract A recombinant antibody-binding protein originating from streptococcal protein G was modified with lipid in a site-directed manner by genetic engineering. The resulting lipoprotein was incorporated into the surface of liposomes by simple mixing. Immunoliposomes were then prepared by binding anti-IgG antibodies molecules onto the surface of proteoliposome via the lipid-anchored streptococcal protein G. Either small fluorophores or fluorescently labeled proteins were encapsulated into prepared immunoliposomes, and these molecular tracers could be delivered into cells whose surfaces were marked with specific antibodies.

Keywords Immunoliposome · Protein G · Drug delivery system · Antibody-binding protein · Lipid-modified protein

Introduction

The selective delivery of drugs to target cells and tissues is an important technique for drug delivery system (DDS). Liposomes have been widely used as a carrier for DDS because of their high encapsulation ability for water-soluble compounds [1]. Immunoliposomes bearing antibody molecules on their surface have been developed to introduce a targeting ability into liposomes [2–4]. To incorporate soluble antibody molecules stably on the surface of liposomes, it is necessary to introduce hydrophobic moieties, e.g., by directly coupling antibody molecules to lipids. Generally, this has been performed by chemical coupling [5, 6]; however, the conjugates produced by this method often form a heterogeneous population in terms of number and location of lipid moieties. This may lead to a loss or decrease of antigen-binding properties, and it is difficult to control the orientation of antibody molecules on the liposome surface.

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To overcome these problems of chemical conjugation, we have exploited the major lipoprotein (lpp) of *E. coli*, which contains a specific lipid modification at its amino terminus which anchors lpp in the bacterial membrane. The determinants for the biosynthetic lipid modification are contained within the first 29 residues of lpp [7]. Laukkanen et al. reported the production of a lipid-tagged single-chain antibody (lpp-scFv) by fusion of genes for a single-chain anti-2-phenyloxazolone antibody and the essential part of lpp required for lipid modification [8]. Immunoliposomes bearing the lpp-scFv were characterized by surface plasmon resonance [9], quartz crystal microbalance [10], and fluoroimmunoassay [11]. However, using this method, the sequences of various scFv were needed to tailor immunoliposomes against various antigens. We note that stable and functional immobilization of an antibody-binding protein on the liposome surface should provide us with a facile method for preparing immunoliposomes against various antigens without the need for antibody engineering. Indeed, in our previous study, the B-domain of protein A was lipid modified in a site-directed manner [12].

Protein A is a cell wall component of *Staphylococcaus aureus* which binds specifically to the Fc portion of immunoglobulin G (IgG) from many mammals [13, 14]. Since the binding does not inhibit antigen–antibody interaction, protein A is widely used to immobilize IgG on solid surfaces. Feng et al. introduced ZZ-His, an Fc-binding motif from protein A, as an adaptor to conjugate antibody to make immunoliposomes [15]. This protein has its limitations, however, because protein A exhibits poor avidity for some antibody classes, such as mouse IgG_1 and goat IgG_1, even though most monoclonal antibodies are generated from mouse. In the present study, we utilized three repeats of the Fc-binding domain of streptococcal protein G (C3), which has more IgG-binding versatility than does protein A [16].

The purpose of the present study is to construct a lipid-modified IgG-binding protein and to demonstrate its application to cell targeting for drug delivery. C3 was modified with lipid in a site-directed manner, and the resulting protein (lpp-C3) was incorporated on the surface of liposomes by mixing in vitro. Immunoliposomes were then prepared by binding anti-IgG antibodies on the surface of proteoliposomes via the C3 moiety, and they were selectively delivered to target cells whose surfaces were marked with specific antibodies.

Materials and Methods

Materials

Commercially available liposomes, COATSOME EL-01-C (Nippon Yushi, Japan), were used for all experiments. Anti-human leukocyte antigen (HLA) antibody (mouse) and anti-mouse IgG antibody were purchased from Sigma and Pierce, respectively. All other chemicals were of analytical grade.

Expression and Purification of Lipid-Tagged Antibody-Binding Protein

The expression plasmid for the lipid-tagged antibody-binding protein, pLpp-C3, encodes the signal peptide and nine mature N-terminal amino acid residues of lpp fused to three repeats of the C-domain of protein G with a hexahistidine tail. As control expression plasmids, pLpp(−)-C3 and pC3 were also constructed.

