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Preparation of cationic immunovesicles containing cationic peptide lipid for specific drug delivery to target cells

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Abstract

The cationic vesicle composed of Span80 and cationic peptide lipid (CPL) was prepared. The cytotoxicity of the Span80-CPL cationic vesicle was very low compared with Span80 vesicle. Antibody was able to be immobilized on vesicle surface by mediation of protein A. The antigen targeting ability of the antibody-immobilized vesicle (immunovesicle) derived from antibody was evaluated. Our results suggested that the Span80-CPL immunovesicles specifically associate with target cells by the antibody mediation, and the substance capsulated in immunovesicle was transferred into the target cells. This means that the Span80-CPL immunovesicle is expected to achieve a high local concentration of an encapsulated drug at the target.

Abbreviations: CPL – cationic peptide lipid; FBS – fetal bovine serum; FITC – fluorescein isothiocyanate; IADE – isothiocyanic acid dodecyl ester; PBS – phosphate buffered saline; PI – propidium iodide; Span80 – sorbitan monooleate; Tween80 – polyoxyethylene (20) sorbitan monooleate

Introduction

Liposomes have been studied as carrier of drug delivery system for cytostatic and cytotoxic drugs. Hydrophilic drugs can be easily capsulated in the inner aqueous phase and hydrophobic drugs can be incorporated into the bilayer of liposomes. The target-specific drug delivery is a potentially attractive for increasing the therapeutic efficiency and reducing side effects of the drugs. There are some reports concerning the antibody-coupled liposomes, termed as immunoliposomes (Maruyama et al. 1997; Kessner et al. 2001; Park et al. 2002). Drug delivery to a specific site by immunoliposomes is a prevalent mode of therapy. For instance, immunoliposomes have been regarded as very effective drug-targeting system for chemotherapeutic cancer treatment (Bendas 2001).

For the purpose of practical use, submicrometer-size cationic lipid vesicles composed of inexpensive, commercially available, artificial amphiphilic lipids have been prepared (Sugahara et al. 2001). In the present work, we developed drug and gene carrier by using of lipid vesicles immobilized antibody. The vesicles composed of non-ionic surfactant, Span80 (sorbitan monooleate), Tween80 (polyoxyethylene (20) sorbitan monooleate), soybean lecithin, cholesterol, and cationic lipids were prepared by modified two-step emulsification procedure (Kato et al. 1999). In terms of some of

physicochemical properties, the resulting vesicles were similar to phospholipids liposomes. They should be regarded as one of liposome family, and inexpensive liposome alternatives (Yoshioka et al. 1994). The lipid vesicles are very useful microcapsule for drug and gene carriers (Kato et al. 1999). In this manuscript, the newly synthesized lipid, cationic peptide lipid (CPL), was applied for preparation of cationic immunovesicles. It has been reported that amphiphiles containing an amino acid residue, interposed between a polar head group and an aliphatic double chain, form stable single-walled vesicles in aqueous media (Murakami et al. 1984a). We adopted *N*,*N*-dihexadecyl-*N* α-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide (CPL) as a vesicle-forming lipid. The physicochemical properties for single-walled vesicles of this lipid, such as vesicle diameter, bilayer thickness, number of lipid molecules in the outer and inner surfaces of the vesicles, and phase-transition behavior, are comparable to those of the naturally occurring phospholipids, egg lecithin liposome, even though the single-walled vesicles formed with CPL are morphologically much more stable than that for egg lecithin. In the present study, we have developed the target-specific drug delivery system by using of Span80-CPL cationic immunovesicles, which are easily and cheaply prepared, compared with immunoliposomes.

Materials and methods

Reagents

Span80 and Tween80 were purchased from Wako Pure Chemistry (Osaka, Japan). Cholesterol was purchased from Sigma (St. Louis, MO, USA). Crude soybean phospholipids was obtained from Wako Pure Chemistry, and purified by acetone precipitation method. The composition of the crude soybean phospholipids was purified and analyzed by HPLC using silica column by Wako Pure Chemistry, yielding 30 wt% phosphatidylcholine, 25 wt% phosphatidylethanolamine, 17 wt% phosphatidylinositol, 11 wt% phosphatidic acid, and 3 wt% lysophosphatidylcholine. The remaining lipids were glycolipids and natural lipids. *N*,*N*-dihexadecyl-*N* α-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide (CPL) was prepared according to the methods reported previously (Murakami et al. 1982, 1984b, 1991). The molecular structure of CPL was shown in Figure 1. Propidium iodide (PI), and protein A were purchased from SIGMA (St Louis , MO, USA), and degraded DNA from salmon roe was derived from Wako Pure Chemistry.

