Drug Delivery By Organ-Specific Immunoliposomes

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List of Abbreviations

Chol:	cholesterol
dpFUDR:	3',5'-O-dipalmitoyl-2'-deoxy-2'-fluoro-uridine
GM ₁ :	ganglioside GM ₁
MST:	mean survival time
NGPE:	N-glutaryl phosphatidylethanolamine
PBS:	phosphate buffered saline (117 mM NaCl, 2.7 mM KCl, 1.5 mM KH ₂ PO ₄ , 0.1 mM Na ₂ HPO ₄ , pH 7.4).
PC:	egg phosphatidylcholine
PS:	phosphatidylserine
RES:	reticuloendothelial system
¹¹¹ In-DTPA-SA:	¹¹¹ Indium diethylenetriamin pentaacetic acid distearylamine complex

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Abstract

Monoclonal antibodies highly specific to the mouse pulmonary endothelial cells were conjugated to liposomes. The resulting immunoliposomes showed high levels of lung accumulation when injected intravenously into mice. Optimal target binding and retention were achieved if the lipid composition included ganglioside GM₁ to reduce the uptake of immunoliposomes by the reticuloendothelial system. Details of the construction and optimization of these organ-specific immunoliposomes are reviewed. The drug delivery potential of this novel liposome system was demonstrated in an experimental pulmonary metastasis model. Immunoliposomes containing a lipophilic prodrug of deoxyfluorouridine effectively prolonged the survival time of the tumor-bearing mice. This and other therapeutic applications of the immunoliposomes are discussed.

Introduction

Liposomes are versatile and efficient carriers for both hydrophilic and hydrophobic drugs. They are biocompatible, biodegradable and usually non-immunogenic. Drugs encapsulated in liposomes are protected from the action of the external enzymes and other inactivation factors. However, liposomes injected systemically localize predominantly in the mononuclear phagocytes of the reticuloendothelial system (RES) primarily in the liver and secondarily in the spleen (reviewed in 1). This natural homing activity to the RES, or so-called "passive targeting", has been exploited to deliver drugs to these cells. For example, antiparasitics encapsulated in liposomes effectively kill the microorganisms residing in the macrophages (2,3). Liposomes containing the macrophage-activating factors are also useful for both antitumor and antiviral activities (4).

Delivery of liposomes to cells, tissues or organs other than the RES is referred to as the "active targeting". It is necessary to conjugate a suitable targeting ligand, such as a monoclonal antibody, to the liposome surface for the target specificity. There are many established methods of preparing the antibody-coated liposomes, i.e. immunoliposomes, and the target-binding specificity of the immunoliposomes has been demonstrated in many cell-free, or tissue culture systems (reviewed in 5). However, efficient target binding and retention of the immunoliposomes in

an animal model, particularly those bearing tumors at a specific site, have not been demonstrated until recently (6,7,8). Two different problems have been identified: RES uptake of the immunoliposomes and failure of immunoliposomes to cross the anatomical barriers (9). The former problem has been solved by the recent development of liposomes with a reduced RES affinity. These liposomes can escape the surveillance of the macrophage and have been nicknamed "stealth liposomes" (13). In this report, we will describe the importance of the stealth lipid composition for the optimal target binding and retention of immunoliposomes (14). The latter problem stems from the fact that liposomes or immunoliposomes are too large to penetrate the endothelial barrier of the vessel wall. Unfortunately many tumor cells, either in a primary or metastatic tumor, are not readily accessible by liposomes. Thus, a direct targeting of immunoliposomes to the tumor cells is probably difficult. As an alternative approach, we have decided to design an organ-specific drug delivery system which allows an efficient binding and retention of immunoliposomes to the vascular endothelial cells of that organ. Drugs released from the bound immunoliposomes need only diffuse a short distance to reach the tumor cells in that organ. This concept is described schematically in Fig. 1.

The mouse pulmonary metastatic tumor lends itself as an ideal model to test the new concept. Experimental metastasis can be easily established by i.v. injection of free tumor cells which usually form numerous metastatic tumor nodules in the lung. It has

been reported that these tumors are either poorly vascularized or not at all (15). One of us (S.J.K.) has isolated two rat monoclonal IgG antibodies which specifically bind with a glycoprotein antigen, gp112, located on the luminal surface of the capillary endothelial cells in the mouse lung (16). gp112 is highly organ-specific; its concentration in the lung is at least 34-fold greater than any other organs in the mouse (15). We have used this model system to test the drug delivery potential of the organ-specific immunoliposomes.