For the expression of proteins, *Escherichia coli* BL21 cells were transformed with pLpp-C3, pLpp(−)-C3, or pC3. Transformants were cultured in LB medium containing

ampicillin at 37 °C to the optical density at 660 nm of 0.6. The expression of each protein was induced with isopropylthio-β-D-galactoside to a final concentration of 1 mM, and growth was continued at 37 °C for another 3 h. The cells were harvested by centrifugation at 2,000×*g* then washed with phosphate buffered saline (PBS). The cell pellet obtained was disrupted in a Bug buster (Novagen) containing benzonase nuclease and lysozyme. After centrifugation, the pellet was dissolved in 8 M urea and the supernatant were applied to a TALON metal affinity resin (Clontech) equilibrated with TALON buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0). The bound proteins were eluted with a buffer containing 500 mM imidazole. The imidazole was subsequently removed by dialysis against PBS buffer.

Preparation of Immunoliposome

Liposome solution was prepared by following the manufacturer's instruction, just adding COATSOME EL-01-C to pure water and stirring gently. The solution of purified lpp-C3 was then added to the liposome solution with stirring at 4 °C to make a proteoliposome. The proteoliposome solution was centrifuged at 90,000×*g* for 5 min to remove free Lpp-C3, and the pellet was resuspended in PBS. To prepare immunoliposomes, anti-IgG antibody was then added to the proteoliposome solution and reacted for 1 h at room temperature. After centrifugation, the pellet was again resuspended in PBS and stored at 4 °C.

Targeting to Cells

For cell targeting, HLA, which is expressed on the surface of HeLa cells, was utilized. First, anti-HLA antibody (mouse) was added to the culture medium of HeLa cells at 9 nM final concentration and incubated for 90 min. After washing with PBS, anti-mouse IgG-displayed liposomes containing fluorescein isothiocyanate (FITC) or Alexa488-labeled streptavidin were added to the culture medium and incubated for 2 h. The cells were suspended in PBS and analyzed by flow cytometry using the FACScan System (Beckton-Dickinson).

Results and Discussion

Expression and Purification of Lipid-Tagged Antibody-Binding Protein

The constructed plasmid pLpp-C3 for the expression of lipid-tagged antibody-binding protein encodes a signal peptide (N9lpp), antibody-binding domains from protein G (C3), and a C-terminal His-tag under the control of the *tac* promoter. The corresponding protein was expressed in an insoluble fraction of *E. coli* BL21 which may be caused by the introduction of hydrophobic lipid molecules. It is suggested that the lipid-modified protein was located at the inner membrane as we described previously [12]. The control plasmid pLpp(−)-C3 is designed to remove one cysteine residue which is the site of lipid modification. The resulting protein was expressed in a soluble fraction. C3 protein without the lpp tag encoded in pC3 was also expressed in a soluble fraction. All three proteins were purified by affinity column chromatography utilizing a common C-terminal His-tag. Purified proteins were analyzed by SDS-PAGE (Fig. 1). Predicted molecular masses of Lpp (−)-C3 and C3 are 23.7 and 22.7 kDa, respectively. The signal sequence of Lpp (sslpp) of Lpp-C3 is removed in *E. coli*, and the molecular mass which is predicted from the amino

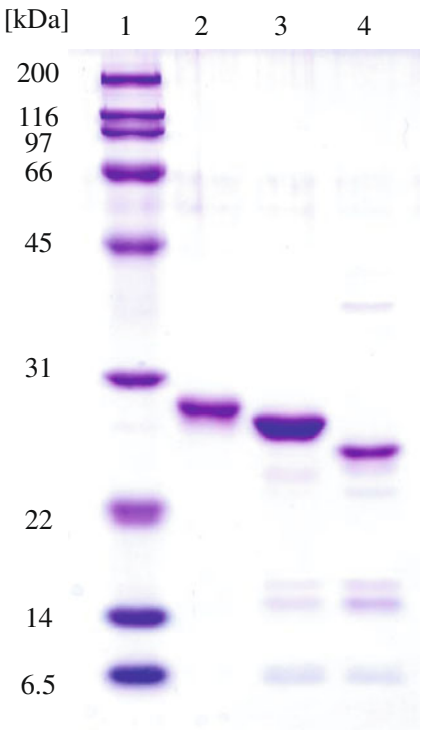

Fig. 1 SDS-PAGE analysis of purified proteins. Purified proteins were separated by electrophoresis in a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. *Lane 1*, molecular weight marker (molecular mass is indicated on the left); *lane 2*, Lpp-C3; *lane 3*, Lpp(-)-C3; *lane 4*, C3

acid sequence is 23.8 kDa. Although the difference between Lpp(−)-C3 and Lpp-C3 was only one cystein residue, the band of Lpp-C3 in SDS-PAGE is a little bigger than the predicted value. This result suggests that lipid molecules are incorporated in the Lpp-C3 in *E. coli* as predicted. Laukkanen et al. reported that the electrophoretic band of lipid-modified protein was shifted ca. 2 kDa [8], which is in agreement with our result.