Preparation of Span80-CPL cationic immunovesicles

The micrometer-sized lipid vesicles were prepared by a two-step emulsification technique as described previously (Kato et al. 1993). Briefly, 4.0 ml of *n*-hexane solution containing 132 mg of Span80, 12 mg of soybean lecithin, 6 mg of cholesterol, and CPL was put in a test tube (20 mm diameter, 95 mm height). Propidium iodide (PI) was mixed with degraded DNA from salmon roe dissolved in phosphate buffered saline (PBS), and the DNA–PI complex was used for fluorescent tracer of vesicles. The DNA–PI complex of 0.3 ml was then added drop by drop and the system was sonicated by a supersonicator, US-150U (Nihon Seiki, Japan) three times at 15 s sonication interval. The inner aqueous phase of a water-in-oil emulsion formed was composed of DNA–PI in PBS. This emulsion was then transferred into a round bottom flask, and *n*-hexane was removed with a rotary evaporator, leading to the formation of a creamy layer of water and lipid, which adhered to the inner wall of the flask. After complete evaporation of *n*-hexane, 4.0 ml of PBS containing 10 mM Tween80 and protein A coupled with isothiocyanic acid dodecyl ester (IADE; Tokyo Chemical Industry, Tokyo, Japan) was added as second emulsifying agent. Protein A–IADE complex was used for immobilizing IgG antibody. The mixture was stirred by homomixer at a constant speed of 3000 rpm for 1 min, and the resulting heterogeneous suspension was transferred to a 5 ml brown bottle and stirred by magnetic stirrer for 3 h. Then the solution was

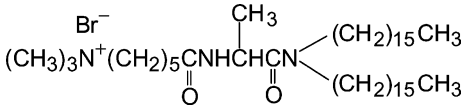

Figure 1. Molecular structure of CPL.

incubated overnight to remove *n*-hexane, completely. Finally, goat IgG antibody specific for human IgM (Biosource International, Camarillo, CA, USA) was added to the vesicle solution and stand for 3 h at room temperature, and immunovesicles were separated from unbound goat IgG on a BioGel A-5m (Bio-Rad, CA, USA).

Cells and cell culture

Human–human hybridoma HB4C5 cells producing IgM were used. HB4C5 cell line was a fusion product of a human B lymphocyte from lung cancer patient and a human fusion partner, NAT-30 (Murakami et al. 1985). HB4C5 cells were cultured in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 10 μg/ml of insulin, 20 μg/ml of transferrin, 20 μM ethanolamine, and 25 nM selenite (ITES-ERDF) at 37 °C under humidified 5% CO_2–95% air (Murakami et al. 1982). Human non-tumorigenic epithelial cell line, MCF10-2A cells were purchased from ATCC and cultured in 5% FBS-ERDF medium.

Flowcytometry analysis

HB4C5 cells and MCF10-2A cells were cultured in 5% FBS-ERDF medium at 37 °C under humidified 5% CO_2–95% air. Span80-CPL immunovesicles immobilized anti-human IgM antibody and encapsulating DNA–PI complex were added to the medium at 5% and cultured for 3 h. Following cultivation, these cells were washed with iced PBS 3-times, and the delivered DNA–PI complex into the cells by immunovesicles was analyzed by FACSCalibur (Becton-Dickinson, USA).

Results and discussion

Cytotoxicity of Span80 and Span80-CPL vesicles

We evaluated the cytotoxicity of Span80 vesicles and Span80-CPL vesicle. Span80-CPL vesicle contained 30 wt% CPL against total lipids. The vesicle density was determined as turbidity at 600 nm. Each vesicle solution was adjusted turbidity at 0.5, and added to the culture medium at various concentrations. Cell viability of HB4C5 cells was determined by trypan blue dye exclusion test. As the result of that, by the addition of Span80 vesicle at 5%, the viability of HB4C5 cells 6 h after inoculation was less than 40% (Figure 2). On the other hand, cytotoxicity of 30 wt% CPL vesicle added at 5% was negligible. It was supposed that the cytotoxicity of Span80 vesicle is derived from its main component Span80. Therefore, this result means that cytotoxicity of Span80 vesicle is mitigated by the addition of CPL instead of 30 wt% of Span80 as the membrane components.

