Liposomes Bearing Polyethyleneglycol-Coupled Transferrin with Intracellular Targeting Property to the Solid Tumors *In Vivo*

Osamu Ishida,[1] Kazuo Maruyama,[1,4] Hiroyuki Tanahashi,[1] Motoharu Iwatsuru,[1] Katsunori Sasaki,[2] Masazumi Eriguchi,[3] and Hironobu Yanagie[3]

Received March 3, 2001; accepted April 2, 2001

Purpose. The purpose of this study was to determine the usefulness of transferrin (TF)-pendant-type polyethyleneglycol (PEG)-liposomes (TF-PEG-liposomes), in which TF was covalently linked to the distal terminal of PEG chains on the external surface of PEG-liposomes as a carrier for *in vivo* cytoplasmic targeting to tumor cells.

Methods. Small unilamellar TF-PEG-liposomes (100–140 nm in diameter) were prepared from DSPC, CH, DSPE-PEG, and DSPE-PEG-COOH (2:1:0.11:0.021, molar ratio), and were conjugated to TF via the carboxyl residue of DSPE-PEG-COOH. The intracellular targeting ability of TF-PEG-liposomes to tumor cells was examined *in vitro* and in Colon 26 tumor-bearing mice.

Results. TF-PEG-liposomes, bearing approximately 25 TF molecules per liposome, readily bound to mouse Colon 26 cells *in vitro* and were internalized by receptor-mediated endocytosis. TF-PEG-liposomes showed 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 microscopic studies in Colon 26 tumor-bearing mice revealed that the extravasated TF-PEG-liposomes were internalized into tumor cells by receptor-mediated endocytosis.

Conclusion. TF-PEG-liposomes had the capabilities of specific receptor binding and receptor-mediated endocytosis to target cells after extravasation into solid tumors *in vivo*. Such liposomes should be useful for *in vivo* cytoplasmic targeting of chemotherapeutic agents or plasmid DNAs to target cells.

KEY WORDS: liposomes; polyethyleneglycol; targeting; transferrin; endocytosis; extravasation.

[1] Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukui-gun, Kanagawa 199-0195, Japan.

[2] Department of Anatomy, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-0802, Japan.

[3] Department of Clinical Oncology, Institute of Medical Science, University of Tokyo, Shiroganedai, Minato-ku, Tokyo 108, Japan.

[4] To whom correspondence should be addressed. (e-mail: maruyama@pharm.teikyo-u.ac.jp)

ABBREVIATIONS: CH, cholesterol; ^{3}H-CHE, ^{3}H-cholesteryl hexadecyl ether; DSPC, distearoylphosphatidylcholine; DSPE-PEG, distearoyl-N-(monomethoxy polyethylene-glycol succinyl)phosphatidylethanolamine; DSPE-PEG-COOH, distearoyl-N-(3-carboxypropionoyl polyethyleneglycol succinyl) phosphatidylethanolamine; Mes, 2-(N-morpholino) ethane-sulfonic acid hemisodium salt; NGPE, N-glutaryl distearoylphosphatidylethanolamine; PBS, phosphate-buffered saline; RES, reticuloendothelial system; TF, human iron-saturated transferrin.

INTRODUCTION

Among the various approaches to active targeting, immunoliposomes using an antibody as a targeting ligand and a lipid vesicle as a carrier for both hydrophobic and hydrophilic drugs, have attracted much attention (1,2). The rationale of this approach has been well established in various *in vitro* systems. Studies *in vivo* have revealed that coating liposomes with antibody leads to enhanced uptake of the immunoliposomes by the reticuloendothelial system (RES) (3,4), and the immunotargeting efficiency depends on the antibody density on the surface (5). Thus, highly efficient targeting and a relatively low level of RES uptake of immunoliposomes are apparently mutually exclusive.