Antibody-binding affinity of Lpp-C3, Lpp(−)-C3, and C3 were analyzed by Western blotting (data not shown). It was confirmed that these proteins including the C3 domain retained sufficient IgG-binding abilities. Lipid modification of C3 had no effect on its binding ability.

IgG-Binding Ability of lpp-C3 Displayed on the Surface of Immunoliposomes

Liposome solution can be prepared by only adding the dried Coatsome to a buffer solution with gentle mixing. The prominent diameter of prepared liposomes was around 200 nm with a distribution from 100 to 400 nm as estimated by dynamic light scattering (data not shown).

Since just the N-terminus of Lpp-C3 was designed to be modified with lipid, only the N-terminus is expected to be buried in the lipid membrane of the liposome surface. In the resulting proteoliposome, Lpp-C3 should be displayed on the surface with the C3 domain freely accessible by the solution phase. Immunoliposomes can be prepared by a simple manner, only adding the antibody to the proteoliposome. Formation of immunoliposomes was confirmed by using a fluorescence-labeled antibody. First, proteoliposome solution was prepared by mixing 4.4 mM of liposome solution and 2.88 μM of Lpp-C3 or the other proteins (Lpp(−)-C3 and C3). Alexa488-labeled anti-mouse IgG antibody was then added to the proteoliposome solution to a final concentration of 1.21 μM. After centrifugation

(90,000×*g*, 5 min) of the solution, the pellet was washed with PBS and resuspended in PBS. A 100-μl aliquot of the solution was pipetted into the well of 96-well microplate, and fluorescence of each well was measured. As shown in Fig. 2, proteoliposomes prepared with Lpp-C3 exhibited high fluorescence intensity. To the contrary, fluorescence of liposomes with Lpp(−)-C3 or C3 was quite low. These results indicate that immunoliposomes were correctly formed only through Lpp-C3.

Optimization of Conditions for Immunoliposome Preparation

To raise the targeting efficiency as a carrier, it is important to display sufficient antibody molecules on the surface of liposome. In order to optimize the conditions for making immunoliposomes, the effect of the concentration of Lpp-C3 was investigated. The proteoliposome was prepared with 230 μM of liposome and various amounts of purified Lpp-C3. After that, 0.26 μM of fluorescence-labeled antibody was added and the fluorescence of the immunoliposome was detected (Fig. 3). The fluorescence increased with the amount of Lpp-C3 and reached a constant value when 2.0 μM of Lpp-C3 was used. At this concentration, incorporation of Lpp-C3 to the liposome was assumed to be saturated. Therefore, this condition was used for the preparation of the proteoliposome.

Next, we investigated conditions for the surface saturation of proteoliposomes with antibodies. Various concentrations of fluorescence-labeled antibody were added to the solution of proteoliposome, and the fluorescence of the liposome complex was measured (Fig. 4). Fluorescence intensity increased with the concentration of antibody and reached a constant value at 100 nM. This condition for the preparation of immunoliposomes was used for further experiments.

Targeting of Immunoliposome Encapsulating FITC to Cells

To confirm the possibility of the application of our immunoliposome preparation to DDS, FITC as a model small molecule was doped into the liposomes. Since the strong fluorescence intensity was observed in the liposome pellet obtained by centrifugation, incorporation of FITC in the liposome was confirmed.