Materials and Methods

Materials

Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS) were obtained from Avanti Polar lipids Inc. (Birmingham, AL) and cholesterol was from Sigma (St. Louis, MO). Ganglioside GM₁, bovine, was obtained from Calbiochem (San Diego, CA). DTPA-SA was synthesized according to (17) and ¹¹¹In-DTPA-SA was prepared as described (7). This lipophilic radiolabel is not transferred to the serum components from liposomes (unpublished data), nor is it rapidly metabolized *in vivo* (7). The synthesis of N-(glutaryl)phosphatidylethanolamine (NGPE) has been described (18). Dipalmitoyl deoxyfluorouridine (dpFUdR) was a gift from Prof. Gerrit Scherphof.

Antibody

Rat monoclonal antibody 34A was purified from nu/nu mouse ascites fluid as described (19). 34A was radiolabeled with ¹²⁵I using IODO-GEN (Pierce, Rockford, IL) method to a specific activity of 2 to 4 x 10⁵ cpm/μg, and conjugated with NGPE as previously described (7).

Preparation of immunoliposomes

34A-immunoliposomes were prepared by a detergent dialysis method as previously described (7). Lipid mixture (with or without dpFUdR) containing ¹¹¹In-DTPA-SA (1%) were dried with N₂ gas. The dried lipid was solubilized with 100 mM octylglucoside in PBS (lipid/octylglucoside = 1:5, m/m). The resultant solution was

mixed vigorously with 34A conjugated with NGPE, and then the mixture was dialyzed against PBS for 16-20 h at 4°C. The immunoliposomes were extruded 4 times through a stack of two Nuclepore membranes (pore size 0.4 μ m). The average size of liposomes was measured using a Coulter N4SD sub-micron particle size analyzer (Hialeah, FL). The immunoliposomes were separated from the unbound antibody (50-70% of the original amount) on a Bio-Gel A1.5M (Bio-Rad) column. The peak liposome fractions were pooled and diluted to 1 mg lipid/ml PBS (pH 7.4). The amount of 34A incorporation into liposomes and the final ratio of antibody to lipid in the 34A-immunoliposomes were calculated from the radioactivities of ^{111}In for lipids and ^{125}I for the antibody.

Biodistribution studies

Liposomes (200 μ g lipid) were injected into Balb/c mice (6-8 weeks old) via the tail vein and the distribution of the liposomes in major organs and blood were examined as a function of time. At the desired time, mice were anesthetized lightly and bled by eye puncture. Blood was collected and weighed. The mice were sacrificed by cervical dislocation and dissected. Organs were collected, weighed, and analyzed for ^{111}In radioactivity in a gamma counter. The results were presented as percent of the total injected dose for each organ. The total radioactivity in the blood was determined by assuming that the total volume of blood was 7.3% of the body weight (20).

Tumor cells

EMT6 cells were grown as a monolayer culture in DMEM medium

containing 20% fetal calf serum (21). Cells were detached from the plate by trypsin-EDTA treatment and washed in PBS. 5×10^3 cells were injected per mouse via the tail vein of Balb/c mice (6-8 weeks old) to induce experimental lung metastatic tumors. Immunoliposomes were injected i.v. 2 and 4 days after the tumor cell injection. The survival of mice was followed over the next 60 days.

Results and Discussion

Optimization of immunoliposome binding to the lung

In order to reduce the uptake of immunoliposomes by the liver and spleen, we have tested the effect of incorporating GM₁ into the liposome membrane on the lung binding of immunoliposomes. We (14,22) and others (10,11,12) have demonstrated that the presence of this monosialoganglioside in the liposomes effectively reduces the RES uptake and prolongs the circulation time of liposomes. Since GM₁ contains one negative charge per molecule, we have included the negatively charged PS in the control liposomes. Also shown in Fig. 2 is the effect of the antibody 34A to lipid ratio of immunoliposomes on the target binding. 34A-liposomes of a high antibody density bound rapidly to the lung; it reached the steady-state level within 5 min of injection. 34A-liposomes of lower antibody densities bound less rapidly and showed a lower steady-state level. Liposomes containing no 34A did not bind to the lung at all. It is also clear from the data in Fig. 2 that 34A-liposomes containing GM₁, bound more effectively to the lung target than the ones containing PS. In a separate experiment, it was shown that the liver uptake rate of the 34A-liposomes containing GM₁ was approximately 10-fold lower than the ones containing PS (14). Thus, immunoliposomes with a reduced RES affinity bind to the target more effectively than the ordinary immunoliposomes.

