Liposomes

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1 Liposomal Drug Delivery Systems for Gene Delivery In Vivo

It was the German bacteriologist Paul Ehrlich who, in the late nineteenth century, coined the term "magic bullet," meaning a chemical that travels through the body and selectively kills diseased cells without harming neighboring healthy ones (Ehrlich 1956). Since then, many different approaches based on various physical and biochemical principles have been examined with the goal of developing systems with a therapeutically acceptable degree of target specificity (Poste and Kirsh 1983; Gupta 1990; Rowlinson-Busza and Epenetos 1992; Cummings and Smyth 1993).

Among the different approaches to drug delivery, immunoliposomes, using an antibody as a targeting ligand and a lipid vesicle, as carriers for both hydrophobic and hydrophilic drugs have attracted much attention. It has been demonstrated that the specific delivery of drugs to target cells is far more efficient with immunoliposomes than with liposomes lacking antibody (Wright and Huang 1989). The success of in vitro delivery to target cells using immunoliposomes (corresponding to type A in Fig. 1) has prompted similar experiments in vivo. However, targeting of immunoliposomes in vivo is far more complicated, as studies in vivo have revealed that bound antibodies lead to enhanced uptake of the immunoliposomes by the reticuloendothelial system (RES) (Aragnol and Leserman 1986; Derksen et al. 1988; Peeters et al. 1987), and that targeting efficiency depends on the antibody density on the immunoliposome surface (Maruyama et al. 1990). In addition, rapid uptake by the RES and endothelial barriers separating blood and tissues largely prevent immunoliposomes from reaching their target cells. Thus, highly efficient targeting and a relatively low level of RES uptake of the immunoliposomes are apparently mutually exclusive. As systemic administration is the most practical route for treatment, immunoliposomes overcoming these physiological barriers are highly desirable. The development of liposomes with RES-avoidance is a necessary first step in this direction.

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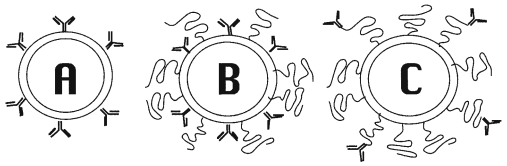

FIG. 1. Schematic illustration of the immobilization of antibody on liposomes. Type A: PEG-free immunoliposomes with antibody covalently linked to the short anchor *N*-glutaryl-phosphatidylethanolamine (NGPE); type B: PEG-immunoliposomes with antibody covalently linked to NGPE; type C: PEG-immunoliposomes with antibody attached to the distal terminal of distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl) phosphatidylethanolamine (DSPE-PEG-COOH), -maleimide or -hydorazide, so-called pendant-type PEG-immunoliposomes

A major development in the last few years has been the synthesis of liposomes with a prolonged circulation time in blood, commonly called long-circulating or sterically stabilized liposomes. Liposomes containing polyethylene glycol derivatives of phosphatidylethanolamine (PEG-lipid) (Klibanov et al. 1990; Blume and Cevc 1990; Allen et al. 1991; Maruyama et al. 1992) are not readily taken up by macrophages in the RES, and hence remain in the circulation for a relatively long period of time. Pharmacokinetic analysis and therapeutic studies in tumor-bearing mice, in which elevated liposome accumulation was shown, have demosntrated the potential of PEG-liposomes as drug carriers for use in cancer therapy (Lasic and Martin 1995; Gabizon and Papahadjopoulos 1988; Unezaki et al. 1995; Unezaki et al. 1994). Furthermore, results from preclinical studies with doxorubicin encapsulated into PEG-liposomes revealed an increased therapeutic efficacy compared to the free drug or drugs encapsulated in conventional liposomes. These new formulations of long-circulating liposomes should allow the development of immunoliposomes with both long survival times in the circulation and effective target recognition in vivo. PEG is very useful because of its ease of preparation, relatively low cost, controllable molecular weight and the ability to link it to lipids or protein (including antibody) by a variety of methods.

Given a suitable antibody with high specificity and affinity for the target antigen, the critical factor is the accessibility of target cells to the immunoliposomes. Efficient target binding of the injected immunoliposomes occurs only when the target cell is in the intravascular compartment or is accessible through leaky vascular structures. Thus, in terms of targeting drug delivery by immunoliposomes, two anatomical compartments can be considered. One is a readily accessible intravascular site, such as the vascular endothelial surface, leukemia cells, T cells, B cells or a thrombus. The other is a much less accessible extravascular site, such as a solid tumor, an infection site, or an inflammation site, where the vascular structure is leaky.