Preparation of Span80-CPL immunovesicles

Span80-CPL immunovesicles targeted to HB4C5 cells producing monoclonal IgM were prepared. Antigen binding site of antibody has to be extrorsely immobilized on vesicle surface. Then, we employed protein A to fix goat IgG specific for human IgM antibody. The posture control of IgG antibody on the vesicle surface is easily achieved by protein A as an anchor of IgG. Protein A which has binding sites to the F_c region of IgG antibody, was immobilized to the vesicle membrane. The F_c region of IgG associates with protein A on the vesicle surface, and the F_{ab} region is oriented to the outer direction of the vesicle surface. For the immobilization of protein A to the vesicle, IADE was used for an anchor (Figure 3). IADE–protein A complex was prepared by mixing for 3 h. The electron micrograph of Span80-CPL immunovesicles was shown in Figure 4.

Association of Span80-CPL immunovesicles with target cells

The electrophoretic analysis revealed that the surface of the lipid vesicles composed of only Span80 is anionic in spite of using non-ionic surfactant as reported previously (Kato et al. 1993). On the other hand, it is well known that surface of animal cells is negatively charged. It is expected from this fact that the cells repel Span80 vesicles because of the electrostatic force. Human hybridoma HB4C5 cells were treated with Span80 immunovesicles or Span80-CPL immunovesicles. Both immunovesicles were immobilized FITC

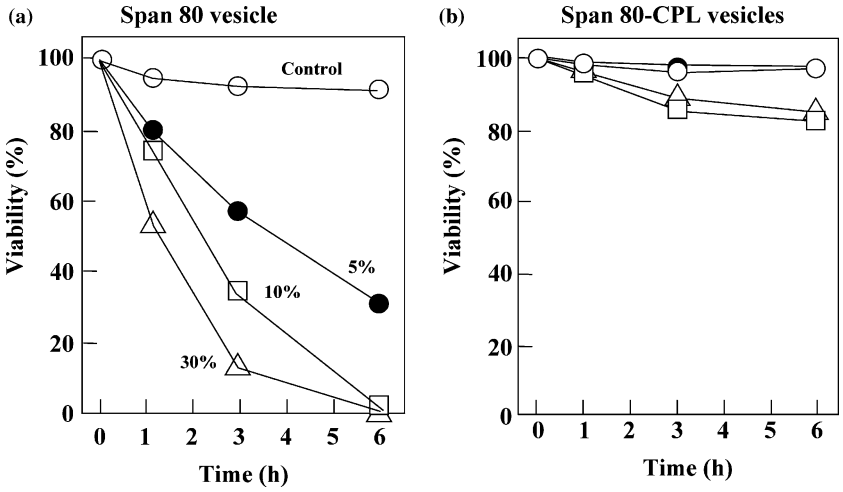

Figure 2. Cytotoxicity of Span80 and Span80-CPL vesicles. Span80 vesicles and Span80-CPL vesicles containing 30 wt% CPL against total lipids were prepared. Vesicle density was adjusted by turbidity 0.5 at 600 nm. HB4C5 cells were cultured at 5×10^5 cells/ml in ITES-ERDF medium and each vesicle solution was added to the medium at 5% (●), 10% (□), and 30% (△). Cell viability was determined by trypan blue dye exclusion test at each culture periods. (a) Span80 vesicle; (b) Span80-CPL vesicles containing 30 wt% CPL against total lipids.

labeled anti-human IgM and encapsulating DNA–PI complex. As shown in Figure 5, the association of Span80 immunovesicles with HB4C5 cells was very slight, and the infusion of the encapsulated DNA–PI into HB4C5 cells was undetectable. On the contrary, Span80-CPL immunovesicles constructed by 30 wt% of CPL and 70 wt% of Span80 were obviously bound to HB4C5 (FITC-conjugated anti-human IgM), and the infusion of contents of vesicles to target cells was also significant (DNA–PI).