These data indicate two kinetically competing processes: binding to the lung target and uptake by the RES. The steady-

state level of the lung accumulation of immunoliposomes is determined by the relative rates of the two processes. Increasing the antibody density of immunoliposomes favors an enhanced rate of target binding. Inclusion of GM_1 in the liposome membrane effectively reduces the rate of RES uptake of immunoliposomes. When the two factors are optimized at the same time, we can routinely obtain 70-75% of the injected dose accumulating in the lung within 15 min of injection.

Target retention of immunoliposomes

Successful therapy mediated by the organ-specific immunoliposomes requires a relatively long residence time of the immunoliposome in the target organ. Fig. 3 shows that 34A-liposomes containing GM_1 stayed in the lung longer than the ones containing PS. Since PS-containing liposomes show a high affinity to macrophages, this result suggests the removal of immunoliposomes from the lung involves the action of macrophages. Alternatively, released immunoliposomes could reenter the circulation and rebind to the target if they are not taken up by the RES. GM_1 -containing 34A-liposomes would show a greater chance of rebinding than the ones containing PS due to the difference in the RES affinity. Also shown in Fig. 3 is that 34A-liposomes more enriched with antibody were retained longer by the target than the ones less enriched. This is understandable because immunoliposomes containing higher numbers of antibody are more multivalent than the ones containing less antibody. Multivalent binders dissociate very slowly from the

receptors (23). These data have taught us that we shall use for the tumor therapy immunoliposomes of high antibody density which also contain GM₁ in the liposome membrane, the so-called "stealth immunoliposomes".

An Experimental pulmonary metastasis model

EMT6 cells were originally derived from a spontaneous breast tumor of a Balb/c female mouse (21). It grows rapidly in tissue culture and can be transplanted subcutaneously to form solid tumor in mice. When 5×10^3 single cells were injected via the tail vein, the mice developed massive tumor nodules in the lung and usually die after 20-22 days. No growth of the tumor was seen in any other organs. The tumor-bearing mice showed a high level of 34A-liposome accumulation in the lung, indistinguishable from the normal mice (data not shown). We have used this experimental model to test the ability of 34A-liposomes to deliver cytotoxic drugs for tumor therapy.

Choice of the drug

Organ-specific immunoliposomes do not directly deliver the encapsulated drug to the tumor cells. Instead, the drug has to diffuse from the bound liposomes to the nearby tumor cells. We have chosen a hydrophobic drug, i.e. 3',5'-O-dipalmitoyl-2'-deoxyfluorouridine (dpFUDR), because a hydrophobic drug is likely to diffuse more efficiently across the cellular barriers. Furthermore, nearly all of the added dpFUDR incorporated into the 34A-liposome membranes. Its presence in the liposome membrane did

not interfere with the incorporation of the 34A antibody into the same liposomes. dpFUdR is rapidly metabolized to FUdR which is cytostatic by inhibiting the cellular DNA synthesis (Fig. 4). dpFUdR is therefore a lipophilic prodrug of FUdR (24). EMT6 cells in culture could be killed by liposomal dpFUdR with a LD₅₀ of 7 ng/ml, indicating that it is a potent cytostatic drug for this tumor system. Waalkes and Scherphof have shown that dpFUdR formulated in antibody-free liposome is extraordinarily toxic to mice, due to the high concentrations of the drug delivered to the liver and the slow elimination of the drug by the animal (25). This drug is, however, suitable for the stealth 34A-liposome system, because the immunoliposomes are supposed to direct the drug away from the major organ which converts the prodrug into the drug, i.e. the liver.