1.1 Targeting to a Readily Accessible Site

1.1.1 In Vivo Immunoliposome Targeting to the Lung Endothelial Surface

Immunoliposomes containing monoclonal antibodies specific for mouse pulmonary endothelium were used as a model system. The antibody 34A binds to a surface gly-

coprotein (gp112) expressed at high concentration in the mouse lung (Kennel et al. 1988). It has been demonstrated that mouse lung contains large amounts of gp112 (500 to 700 ng/mg protein), whereas other organs have very low (spleen, uterus) or undetectable (liver) levels. The anatomy and physiology of the lung favor its use as a model system for a number of reasons: all the blood circulates through the lung capillaries, the flow rate of blood in the lung is relatively slow for the purpose of oxygen and carbon dioxide exchange, and the largest capillary bed in the body is found in this organ. This convenient model has allowed us to investigate the effects on target binding of various physical parameters of immunoliposomes, such as the antibody-to-lipid ratio, liposome size, and injection dose.

Liposomes composed of egg phospahtidylcholine (PC), cholesterol (Chol) and *N*-glutaryl-phosphatidylethanolamine (NGPE) with a molar ratio of 5:5:1 were conjugated to the antibodies via NGPE (Maruyama et al. 1990). The resulting 34A-immunoliposomes (corresponding to type A in Fig. 1) were then injected into mice (Balb/c, 6–8 weeks old, male) via the tail vein.

Among the several parameters examined, the most important one seems to be the antibody-to-lipid ratio of the immunoliposomes. As shown in Table 1, immunoliposomes (Maruyama et al. 1990) with antibody 34A were prepared with various initial antibody-to-lipid ratios. The results clearly showed that there was a direct relationship between the antibody density of the immunoliposomes and the extent of their binding to the lung target. Approximately 60% of the injected dose was found in the lung within 15 min after injection when the immunoliposomes contained an average of 935 antibody molecules per liposome. Uptake by the liver and spleen followed an opposite trend from that of the lung, i.e. the accumulation of liposomes in the liver and spleen decreased as the antibody-to-lipid ratio increased, suggesting that uptake of immunoliposomes by the liver and spleen might limit the binding of immuno-

Table 1. Effect of antibody-to-lipid ratio of immunoliposomes on biodistribution. Immunoliposomes with different antibody-to-lipid ratio were prepared by employing different input ratios of antibody to lipids. The number of antibody molecules per liposome was calculated from the known conjugation efficiency for each preparation of immunoliposomes and the average diameter of the liposomes. The number of lipid molecules per liposome at a given diameter (~250 nm) was estimated according to the method of Enoch and Strittmatter (1979). Data represent the biodistribution of liposomes in the mice 15 min after liposome administration (i.v.). The percent of injected dose value for each ratio of antibody to lipid is the average ± S.D. of three mice. *BLP*, bare liposomes; *34A-LP*, 34A-immunoliposomes

Characteristic	BLP	34A-LP			
Initial antibody-to-lipid ratio (w/w)	—	1:50	1:10	1:5	1:1
Conjugation (%)	—	60	53	57	48
Size (nm)	250	224	236	234	247
No. antibody molecules/liposome	0	24	101	219	935
Organ	Percent injected dose				
Lung	0.4 ± 0.0	3.0 ± 0.3	20.0 ± 1.2	35.3 ± 2.4	59.8 ± 0.4
Blood	10.9 ± 0.7	7.9 ± 0.8	4.8 ± 0.2	2.9 ± 0.0	2.1 ± 0.1
Liver	49.7 ± 1.6	55.3 ± 1.7	41.7 ± 1.8	34.2 ± 2.5	12.1 ± 0.3
Kidney	1.2 ± 0.0	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.3 ± 0.1
Spleen	4.3 ± 0.6	3.7 ± 1.0	3.3 ± 0.2	1.8 ± 0.3	0.6 ± 0.1

liposomes to the lung target. Thus, antibody density is an important factor for target binding in the blood.

1.1.2 The Influence of Prolonged Circulation Time of Liposomes on the Efficiency of Immunoliposome Targeting

The addition of PEG-lipid to liposomes can significantly prolong their circulation time in the blood when the conjugate is incorporated into the liposome membrane (Blume and Cevc 1990; Allen et al. 1991; Maruyama et al. 1992; Lasic and Martin 1995). The role of PEG in 34A-immunoliposome binding to the lung target was examined in the above animal model system. A series of PEG-immunoliposomes were designed, as shown in Fig. 1 (Maruyama et al. 1995). Type A consisted of PEG-free immunoliposomes with the antibody covalently linked to a short anchor. In type B PEG-immunoliposomes, the antibody was covalently linked to the short anchor such that antibody molecules and PEG molecules coexisted on the liposome surface. Type C consisted of a new type of PEG-immunoliposome, in which the antibody or antibody fragments were attached to the PEG terminus, forming so-called pendant-type PEG-immunoliposomes. The location of antibodies on the surface of the liposome can be clearly identified in the backscatter electron microscopy image (Takizawa et al. 1998). For the preparation of type C immunoliposomes, several functionalized PE-PEG derivatives, such as those bearing a carboxyl residue, maleimide residue, or hidorazide residue, at the end of the PEG molecule, were newly synthesized. Ideally, the coupling method should be both simple and rapid, producing a stable, non-toxic bond.

