Immunoliposomes bearing polyethyleneglycol-coupled Fab′ fragment show prolonged circulation time and high extravasation into targeted solid tumors in vivo

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Abstract We have developed a new type of long-circulating immunoliposome (Fab′–PEG immunoliposomes) which is efficiently extravasated into the targeted solid tumor in vivo. Small unilamellar liposomes (100–130 nm in diameter) were prepared from distearoylphosphatidylcholine (DSPC), cholesterol (CHOL) and a dipalmitoylphosphatidylethanolamine derivative of PEG with a terminal maleimidyl group (DPPE-PEG-Mal), and conjugated Fab′ fragment of antibody. Inclusion of DPPE-PEG-Mal and linkage of the Fab′ fragment instead of intact antibody to PEG terminals allowed the liposomes to evade RES uptake and remain in the circulation for a long time, resulting in enhanced accumulation of the liposomes in the solid tumor. Because of the ability of such Fab′–PEG immunoliposomes to target solid tumors, they appear highly attractive as carriers of not only chemotherapeutic agents, but also of macromolecular drugs.

Key words: Liposome; Immunoliposome; Fab′ fragment; Polyethyleneglycol; Drug delivery system

1. Introduction

Drug delivery to a specific site by immunoliposomes represents a potentially attractive mode of therapy. However, although immunoliposomes bind effectively and specifically to target cells in vitro, their targeting efficiency in vivo is relatively low. Studies in vivo have revealed that coating liposomes with antibody leads to enhanced uptake of the immunoliposomes by the reticuloendothelial system (RES) [1,2] and the immunotargeting efficiency depends on the antibody density on the surface [3]. Thus, highly efficient targeting and a relatively low level of RES uptake of the immunoliposomes are apparently mutually exclusive. Recently, RES-evading, long-circulating liposomes have been prepared by coating the liposome surface with amphipathic polyethyleneglycols (PEG) [4–7].

We have reported a new type of PEG immunoliposome which can effectively bind to target cells in vivo [8]. This was achieved by the use of newly synthesized distearoyl-*N*-(3-carboxypropionoyl)polyethyleneglycol phosphatidylethanolamine (DSPE-PEG-COOH) to couple antibodies directly to the distal terminal of PEG chains. The conjugation of a monoclonal IgG antibody, 34A, which is highly specific to pulmonary endothelial cells, to the PEG terminal enabled excellent target binding and retention of the immunoliposomes. However, it should be noted that the target sites for 34A–PEG immunoliposomes are readily accessible, i.e. on the vascular endothelial surface. For a much less accessible target, such as tumor cell surface antigens in a solid tumor, the targeting efficiency of immunoliposomes is likely to be low.

Nevertheless, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal organs [9]; we and others have demonstrated that long-circulating liposomes of small size ($\approx$100 nm mean diameter) and rigid lipid composition are significantly accumulated in solid tumors [10–12]. Further, the employment of antibody Fab′ fragments instead of whole IgG, would reduce RES uptake via the Fc receptor-mediated mechanism. Since the thiol residues of Fab′ fragments permit conjugation with retention of the antigen-binding activity of the intact antibody, it has been proposed that small PEG immunoliposomes bearing antibody Fab′ fragments at the PEG terminals might exhibit prolonged circulation and pass fairly readily through the leaky tumor endothelium by passive convective transport.

In order to test this hypothesis, we have synthesized a dipalmitoyl phosphatidylethanolamine derivative of PEG with a terminal maleimidyl group for the preparation of Fab′–PEG immunoliposomes (Fig. 1). The monoclonal antibody 21B2 (IgG1), specific for the human carcinoembryonic antigen (CEA), was used in this study [13]. The targeting ability of Fab′–PEG immunoliposomes was examined by using CEA-positive human gastric cancer strain MKN-45 cells inoculated into BALB/c *nu/nu* mice.

2. Materials and methods

2.1. Materials

DSPC and DPPE were kindly donated by Nippon Oil and Fats (Tokyo, Japan). CHOL was purchased from Wako Pure Chemicals (Osaka, Japan). DPPE-PEG-COOH was synthesized as described [8]. The average molecular weight of PEG was 3000.