Tumor therapy by organ-specific immunoliposomes

Immunoliposomes containing PC:chol:GM₁:dpFUdR = 10:5:1:0.5 and 34A antibody (protein/lipid ratio = 1:17, w/w) were prepared by a detergent-dialysis method (see Materials and Methods). Immunoliposomes containing dpFUdR were i.v. injected at day 3 and day 5 at 2 mg drug/kg body weight. The survival time of the treated mice was measured over a 60-day period. Fig. 5 shows the therapeutic effect of the immunoliposomes. It is clear that mice received the 34A-liposomes containing drug showed a significantly prolonged mean survival time (MST) of 33 days. Mice treated with free drug formulated in a vegetable oil emulsion, or with the drug-

loaded liposomes without the antibody, or with 34A-liposomes containing drug but no GM₁, did not show any therapeutic effect as their MST's were indistinguishable from those treated with PBS, i.e. approximately 20-22 days. These data indicate that the organ-specific immunoliposomes are effective for the target-specific therapy of a metastatic tumor. These results, although preliminary, are very encouraging.

Other potential advantages of organ-specific immunoliposomes

Since the immunoliposomes are designed to target to the normal, healthy cells, and are not tumor cell-specific, this drug delivery system should be theoretically effective in treating any tumor cells grown in the lung, thus providing a general delivery system for the organ. The drug delivery activity of the immunoliposome should not be limited to the anticancer drugs; drugs efficacious in treating other pulmonary diseases can also be delivered. In fact, the same 34A-liposome system effectively delivers acyclovir, an antiviral drug, for the treatment of an experimental viral pneumonia model (unpublished data). These advantages will be explored further in the future experiments.

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Figure Legends

Fig. 1. Schematic description of organ-specific immunoliposomes.

Fig. 2. Lung uptake of GM₁-containing or PS-containing liposomes. Liposomes (200 µg lipid) were injected via tail vein. Bar is S.D. (n=3). Data taken from ref. 14 with permission.

■; 34A-liposomes (PC:chol:GM₁ = 10:5:1), Ab:lipid=1:11 (w/w), 297 nm in average diameter.

▲; 34A-liposomes (PC:chol:GM₁10:5:1) Ab:lipids=1:37 (w/w), 292 nm in average diameter.

◆; liposomes (PC:chol:GM₁ = 10:5:1), without antibody, 268 nm in average diameter.

□; 34A-liposomes (PC:chol:PS = 10:5:1), Ab:lipid = 1:8 (w/w), 253 nm in average diameter.

△; 34A-liposomes (PC:chol:PS = 10:5:1), Ab:lipid = 1:31 (w/w), 255 nm in average diameter.

◇; liposomes (PC:chol:PS = 10:5:1) without antibody, 235 nm in average diameter.

Fig. 3. Retention of immunoliposomes in lung. Immunoliposomes (200 µg lipid) labeled with ¹¹¹In-DTPA-SA were injected i.v. & initial accumulation in lung was calculated at indicated time intervals. Bar is S.D. (n=3). Data taken from ref. 14 with permission.

■; 34A-liposomes (PC:chol:GM₁=10:5:1), Ab:lipid = 1:11 w/w, 297 nm

in average diameter.

▲ ; 34A-liposomes (PC:chol:GM₁ = 10:5:1), Ab:lipid = 1:37 w/w, 292 nm in average diameter.

□ ; 34A-liposomes (PC:chol:PS = 10:5:1), Ab:lipid = 1:8 w/w, 253 nm in average diameter.

Δ ; 34A-liposomes (PC:chol:PS = 10:5:1), Ab:lipids = 1:31 w/w, 255 nm in average diameter.

Fig. 4. Structure and metabolism of dpFudR.

Fig. 5. Mean survival time of mice treated with dpFudR in different liposome formulations. PBS:phosphate buffered saline, free dpFudR:free drug formulated in a vegetable oil-tween emulsion, 34A-dpFudR-GM₁-LP:34A-liposomes containing PC:chol:GM₁:dpFudr (10:5:1:0.5), dpFudR-GM₁-LP:liposomes containing PC:chol:GM₁:dpFudR (10:5:1:0.5), 34A-dpFudR-LP:34A-liposomes containing PC:chol:dpFudR (10:5:0.5), dpFudR-LP:liposomes containing PC:chol:dpFudR (10:5:0.5).

3',5'-O-dipalm-FUdR

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