Plain liposomes for the preparation of type A or type C immunoliposomes were composed of egg PC and Chol (2:1, m/m) with 6 mol% NGPE or DSPE-PEG-COOH (distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl) phosphatidylethanolamine), each with an average molecular weight of 3,000. The plain liposomes for type B were composed of egg PC and Chol (2:1, m/m) with 6 mol% NGPE and 6 mol% DSPE-PEG with an average molecular weight of 3,000. Small unilamellar vesicles (SUVs) (90–130 nm in diameter) were prepared by the Reversed-phase evaporation vesicle (REV) method followed by extrusion through Nuclepore filters (0.1 μm). The carboxyl residues in the plain liposomes were activated and coupled to 34A antibodies.

In order to test the effects of antibody position and steric hindrance by the PEG chains on the behavior of immunoliposomes in vivo, three different types of 34A-immunoliposomes with similar numbers of antibody molecules per liposome were prepared and their targeting ability and biodistribution were evaluated in mice. As summarized in Table 2, type A, B and C 34A-immunoliposomes, containing approximately 35, 30 and 30 antibody molecules per liposome, respectively, were prepared and injected into mice via the tail vein (Fig. 2). Using the 34A-type A liposomes, with an average of 35 antibody molecules per liposome, 42.5% of the injected dose accumulated in the lung. The 34A-type B immunoliposomes showed a lower level of target binding and a significantly higher blood level than those of type A. In the case of 34A-type C, containing 30 antibody molecules per vesicle, the degree of target binding to the lung was 56.6% of the injected dose, 1.5-fold higher than that of type A. The target binding of 34A-type B is comparable with that of 34A-G_{M1}-immunoliposomes. Although long-circulating liposomes can also be obtained by incorporating PE-PEG

TABLE 2. Characteristics of 34A-immunoliposomes. Plain liposomes used for preparing type A or type C consisted of egg phospahtidylcholine:cholesterol (PC:Chol) (2:1, m/m) with 6 mol% *N*-glutaryl-phosphatidylethanolamine (NGPE) or distearoyl-*N*-(3-carboxypropionoyl poly(ethylene glycol) succinyl) phosphatidylethanolamine (DSPE-PEG-COOH) of average molecular weight 2000, respectively. The plain liposomes for type B were composed of egg PC:Chol (2:1, m/m) with 6 mol% NGPE and DSPE-PEG of average molecular weight of 2000. The average number of antibody molecules per liposome was estimated according to the method of Enoch and Strittmatter (1979)

	34A-type		
	A	B	C
PEG content (mol% of total lipid)	0	6	6
Mean diameter (nm)	121	111	122
Initial antibody:lipid ratio (w/w)	1.6	1.6	1.5
Conjugation efficiency (%)	35.6	31.8	24.8
Number antibody molecules/liposome	35	30	30

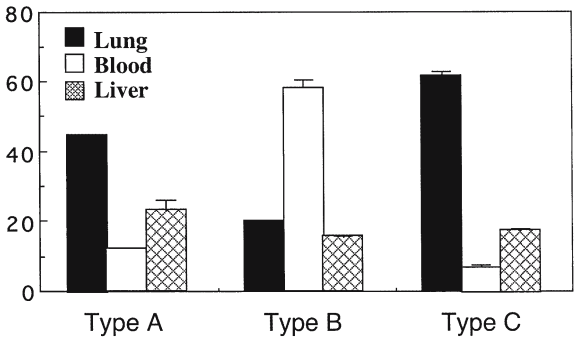

FIG. 2. Comparison of target binding to the lung and biodistribution (blood and liver) of the three different types of 34A-immunoliposomes. 34A-type A, B, and C conjugates contained approximately 35, 30, and 30 antibody molecules per liposome, respectively. Biodistribution (% of dose) was measured 30 min after intravenous injection. For details, see Table 2

derivatives in place of G_{M1}, inclusion of DSPE-PEG with an average molecular weight of 3000 reduced the target binding of 34A-type B. This effect depended on the chain length of PEG, suggesting that, although PEG prolongs the circulation time of immunoliposomes, it sterically hinders the binding of immunoliposomes to their target sites (Klibanov et al. 1991; Mori et al. 1991). Therefore, it was proposed that the use of longer-chain PEG with antibodies attached at the distal terminal of the PEG chain (type C) would afford immunoliposomes with both prolonged circulation time and good target binding (Klibanov et al. 1991; Klibanov and Huang 1992).