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Abbreviations: 21B2, monoclonal antibody IgG1 antibody, specific for the human carcinoembryonic antigen; CEA, human carcinoembryonic antigen; CHOL, cholesterol; DPPE, dipalmitoylphosphatidylethanolamine; DPPE-PEG, dipalmitoyl-*N*-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine; DPPE-PEG-COOH, dipalmitoyl-*N*-(3-carboxypropionoyl polyethyleneglycol succinyl)phosphatidylethanolamine; DPPE-PEG-Mal, dipalmitoylphosphatidylethanolamine derivative of PEG with a terminal maleimidyl group; DSPC, distearoylphosphatidylcholine; MKN-45, CEA-positive human gastric cancer cells; RES, reticuloendothelial system

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2.2. Preparation of DPPE-PEG-Mal

The dipalmitoylphosphatidylethanolamine derivative of PEG with a terminal maleimidyl group (DPPE-PEG-Mal, Fig. 1) was synthesized according to [14] with minor modifications. Briefly, an aliquot of polyoxyethylene bis(amine) with an average molecular weight of 3350 was dissolved in chloroform, and *N*-(ε-maleimidocaproyloxy)-succinimide (Dojin Lab. Kumamoto) was added thereto. The reaction was allowed to continue until the ninhydrin reaction became negative. The resulting dimaleimide PEG was purified on a silica gel column with a chloroform–methanol gradient. Two molar equivalents of dimaleimide PEG was reacted with a thiol-modified DPPE, which was synthesized by adding 20 mg of iminothiolane (Sigma) and 25 μl of triethylamine to DPPE (84 mg) dissolved in a mixture of chloroform–methanol (1:1, v/v), under nitrogen gas (conversion of the amino group to a thiol group was confirmed by the negative ninhydrin reactivity and the acquired reactivity with fluorescein maleimide (Funakoshi, Tokyo)). The reaction mixture was evaporated, dissolved in chloroform and passed through a silica gel column as above. The fraction containing DPPE-PEG-Mal was dried in vacuo and dispersed in a small amount of water to form micelles. The DPPE-PEG-Mal solution was ultrafiltered with an Amicon membrane (XM-50) to remove non-micelle-forming impurities and the product was lyophilized. The yield of DPPE-PEG-Mal was approximately 20%. The formation of DPPE-PEG-Mal was confirmed by NMR.

2.3. Preparation of anti-human CEA monoclonal antibodies and their Fab′ fragments

The monoclonal antibody 21B2 (IgG1), specific for the human carcinoembryonic antigen (CEA), was prepared as described [13]. 21B2 was isolated from BALB/c mice after immunization with human CEA antigen purified from a CEA-producing human gastric cancer strain MKN-45 cells. Fab′ fragments of 21B2 were prepared by pepsin digestion of the antibody and 2-aminoethanethiol reduction of the $F(ab')_2$ fragments, as described previously [15]. Antibodies and fragments were radiolabeled with ^{125}I, using the IODO-GEN method, to a specific activity of $2–5\times10^5$ cpm/μg. The antigen-binding activity of the Fab′ fragment was confirmed by cell binding assay with MKN-45 cells in vitro.

2.4. Preparation of immunoliposomes

PEG–liposomes were composed of DSPC/CHOL/DPPE-PEG-Mal (2:1:0.2, molar ratio) with [^{3}H]cholesteryl hexadecylether (NEN, Boston, MA) as an inert membrane marker [2]. Small unilamellar liposomes (SUV) were prepared by reverse-phase evaporation [16] followed by extrusion (Lipex Biomembranes, Canada) through two stacked polycarbonate membrane filters (Nuclepore Co., CA) of 0.1 μm diameter. PBS (pH 6.5) was used as the liposomal aqueous phase. The extruded liposomes were centrifuged at $200\,000\times g$ for 15 min at 4°C (Hitachi CS120, S100AT5 rotor), and then resuspended in PBS (pH 6.5). To 1 ml of PEG–liposomes (2 mg lipids (DSPC+CHOL)), the Fab′ fragments (0.3–1 mg) with a trace amount of ^{125}I-labelled Fab′ were added, and the mixture was incubated for 2 h at room temperature. The immunoliposomes were separated from unbound protein on a BioGel A15m column preequilibrated with saline. As control immunoliposomes, IgG–PEG immunoliposomes were prepared by conjugation of the intact antibody to liposomes composed of DSPC, Chol, and DPPE-PEG-COOH (2:1:0.2, molar ratio) as described previously [8]. Liposome size was measured by a Nicomp 370 HPL submicron particle analyzer (HIAC Pacific Scientific, CA). All liposomes were prepared to be of similar, homogeneous, small size (100–130 nm in diameter). The lipid concentration and the coupling efficiency of Fab′ fragment were estimated by phosphorus assay [17] and from the radioactivity of ^{125}I, respectively. The average number of antibody molecules per liposome was calculated as described previously [8]. The coupling efficiency of the whole antibody of 21B2 to PEG-COOH liposomes was approximately 20–30%. In contrast, that of Fab′ fragment to PEG-Mal liposomes was approximately 60–70%.