Specific binding of Span80-CPL immunovesicles to HB4C5 cells

The specific binding between Span80-CPL immunovesicles and HB4C5 cells was investigated. Span80-CPL immunovesicles targeting human IgM were prepared. Span80-CPL immunovesicles were composed of 30 wt% CPL and immobilized anti-human IgM, encapsulating DNA–PI complex. As the results of flowcytometric analysis, CPL immunovesicles transported encapsulated

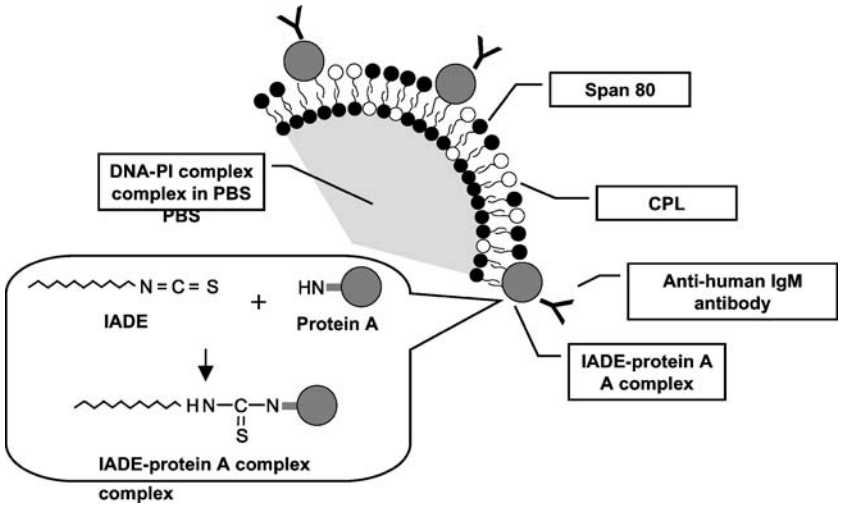

Figure 3. Schematic illustration of Span80-CPL immunovesicles.

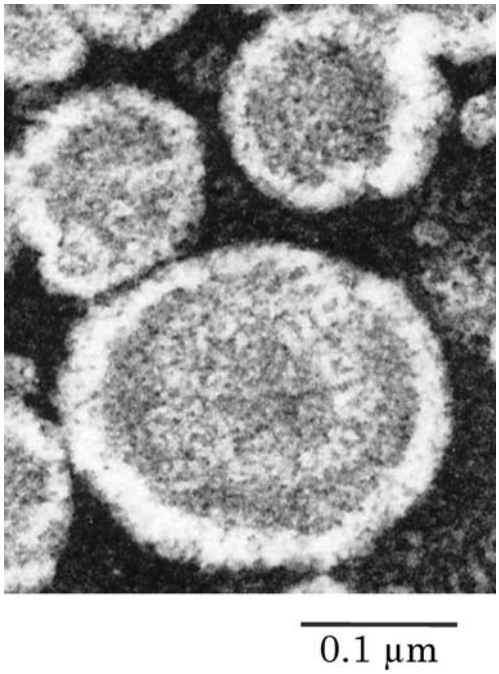

Figure 4. Electron micrograph of Span80-CPL immunovesicles. Span80-CPL immunovesicles were treated with 2% uranyl acetate for 2 min and observed by transmission electron microscope (Hitachi H-800, Japan).

materials (DNA–PI) into HB4C5 cells (Figure 6a). However, Span80-CPL immunovesicles were not able to interact with HB4C5 treated with anti-human IgM prior to inoculation with the immunovesicles (Figure 6b). Moreover, the immunovesicles did not interact with MCF10-2A