The efficiency of 34A-type C binding to the target as a function of the antibody content was evaluated A series of 34A-type C immunoliposomes was prepared with

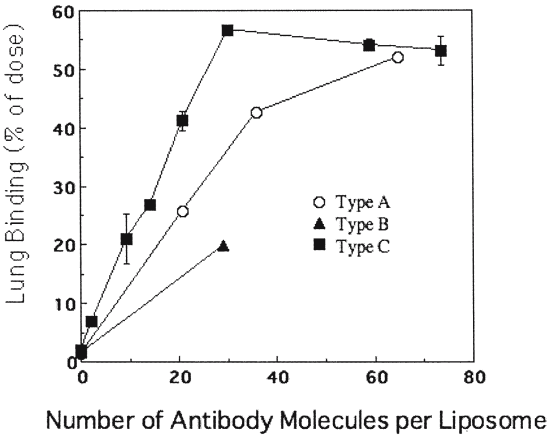

FIG. 3. Effect of antibody density on the lung targeting of 34A-type A, B, and C immunoliposomes. Lung binding (% of dose) was measured 30 min after injection

various initial antibody-to-lipid ratios. The final number of antibody molecules per liposome varied from 0 to 74, but the average sizes of the immunoliposomes were approximately the same, 90–130 nm in diameter. It is clear from the data in Fig. 3 that the efficiency of lung targeting was dependent on the antibody content of the immunoliposomes. Liposomes containing small numbers of antibody molecules per liposome accumulated in the lung at low levels and were retained in the blood at high levels. By contrast, linking an average of 74 antibody molecules per liposome on the PEG terminals resulted in the accumulation of 53% of the injected dose in the lung and only 7% in the blood. Lung binding reached a plateau at about 30 antibody molecules per liposome and a further increase in antibody content caused increased liver uptake. Thus, antibody density is an important factor for target binding even in the targeting of type C immunoliposomes. At low numbers of antibody molecules per liposome, such as 2 and 9 molecules, free PEG favors the evasion of RES uptake of the liposomes. This led to the high blood residence observed for 34A-type C immunoliposomes and a low efficiency of target binding due to the low antibody content. Type C liposomes showed higher immunotargetability than those of type A and type B at low antibody content (less than 30 antibody molecules per vesicle). Thus, type C is accumulated more effectively in the lung than the other immunoliposomes, in spite of the low antibody content.

1.1.3 Lymphoblastic Leukemia Cells

Patients with Philadelphia-chromosome-positive acute lymphoblastic leukemia (Ph^+ ALL) have a poor prognosis despite intensive therapeutic intervention. Recently, imatinib, a BCR-ABL tyrosine kinase inhibitor, has been proven to be an effective treatment for Ph^+ ALL, but nearly all patients rapidly acquire resistance. While high-dose imatinib administration might overcome this resistance, systemic toxicities would likely limit this approach. Therefore, a new delivery system allowing for the specific targeting of imatinib is needed. Since almost all Ph^+ ALL cells express CD19 on their surfaces, an immunoliposome carrying anti-CD19 antibody (CD19-type C) was developed (Harata et al. 2004). The internalization efficiency of CD19-type C approached

100% in all Ph^+ ALL cells but was very low in CD19-negative cells. The cytocidal effect of imatinib-encapsulated CD19-type C on Ph^+ ALL cell lines and primary leukemia cells from Ph^+ ALL patients was much greater than that of imatinib with or without control liposomes. Importantly, imatinib- CD19-type C did not affect colony formation by CD34-positive hematopoietic cells, even at inhibitory concentrations of free imatinib. Taken together, these data clearly demonstrates that the imatinib- CD19-type C induces the specific and efficient death of Ph^+ ALL cells. This new therapeutic approach may be an effective therapy for patients with Ph^+ ALL, with fewer side effects than free imatinib.

2 Targeting to a Less Accessible Site (Solid Tumor)

2.1 The Influence of the Prolonged Circulation Time of Liposomes on Their Extravasation into Solid Tumor Tissue

Generally, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal tissues outside the RES, which have continuous and nonfenestrated vascular endothelia, and the extravasation of macromolecules or liposomes is greatly limited (Jain and Gerlowski 1986). By contrast, the extravasation of circulating molecules from blood vessels to tumor tissue is a function of both local blood flow and microvascular permeability (Dvorak et al. 1988). In addition, since there is little or no lymphatic drainage in tumor tissues, accumulated macromolecules are retained in the tumor interstitium for a prolonged period of time. This phenomenon, termed the enhanced permeability and retention (EPR) effect, has been shown to occur universally in tumors (Maeda 1992; Maeda et al. 1992), and it is conceivable that long-circulating liposomes could take advantage of the EPR effect for efficient target binding in tumors.