We have reported a new type of PEG-immunoliposomes, so-called pendant-type PEG-immunoliposomes, in which antibodies are coupled to the extremities of surface-grafted PEG chains (6,7). This was achieved by the use of functionalized PEG derivatives to couple antibodies directly to the distal terminal of PEG chains incorporated in liposomes. 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 (6). The coupling of the Fab′ fragment instead of intact antibody, 21B2, specific for the human carcinoembryonic antigen (CEA), 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 (7). Therefore, with the availability of suitable ligands, this approach should produce promising drug carrier systems for targeting tumor cells.

In the present study, we have explored the possibility of using transferrin (TF) for developing an intracellular drug delivery system. TF is a glycoprotein, which transports ferric ion in the body. It is well known that the TF receptor concentration on tumor cells is much higher than that on normal cells (for review see [8]). Transferrin receptor-mediated endocytosis is a normal physiological process by which transferrin delivers iron to the cells (9,10). TF was coupled to the distal terminal of the PEG chains of PEG-liposomes to prepare pendant-type PEG-liposomes. TF-PEG-liposomes were then injected intravenously into tumor-bearing mice. We provide evidence of extravasation from the blood circulation to the solid tumor region, followed by specific binding and internalization of TF-PEG-liposomes into tumor cells, leading to the delivery of their content into the cytoplasm.

MATERIALS AND METHODS

Materials

DSPC (COATSOME MC–8080), DSPE (COATSOME ME–8080), monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu), and polyethyleneglycol bis(succinimidyl succinate) (PEG-2OSu) were kindly donated by Nippon Oil and Fats Co. (Tokyo, Japan). The values of the number-average molecular weight of PEG-OSu and PEG-2OSu were 2219 and 3230, respectively, and their polydispersities were 1.03 and 1.04, respectively, as measured by means of gel permeation chromatography. CH and triethylamine were purchased from Wako Pure Chemicals (Osaka, Japan).

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1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (S-NHS) and IODO-GEN were obtained from Pierce (Rockford, IL). $Na^{125}I$ and ^3H-CHE were obtained from New England Nuclear Japan (Tokyo). Human iron-saturated TF and pronase were purchased from Sigma Chem. (St. Louis, MI). All other chemicals were of the highest grade commercially available. DSPE-PEG, DSPE-PEG-COOH, and NGPE were synthesized as previously described (6). TF was radio labeled with ^{125}I, using the IODO-GEN method, to a specific activity of 1–4 $\times$ 10^5 cpm/μg.

Preparation of TF-Conjugating Pendant-Type PEG-Liposomes

PEG-liposomes were prepared from DSPC, CH, DSPE-PEG, and DSPE-PEG-COOH (2:1:0.11:0.021, molar ratio), and if necessary, ^3H-CHE as an inert membrane marker was added in a trace amount. Small unilamellar liposomes (SUV, 90-130 nm in diameter) were prepared by reverse-phase evaporation (11) followed by extrusion (Lipex Biomembranes, Canada) through two stacked polycarbonate membrane filters (Nucleopore, Clifton, NJ) of 0.1 μm diameter. Mes buffer (10 mM Mes/150mM NaCl, pH 5.5) was used as the liposomal aqueous phase. The extruded liposomes were centrifuged at 200,000 g for 20 min at 4°C (Hitachi CS120, S100AT5 rotor), and then resuspended in Mes buffer (pH 5.5).

TF-pendant-type PEG-liposomes (TF-PEG-liposomes) were prepared by coupling of TF to PEG-liposomes as described previously (6). Briefly, to 1 ml of PEG-liposomes (5 μmole lipids) in Mes buffer (pH 5.5), 21 μmole of EDC and 28 μmole of S-NHS were added, and the mixture was incubated for 15 min at room temperature. The mixture was applied to a Sephadex G-25 column equilibrated with Mes buffer (pH 5.5) and the liposome fractions were collected. The desired amount of TF with a trace amount of ^{125}I-labeled TF was then added to the liposome solution and the whole was incubated for 3 h at room temperature with gentle stirring. The mixture was centrifuged at 200,000 g for 30 min at 4°C, and then resuspended in PBS (pH 7.4). To this suspension, $FeCl_3$-nitrilotriacetic acid solution was added to obtain diferric TF (12). The whole was centrifuged at 200,000 g for 30 min at 4°C, and the precipitated TF-PEG-liposomes were resuspended in PBS (pH 7.4). For the electron microscopic observation, TF-PEG-liposomes entrapping colloidal gold were prepared by a modified procedure according to Huang *et al.* (13).