2.5. In vitro and in vivo studies

The human CEA-positive gastric cancer cell line, MKN-45, was maintained in culture flasks in complete E-RDF medium (Kyokuto Co., Tokyo) supplemented with 10% FCS (Filtron Pty Ltd., Australia). In vitro cell binding was studied using [^{3}H]cholesteryl hexadecylether-labeled PEG immunoliposomes. MKN-45 cells, grown as monolayers in 35 mm culture dishes, were incubated with liposomes at varying lipid concentrations for 90 min at 4°C. After the incubation, cells were washed, trypsinized, and solubilized with Soluene 350 (Packard Instruments, IL) then the radioactivity was counted in Hionic-Fluor scintillation mixture in an Aloka LSC-3000 counter (Aloka Co., Tokyo).

The tissue distribution of PEG immunoliposomes was examined by using MKN-45-bearing BALB/c *nu/nu* mice. Two million cells in 100 μl were inoculated into the back of female BALB/c *nu/nu* mice (Nihon Clea Co., Tokyo). When the estimated tumor weight (calculated as $1/2\times\text{length}\times\text{width}^2$) [18] reached about 300 mg, the mice were used for experiments. [^{3}H]cholesteryl hexadecylether-labeled PEG immunoliposomes were injected into MKN-45-bearing BALB/c *nu/nu* mice (3–5/group) via the tail vein. At selected time intervals after administration, mice were lightly anesthetized, bled

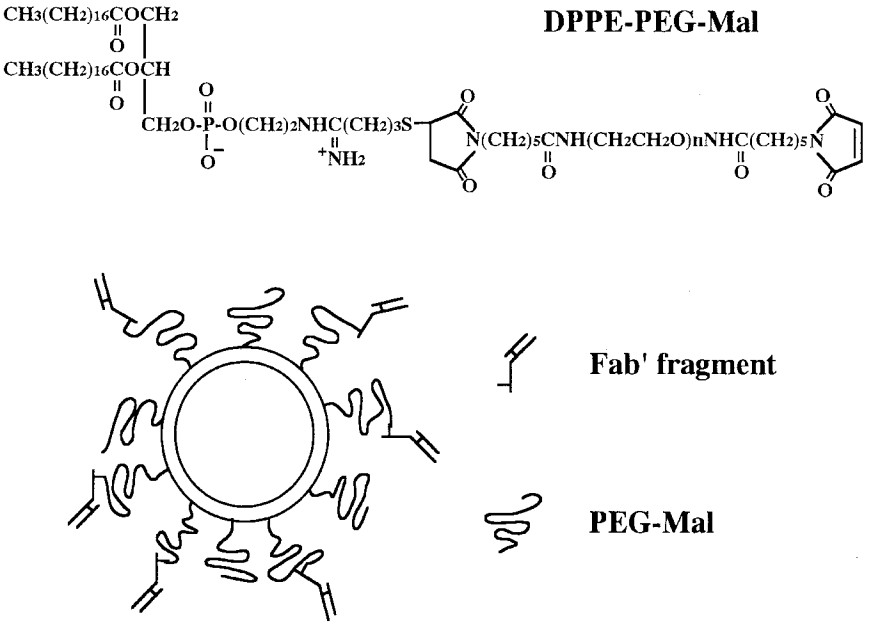

Fig. 1. Structure of DPPE-PEG-Mal and schematic illustration of Fab′–PEG immunoliposome.