The extravasation of liposomes of different sizes into solid tumors was examined in various tumor models in mice (Unezaki et al. 1995, 1996). As shown in Fig. 4, long-

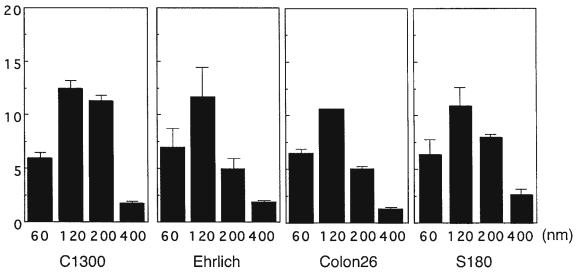

Fig. 4. Effect of liposomal diameter on the accumulation of PEG-liposomes in various implanted tumors in mice. PEG-liposomes composed of DSPC : Chol : DSPE-PEG (1 : 1 : 0.13, m/m) and containing ^{67}Ga-deferoxamine were intravenously injected into tumor-bearing mice. The mice were prepared by inoculating the following cells (1×10^7 cells) into the hind foot and used when the tumor had reached a diameter of 8 mm: mouse C-1300 neuroblastoma to A/J Sic mouse, mouse colon 26 adenocarcinoma to BALB/c mouse, mouse Ehrlich (1×10^7 cells) to ddY mouse or mouse sarcoma 180 to ddY mouse. Biodistribution was estimated at 6 h after injection

circulating liposomes composed of DSPC/Chol/DSPE-PEG (1:1:0.13, m/m), with an average diameter of 100–200 nm, accumulated efficiently in all tumor tissues examined. Clearly, liposome size is also an important factor for extravasation. Observations using fluorescence microscopy have shown that PEG-liposomes can indeed extravasate beyond the endothelial barrier, mainly through postcapillary venules (Unezaki et al. 1996; Huang et al. 1992; Ishida et al. 1999). Due to the increased circulation time of liposomes containing PE-PEG and the leaky structure of the microvasculature in solid tumor tissue, these liposomes accumulate preferentially in the tumor tissue. Thus, under physiological tumor conditions, only small liposomes ranging from 100–200 nm in diameter with a prolonged circulation half-life have a high probability of encountering the leaky vessels of the tumor tissue.

Recent studies have shown that PEG-liposomes encapsulating anthracyclines such as doxorubicin (DXR) exhibit improved therapeutic efficacy and reduced toxicity after i.v. injection into solid-tumor-bearing mice (Unezaki et al. 1995; Papahadjopoulos et al. 1991; Gabizon 1992).

2.2 Characterization of In Vivo Immunoliposome Targeting to Solid Tumors

In order to study whether immunoliposomes injected intravenously can extravasate into solid tumors and bind to tumor cells, we established a models system consisting of the monoclonal antibody 21B2, specific for human carcinoembryonic antigen (CEA), and mice bearing CEA-positive human gastric cancer strain MKN-45 (Uyama et al. 1994). Antibody 21B2 was isolated from BALB/c mice after immunization with human CEA antigen, purified from cells of a CEA-producing human gastric cancer line, MKN-45. Fab′ fragments of 21B2 were prepared by pepsin digestion of the antibody and 2-aminoethanethiol reduction of the F(ab′)2 fragments (Ishikawa et al. 1983). DPPE-PEG with a terminal maleimidyl group was synthesized for the preparation of Fab′-type C immunoliposomes (Maruyama et al. 1997a, 1997b). Two million MKN-45 cells were inoculated into the back of female BALB/c nu/nu mice. When the estimated tumor weight reached about 300 mg, the mice were used for experiments.

As shown in Fig. 5, PEG-Mal liposomes without the 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 DSPE-PEG, DSPE-PEG-COOH and DPPE-PEG-Mal, consistent with previous results (Rowlinson-Busza and Epenetos 1992; Aragnol and Leserman 1986; Derksen et al. 1988). The 21B2-type C conjugates, bearing approximately 51 whole antibody molecules per liposome, were rapidly cleared from the blood and were found entirely in the liver. In contrast, Fab′-type C conjugates, bearing approximately 517 Fab′ molecules per liposome, were retained longer in the circulation with a concomitant decrease in liver uptake compared with 21B2-type C. These results indicated that linkage of whole 21B2 antibodies to the PEG terminal enhanced RES uptake via a Fc-receptor-mediated mechanism (Aragnol and Leserman 1986; Derksen et al. 1988). This problem can be overcome by using the Fab′ fragment. In the case of Fab′-type C, the absence of the Fc portion and the presence of free PEG-Mal (not linked to the Fab′ fragment) may play a role in the prolonged circulation of the liposomes. Thus, the Fab′ fragment is much better than whole IgG in terms of