In this experiment, HLA expressed on the surface of HeLa cells was selected as the antigen for cell targeting. Anti-HLA antibody (mouse) was bound to the surface of HeLa

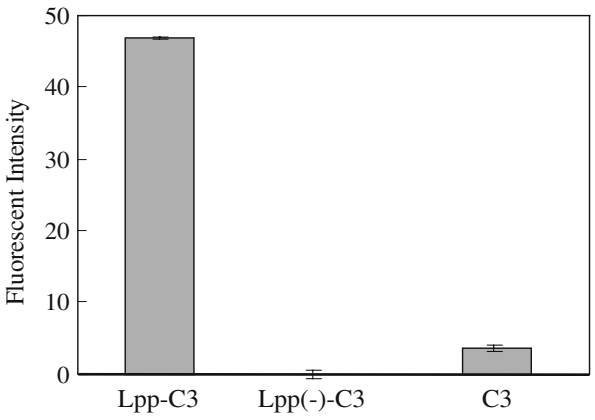

Fig. 2 Formation of immunoliposomes. To prepare proteoliposomes with antibody-binding ability, liposomes were mixed with each protein. After centrifugation to eliminate free proteins, the pellet was resuspended in PBS. Alexa488-labeled anti-mouse IgG was then bound and the fluorescence of liposomes was measured

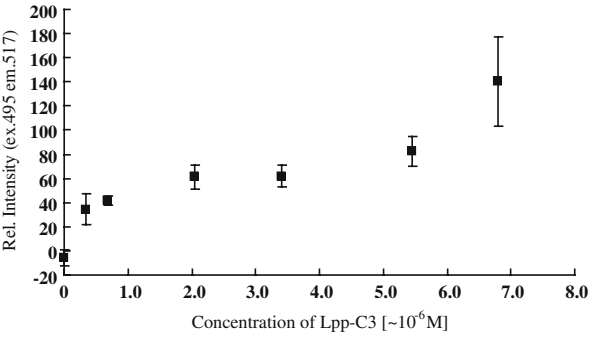

Fig. 3 Relationship between relative fluorescence intensity and concentration of Lpp-C3 for proteoliposome preparation. The proteoliposomes were prepared with 230 μM of liposome and varying concentrations of purified Lpp-C3. Alexa488-labeled anti-mouse IgG was bound and the fluorescence of liposomes was measured

cells, and the FITC-anti-mouse IgG immunoliposomes were added to the cells. The cells were then analyzed by FACS (Fig. 5). When the anti-mouse IgG antibody-displayed immunoliposomes were added to cells, the number of cells with higher fluorescence intensities increased compared with cells treated with non-specific IgG-incorporated immunoliposomes. Analyses by confocal microscopy also showed that the strong fluorescence was observed only in cells which were treated with anti-mouse IgG antibody-displayed immunoliposome (data not shown).

These results suggest that FITC-doped immunoliposomes efficiently target cells in an antigen-specific manner, and the possibility of the application of this methodology to the delivery of drugs with low molecular weights was demonstrated.

Targeting of Immunoliposome Encapsulating Protein to Cells

The cell targeting of immunoliposomes containing protein cargo was also investigated. As a model case, Alexa488-labeled streptavidin was loaded into preteoliposomes. Then anti-mouse IgG antibody was incorporated onto the surface of the liposome for targeting. Fluorescence of Alexa488 was observed in the immunoliposome precipitated by centrifugation. This result indicated streptavidin was loaded into the immunoliposome.

To confirm the cell-targeting ability, the solution of immunoliposome was added to the culture medium of HeLa cells whose surface was marked with anti-HLA antibodies as described in the previous section, and the cells were analyzed by FACS as shown in Fig. 6. Anti-HLA antibody-attached HeLa cells treated with anti-mouse IgG antibody-displayed immunoliposome showed much stronger fluorescence intensities compared with those of cells treated with non-specific IgG-displayed immunoliposome. When the cells were

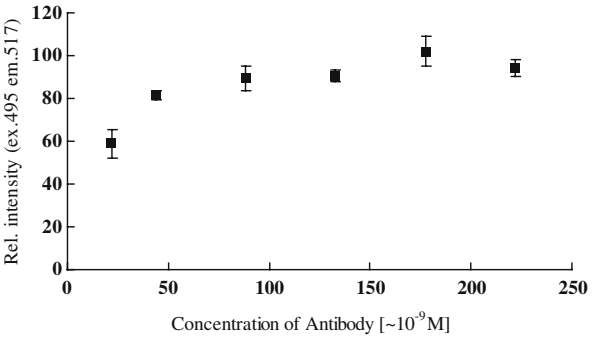

Fig. 4 Relationship between relative fluorescence intensity and concentration of antibody. Various concentrations of Alexa488-labeled antibody were added to the solution of proteoliposomes, and fluorescence of the liposomes was measured