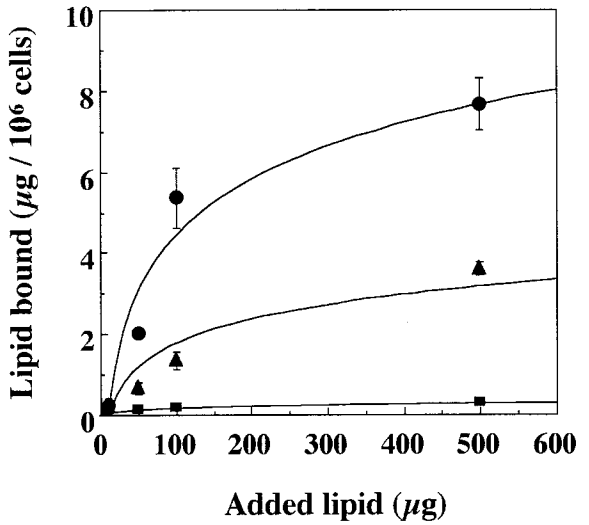

Fig. 2. In vitro cell binding of PEG immunoliposomes. Binding of PEG-Mal liposomes (■), IgG-PEG immunoliposomes ([70], ▲) or Fab′–PEG immunoliposomes ([517], ●) was plotted as a function of added lipid. Liposomes were incubated at varying lipid concentrations with MKN-45 cells for 90 min at 4°C. The number in square brackets represents the average number of antibody or Fab′ molecules per liposome.

by eye puncture, killed by cervical dislocation and dissected. The radioactivity of each internal organ was determined as described above.

The stability of IgG–PEG-COOH conjugation or Fab′–PEG-Mal conjugation in immunoliposomes was checked in 90% mouse serum at 37°C. ^{125}I-labeled antibodies and [^{3}H]cholesteryl hexadecylether-labeled immunoliposomes were used. Liposomal samples from the incubation mixture were separated by the a BioGel A15m column and the cleavage of conjugation was determined from the ratio of radioactivity.

3. Results and discussion

As specific association of immunoliposomes with their target cells is a necessary requirement for target-specific drug delivery, we first tested whether these PEG immunoliposomes specifically bound to the target cells in vitro. As shown in Fig. 2, PEG immunoliposomes conjugated with either whole antibody (IgG) or Fab′ fragment readily bound with MKN-45 cells. The degree of target cell binding of immunoliposomes was superior to that of nontargeted PEG-Mal liposomes. The differences in cell binding between IgG–PEG and Fab′–PEG immunoliposomes were due to the variation of coupled number of antibody or Fab′ fragment. These results revealed that free PEG (not linked to antibody) in liposomes does not interfere sterically with antigen-binding of the antibody or Fab′ fragment.

The cleavage of IgG–PEG-COOH conjugation or Fab′–PEG-Mal conjugation in mouse serum was not observed over 12 h in incubation with mouse serum at 37°C (data not shown). Both conjugation may be stable in vivo and antibodies and fragments remain with the liposome after i.v. injection. We investigated whether the use of Fab′ fragment instead of whole antibody (IgG) would modify the pattern of tissue distribution of immunoliposomes. As shown in Fig. 3, PEG-Mal liposomes without antibody showed prolonged residence in the circulation and low liver uptake, regardless of the presence of the terminal maleimidyl group. There were no marked differences in tissue distribution among liposomes containing DPPE-PEG, DPPE-PEG-COOH and DPPE-PEG-Mal, taking into account our previous results [4,7,8]. DPPE-PEG has an -OCH_3 residue at the terminal and is used for the preparation of conventional PEG–liposomes. IgG–PEG immunoliposomes, bearing approximately 51 whole antibody molecules per liposome, were rapidly cleared from the blood and were found entirely in the liver. In contrast, Fab′–PEG immunoliposomes, bearing approximately 517 Fab′ molecules per liposome, were retained longer in the circulation with a concomitant decrease in the liver uptake compared with IgG–PEG immunoliposomes. These results indicate that the linkage of whole IgG to the PEG terminal enhances RES uptake via the Fc receptor-mediated mechanism [1,2]. In the case of Fab′–PEG immunoliposomes, the absence of the Fc portion and the presence of free PEG-Mal (not linked to Fab′ fragment) may both play a role in the prolonged circulation of the liposomes. As we pointed out previously [8], Fab′ fragment is much better than whole IgG in terms of designing PEG immunoliposomes with a prolonged circulation time. Further, the usage of Fab′ fragment should greatly reduce the antigenicity.