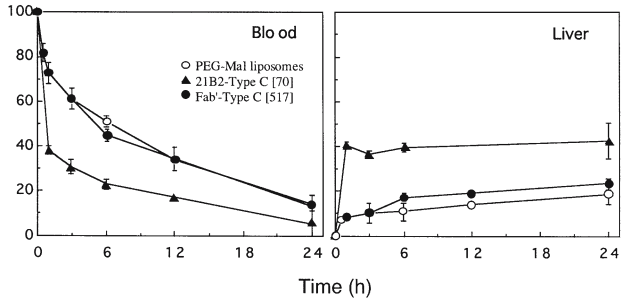

FIG. 5. Time course of blood residence and liver uptake of PEG-Mal liposomes, 21B2-type C [51] or Fab′-type C [517] in MKN-45-bearing BALB/c *nu/nu* mice. Two million MKN-45 cells were inoculated into the backs of female BALB/c *nu/nu* mice. The numbers in square brackets represent the average numbers of antibody or Fab′ molecules per liposome

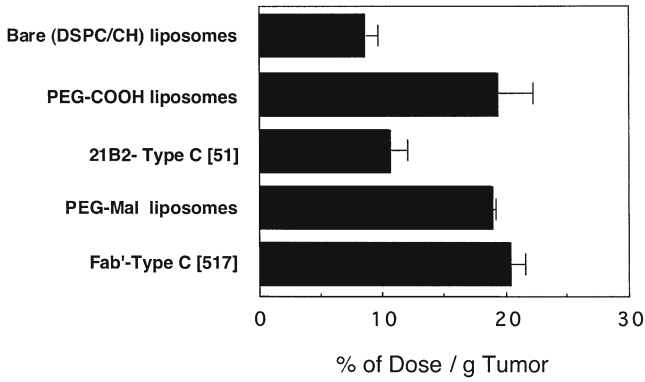

FIG. 6. Accumulation of immunoliposomes in solid tumors in MKN-45-bearing BALB/c *nu/nu* mice at 24 h after injection. Two million MKN-45 cells were inoculated into the backs of female BALB/c *nu/nu* mice. The numbers in square brackets represent the average numbers of antibody or Fab′ molecules per liposome

designing PEG-immunoliposomes with a prolonged circulation time. Furthermore, using the Fab′ fragment should greatly reduce the antigenicity.

The next question to arise was whether Fab′-type C could extravasate into solid tumor tissue and bind to tumor cells. Figure 6 shows the accumulation of Fab′-type C, 21B2-type C and comparable PEG-liposomes, with an average diameter of 100–130 nm, in MKN-45 solid tumor in mice at 24 h after injection. A relatively high accumulation was obtained with PEG-COOH liposomes, PEG-Mal liposomes and Fab′-type C immunoliposomes. These results clearly correlated with the prolonged circulation time. The accumulation rate of Fab′-type C was two-fold higher than that of 21B2-type C or bare liposomes, and equal to that of PEG-Mal and PEG-COOH liposomes.

2.3 Transferrin-Type C In Vivo Intracellular Targeting to Solid Tumors

Transferrin (TF) is a glycoprotein that transports ferric ion in the body. TF receptors are abundant in cancerous tissues and reflect the growth potential of the tumor. It is therefore reasonable to assume that TF receptors might be available as a target molecule for therapy (for review see Wagner et al. 1994). TF-receptor-mediated endocytosis is a normal physiological process by which TF delivers iron to cells (Huebers and Finch 1987; Aisen 1994). TF-type C conjugates, bearing approximately 25 TF molecules per liposome, readily bound to mouse Colon 26 cells in vitro, and were internalized by receptor-mediated endocytosis. Coupling of TF molecules did not cause enhanced RES uptake of liposomes, presumably because TF is an abundant serum glycoprotein. TF-PEG-liposomes had a prolonged residence time in the circulation and low RES uptake in Colon 26 tumor-bearing mice, resulting in enhanced extravasation of the liposomes into the solid tumor tissue. Electron microscopy studies in Colon 26 tumor-bearing mice revealed that the extravasated TF-PEG-liposomes were internalized into tumor cells by receptor-mediated endocytosis. TF-PEG-liposomes were taken up into endosomal-like intracellular vesicles, as visualized by transmission electron microscopy (data not shown) and maintained a high drug level in the tumor for over 72 h after injection. This high retention indicates cellular uptake of the extravasated TF-PEG-liposomes by TF-receptor-mediated endocytosis (Ishida et al. 2001; Maruyama et al. 2004). Thus, TF-PEG-liposomes are potential tools for in vivo cytosolic delivery of active macromolecules, such as genes or oligonucleotides.