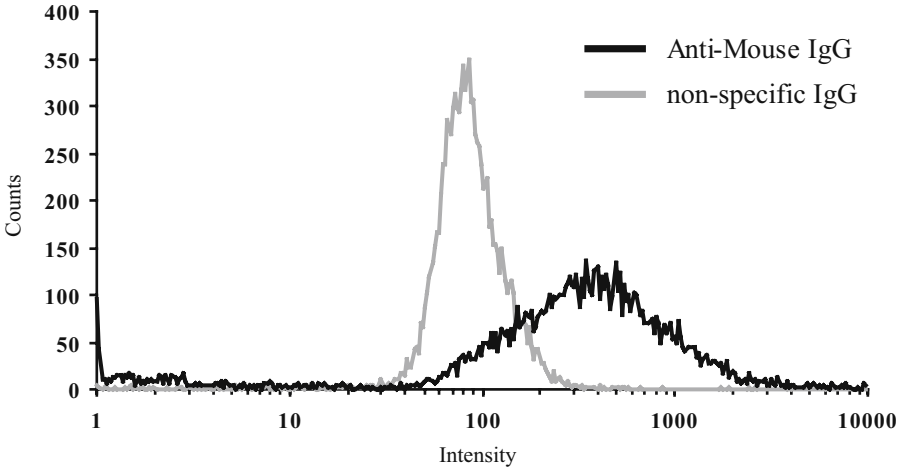

Fig. 5 FACS analyses of anti-HLA antibody-marked cells treated with immunoliposomes containing FITC. HeLa cells were marked with anti-HLA antibody (mouse) and were treated with anti-mouse IgG antibody- or non-specific IgG-displayed immunoliposomes containing FITC

observed by confocal microscopy, the strong fluorescence was observed even inside of cells (data not shown). This result suggests that streptavidin encapsulated in the liposome was delivered into cells by endocytosis after targeting to the cell surface via antibody. These results indicate that the cell-targeting delivery of proteins in liposomes could be performed by introducing specific antibodies on the surface of liposomes.

In further cell-targeting experiments, anti-HLA antibodies were directly displayed on the surface of proteoliposomes. However, the resulting fluorescence signals of HeLa cells

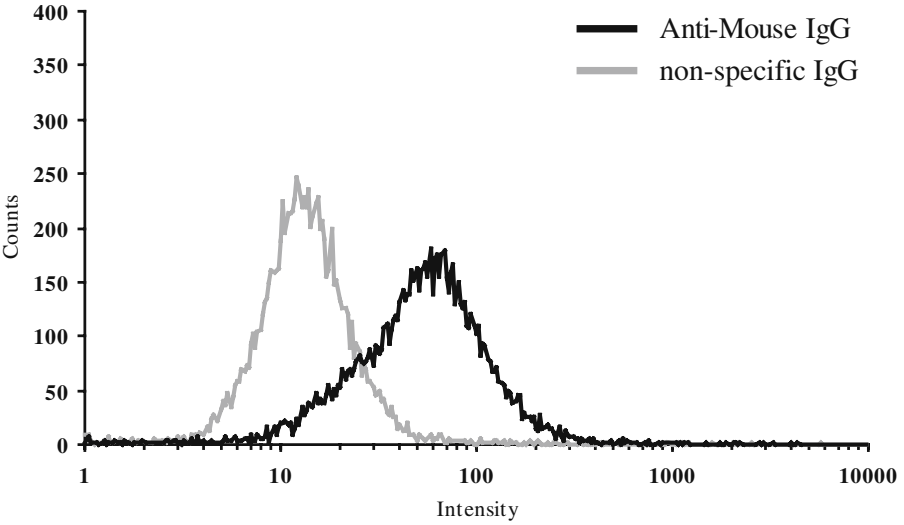

Fig. 6 FACS analyses of anti-HLA antibody-marked cells treated with immunoliposomes containing Alexa488-labeled streptavidin. HeLa cells were marked with anti-HLA antibody (mouse) and they were treated with anti-mouse IgG antibody- or non-specific IgG-displayed immunoliposomes containing Alexa488-labeled streptavidin

analyzed by FACS were weak. This result may depend on the properties of the anti-HLA antibody. Our use of anti-IgG antibody-displayed immunoliposomes may have provided an unanticipated opportunity for signal amplification. Currently, we are trying to optimize a delivery system using immunoliposomes with displayed antibodies which are specific for cell surface antigen. Regardless, it is demonstrated that lipid-modified antibody-binding protein, lpp-C3, was useful for preparation of immunoliposomes for targeting to cells.

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