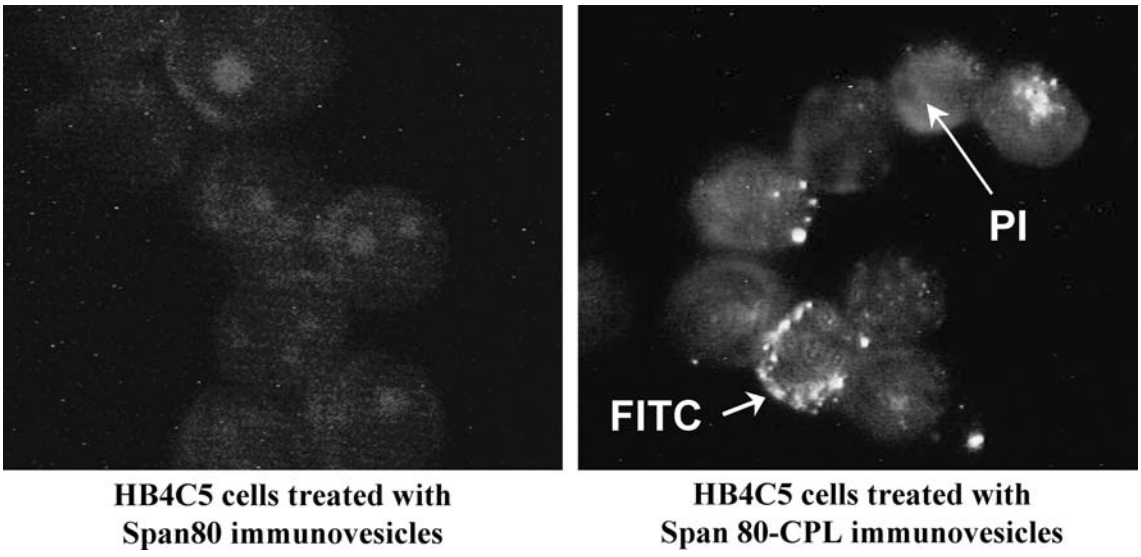

Figure 5. Association of Span80-CPL immunovesicles with target cells. HB4C5 cells were cultured in ITES-ERDF medium containing 5% of Span80 immunovesicles or Span80-CPL immunovesicles immobilized FITC-conjugated anti-human IgM antibody and encapsulating DNA–PI complex. Following cultivation for 3 h, cells were washed with iced PBS 3-times and fluorescent microscopic analysis was carried out.

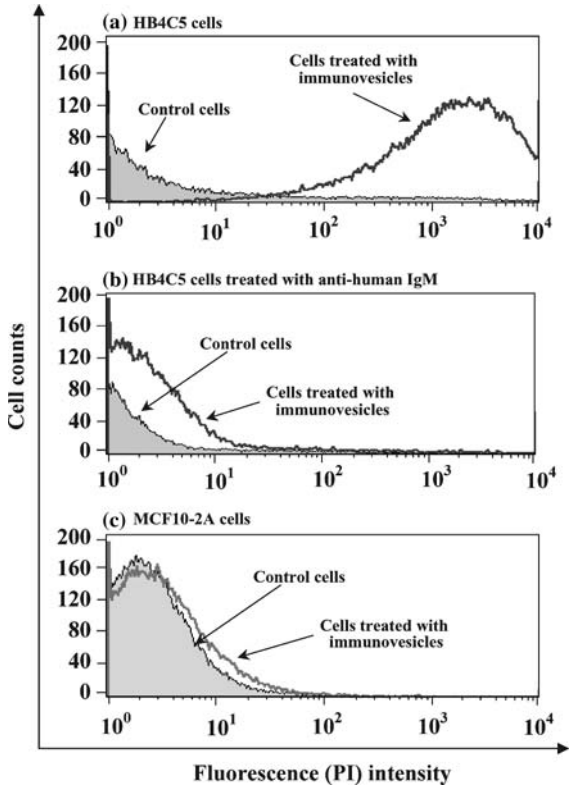

Figure 6. Specific binding of Span80-CPL immunovesicles to HB4C5 cells. Span80-CPL immunovesicles immobilized anti-human IgM antibody and encapsulating DNA–PI complex were prepared. HB4C5 cells and MCF10-2A cells were cultured in 5% FBS-ERDF medium at 37 °C under humidified 5% CO_2–95% air. The immunovesicles were added to the medium at 5% and cultured for 3 h. Following cultivation, these cells were washed with iced PBS 3-times, and flowcytometric analysis was carried out. (a) HB4C5 cells; (b) HB4C5 cells treated with anti-human IgM prior to treatment of immunovesicles; (c) MCF10-2A cells which have no antigenic substance against anti-human IgM antibody.

cells possessing no antigens (human IgM) on cell surface (Figure 6c). These findings suggest that the association of Span80-CPL immunovesicles with HB4C5 cells is mediated by antigen specific binding of antibodies immobilized on vesicle surface.

In summary, we have designed immunovesicles as a novel target-specific drug carrier. Uptake of vesicles by cells is considered as endocytosis. Therefore, it is very important for endocytic internalization to achieve high concentration of vesicles on the surface of target cells. The advantage of our strategy is application of protein A as an anchor of IgG specific for the antigen on target cell surface. By using of protein A, target specific IgG was efficiently and securely immobilized on the vesicle surface. As demonstrated above, we successfully developed immunovesicles composed of Span80 and CPL. Our data suggested that the Span80-CPL immunovesicles are expected to be able to achieve a high local concentration of an encapsulated drug at the target. Moreover, the immunovesicles prepared here are pliable and moderate against immunoliposomes composed of natural phospholipids.

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