3 Liposomal Gene Delivery Systems

The field of non-viral vector-mediated gene therapy, and particularly the use of cationic liposomes, has made great strides between the initial report by Felgner et al., in 1987, and their use in the world's first human gene-therapy clinical trial by Nabel et al. in 1992 (Nabel et al. 1993). Since then, various formulations of cationic liposomes have been developed for gene transfer and have been widely used for the in vitro transfection of eukaroytic cells (Li et al. 1999).

Cationic lipids are an interesting alternative to viral vector-mediated gene delivery in vitro and in vivo, and a large number of families of cationic vectors have been synthesized (Miller 1998). These vectors are easily prepared, and their production can be scaled-up. Most of them aim for in vitro gene delivery of plasmid DNA to give transient expression using cultured cells in culture Transfection kits for the in vitro delivery of genes or oligonucleotides into cells are important research reagents in many fields, including agriculture, medicine, and biotechnology, and are marketed worldwide.

Our goal is to deliver therapeutically active macromolecules as genes or oligonucleotides into the cytosol of target cells. The persisting key research issues is how to design the gene delivery systems to achieve significant cytosolic delivery of plasmic DNA. Many small drug molecules are readily taken up into cells by passive diffusion across cell membranes, but a growing number of pharmacologically active agents emerging from the field of biotechnology, such as proteins, peptides and nucleic acids

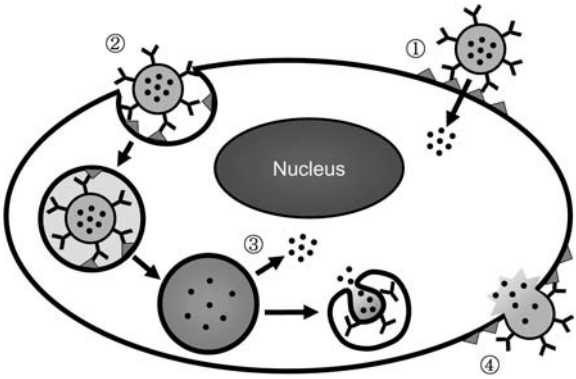

Fig. 7. Schematic representation of potential way by which targeted liposomes can achieve cytosolic drug delivery. *1* Transfer of lipophilic compounds from the liposomal bilayer to the plasma membrane. *2* Receptor-mediated endocytosis of immunoliposomes with subsequent cytosolic release of liberated drug molecules by passive diffusion out of the endosomes. *3* Cytosolic release of liberated drug molecules following low-pH-induced endosomal membrane destabilization or fusion. *4* Fusion of the liposomal bilayer with the plasma membrane

cannot easily pass biological membranes due to their unfavorable physicochemical characteristics. Therefore, cytosolic targeting of novel classes of drugs relies on delivery systems that allow translocation through cellular membranes.

The cytosolic delivery of liposome-entrapped drugs may be obtained in several different ways, as illustrated in Fig. 7. From these potential delivery routes, receptor-mediated endocytosis has been the focus of our research activities on the delivery of membrane-impermeable proteins and plasmid DNA (options 3 and 5, Fig. 7). This liposomal tactic involves: (1) specific targeting of liposomes to an internalizing receptor on the target cell population, leading to receptor-mediated endocytosis of ligand-directed liposomes, and (2) release of drugs from liposomes residing in endosomal compartments by membrane fusion/destabilization events triggered by the acidic environment within the endosomes.

3.1 Encapsulation of Plasmic DNA into Liposomes

When using liposomes as DNA carriers for in vivo gene therapy, an important research problem is how to encapsulate the DNA into liposome, since plasmid DNA is large. Improved formulations of cationic lipids have been used for the efficient delivery of DNA to cells in tissue culture (Felgner et al. 1987; Felgner et al. 1994). Much effort has also been devoted to the development of cationic lipids for the efficient delivery of nucleic acids in both animals and humans (Liu et al. 1997; Aksentijevich et al. 1996; Templeton et al. 1997).