Fig. 4 shows the accumulation of liposomes, with an average diameter of 100–130 nm, the solid tumor at 24 h after injection. Relatively high accumulation into the tumor tissue was obtained with PEG-COOH liposomes, PEG-Mal liposomes and Fab′–PEG immunoliposomes. These results were clearly correlated to the prolonged circulation time (Fig. 3). The accumulation of Fab′–PEG immunoliposomes was 2-fold

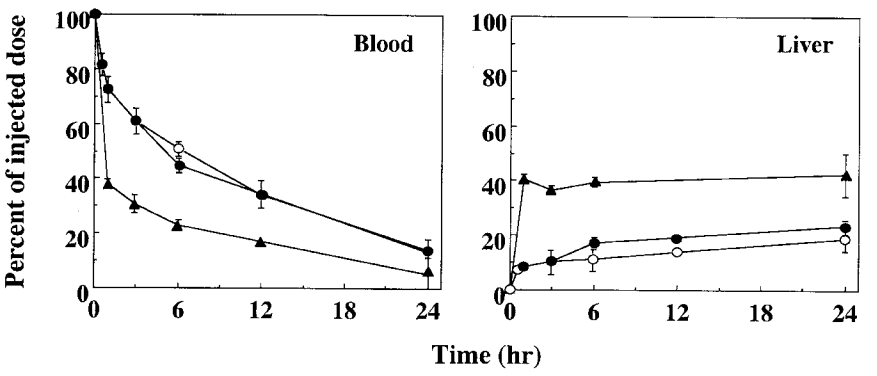

Fig. 3. Time course of blood residence and liver uptake of PEG-Mal liposomes (○), IgG–PEG immunoliposomes ([51], ▲) or Fab′–PEG immunoliposomes ([517], ●).

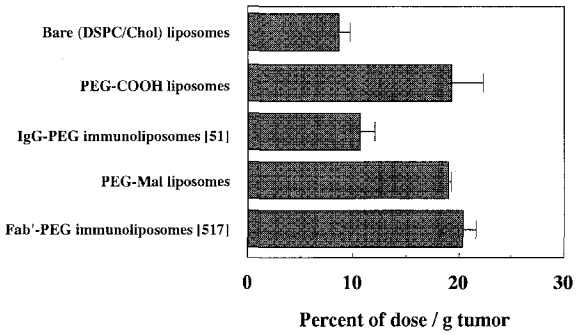

Fig. 4. Accumulation of immunoliposomes in solid tumor in MKN-45-bearing BALB/c *nu/nu* mice at 24 h after injection. The number in square brackets represents the average number of antibody or Fab′ molecules per liposome.

higher than that of IgG–PEG immunoliposomes or bare liposomes, and equal to that of PEG-Mal or PEG-COOH liposomes. The permeability of tumor vasculature is generally increased as compared to normal tissue [9,19], so Fab′–PEG immunoliposomes could extravasate through the leaky endothelium by passive convective transport. It is known, however, that the size limitation of such liposomes is of practical significance. Under physiological tumor condition, only liposomes with small size and prolonged circulation time have a sufficient chance to encounter the leaky vessel of the tumor. The higher concentration and longer blood residence time of Fab′–PEG immunoliposomes may have improved the efficiency of extravasation. IgG–PEG immunoliposomes showed a short circulation time due to a high liver uptake as shown in Fig. 3, so that they have not sufficient concentration and residence time for extravasation through the leaky endothelium. Though there were no differences in accumulation into solid tumor between Fab′–PEG immunoliposomes and PEG liposomes without antibodies, only Fab′–PEG immunoliposomes stand a better chance of binding to MKN-45 cells. This is considerable for the extravasated Fab′–PEG immunoliposomes, because Fab′–PEG immunoliposomes can bind readily with MKN-45 cells in vitro binding assay shown in Fig. 2. This is particularly important for many endocytotic internalization of bioactive materials. Its use in receptor-mediated drug targeting should be investigated.

In summary, we have designed a new type of long-circulating immunoliposome which can be extravasated to the targeted solid tumor in vivo. The formulation retains specific antigen-binding ability to target cells after extravasation. Such liposomes should be able to provide a high local concentration of an encapsulated drug at the target site. Uptake of immunoliposomes by target cells is considered to occur via receptor-mediated endocytosis. A future possibility is to conjugate other ligands to the free PEG terminals of Fab′–PEG immunoliposomes, in order to direct the liposomes to surface receptors known to be internalized at a high rate by cells. Such liposomal formulations should be useful for endocytotic internalization of plasmid DNA.

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