Templeton et al. (1997) developed a unique cationic lipid structure, so-called BIV, using DOTAP, cholesterol and a novel formulation procedure. Nucleic acids are efficiently encapsulated between two bilamellar invaginated structures, BIVs. This procedure is different because it includes a brief, low-frequency sonication followed by manual extrusion through filters of decreasing pore size. The 0.1- and 0.2-μm filters

consist of aluminum oxide membranes that contain a large number of pores per surface area, including evenly spaced and sized pores, and pores with straight channels. The bilamellar invaginated structures condense unusually large amounts of nucleic acids of any size. Furthermore, addition of DNA-condensing agents, including polymers, is not necessary. Encapsulation of nucleic acid by these bilamellar invaginated structures alone is spontaneous and immediate, and therefore cost-effective, requiring only one step of simple mixing. The extruded DOTAP:Chol-nucleic acid complexes are also large enough so that they are not cleared rapidly by Kupffer cells in the liver and yet extravasate across tight barriers and diffuse the target organ efficiently, which is critical for the gene to be expressed at a therapeutic level.

Gregoriadis and coworkers (Gregoriadis et al. 1998; Perrie and Gregoriadis 2000) have developed a dehydration-rehydration method to entrap plasmid DNA into liposomes. This is consistent with the notion that most of the DNA is incorporated within multilamellar vesicles and largely protected from nucleases by the bilayers. The potential of applying such liposome DNA carriers as oral delivery system for DNA vaccines was also assessed.

Wheeler et al. (1999) established "stabilized plasmid-lipid particles" (SPLP), produced by detergent dialysis employing a POPC/DODAC/PEG-CerC_{20} lipid mixture. The features of SPLP are the extended circulation lifetime of systemically administered gene therapy vectors and their accumulation at a distal tumor site. A detailed protocol for producing SPLP is provided in Fenske et al. (2002).

Liposome-protamine-DNA complexes (LPDs) for use as novel non-viral vectors, were developed by Huang's group on the basis of understanding the cellular and molecular barriers to cationic-lipid-based gene delivery systems (Fig. 8) (Sorgi et al. 1997; Li et al. 1998). Protamines are a group of small peptides (MW, 4000–4250) that play a unique role in condensing DNA to form a compact structure in the sperm and in delivering the sperm DNA into the nucleus of the egg after fertilization. They are highly positively charged because of their high arginine content. In addition, protamine sulfate is a USP and JP compound that is used clinically as an antidote to heparin-induced anti-coagulation. Thus, protamine sulfate may be a safe and efficient condensing agent in non-viral gene delivery systems. Since, however, it was found that positively charged liposomes are not suitable for active targeting to tumor cells, a novel formulation of anionic liposome-entrapped protamine-condensed DNA (LPD-II) was developed for gene targeting. The coupling of folate to LPD-II was optimized for systemic delivery of DNA to squamous cell carcinomas of the head and neck, and

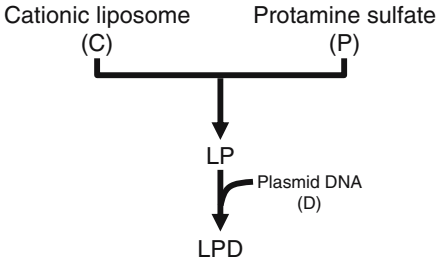

FIG. 8. Preparation of cationic liposome-protamine-DNA (LPD)

breast cancer xenografts (Xu et al. 1999). TF-coupled LPD-II liposomes were selectively targeted to myeroblast cells in vitro (Feero et al. 1997).

3.2 Liposomal Cytosolic Delivery

Receptor-mediated endocytosis would be expected to function as a pathway for the intracellular delivery of anticancer drugs in cancer chemotherapy as well as of nucleic acids in gene therapy (Harashima et al. 2001) (Fig. 7). However, once endocytosed, the control of intracellular trafficking is difficult, since it is under cellular regulation. Therefore, in order to optimize intracellular trafficking after receptor-mediated endocytosis to a target site, such as the cytosol or the nucleus, artificial sorting devices are required (Kamiya et al. 2001).

TF-receptor-mediated endocytosis as an entering pathway was chosen, these receptors are expressed in all nucleated cells in the body and in malignant cells, which have elevated receptor levels due to the high requirement of iron for growth. Once the TF-bearing liposomes are effectively internalized to the target cells, another sorting device is required to permit the liposomes, or their contents, to escape from endosomes to the cytosol. Based on results obtained with fusion peptides, as reported for viruses, GALA was examined, as the second sorting device. GALA is an artificially designed and pH-dependent fusogenic peptide composed of 30 amino acids (Subbarao et al. 1987; Nir and Nieva 2000). A highly efficient cytosolic release was achieved when the GALA peptide was present on the liposomal surfaces, which was attained by anchoring the GALA peptide to the liposomal membrane using a cholesterol derivative (Chol-GALA). Unexpectedly, there was also little escape from endosomes in the presence of GALA, which was introduced into the aqueous phase. Thus, intracellular trafficking can be controlled using TF-attached liposomes containing Chol-GALA via the TF-mediated endocytic pathway and can be applied for selective tumor targeting system (Kakudo et al. 2004).

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