TF-liposomes with TF covalently linked to the short anchor NGPE instead of DSPE-PEG-COOH were prepared in the same manner as TF-PEG-liposomes, and DSPE-PEG was excluded from the lipid components. TF-liposomes are the usual type of ligand-conjugating normal liposomes (5).

The lipid concentration and the coupling efficiency of TF were estimated by phosphorus assay (14) and by measuring radioactivity of ^{125}I, respectively. The average number of TF molecules per liposome was calculated by using the above values and the assumption that the molecular weight of TF is 80,000; the average number of phospholipid molecules per liposome was estimated by the method of Enoch and Strittmatter (15). Liposome size was measured with an electrophoretic light scattering spectrophotometer (ELS-700, Otsuka Electronics, Tokyo).

Cells

Mouse colon carcinoma (Colon 26) was grown in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Gaithersburg, MD). The TF binding assay was performed at 4°C with ^{125}I-labeled TF and TF binding sites and affinities were determined by Scatchard analysis, as previously described (12,16).

Liposome-Cell Association Assay

For binding experiments, Colon 26 cells were washed twice in PBS and 1 $\times$ 10^6 cells were resuspended in 1 ml of RPMI 1640 medium with 10% fetal bovine serum or 10% mouse serum containing 500 μg lipid of ^3H-CHE-labeled TF-PEG-liposomes. The mixture was incubated for 1 h at 4°C or 37°C. To examine the specificity of the TF-receptor mediated binding, cells were incubated with excess dose of TF (50 μg) at 4°C for 20 min, and then binding assays of TF-PEG-liposomes were performed in medium containing excess dose of TF (50 μg) at 4°C. For internalization experiments, Colon 26 cells (5 $\times$ 10^6 cells) were preincubated for 1 h at 4°C with ^3H-CHE labeled TF-PEG-liposomes (500 μg lipid) in RPMI 1640 medium with 10% fetal bovine serum, and then incubated for the indicated time at 37°C. The incubation was stopped by adding 1 ml of ice-cold PBS to 100 μl of reaction mixture and centrifuging at 3000 rpm. The cell pellets were incubated for 30 min at 4°C with 500 μl of a 2 mg/ml pronase (Boehringer Mannheim Japan, Tokyo) solution, to remove the surface-bound TF. After the incubation, cells were washed, and solubilized with Soluene 350 (Packard Instruments, Meridem, CT), then the radioactivity was counted in Hionic-Fluor scintillation mixture in an Aloka LSC-3000 counter (Aloka Co., Tokyo).

Animal Experiments

Tumor-bearing mice were prepared by inoculating s.c. a suspension (~1 $\times$ 10^7 cells) of Colon 26 cells directly into the hind leg of male BALB/c mice (7 weeks old, weighing 20–25 g, Sankyo, Tokyo). Liposome biodistribution experiments were performed when the tumor size was in the range from 6 to 8 mm in diameter. ^3H-CHE labeled TF-PEG-liposomes (500 μg lipid/200 μl) were injected into tumor-bearing mice (3–5 per group) *via* the tail vein. At selected time intervals after administration, mice were lightly anesthetized, bled by eye puncture, killed by cervical dislocation, and dissected. The radioactivity of each tissue was counted as described above.

Transmission Electron Microscopy Studies

Preparation of PEG-liposomes entrapping colloidal gold was done as described previously (13,17). The average size of the liposomes was 132 ± 38 nm in diameter, as determined with a submicron particle analyzer. Colloidal gold-TF-PEG-liposomes (500 μg total lipid) were injected into Colon 26 tumor-bearing mice *via* the tail vein. At 24 h after injection, the mice were retrogradely perfused with 80 mM cacodylate buffer (pH 7.4) solution containing heparin (0.25 g/l) and procaine hydrochloride (5 g/l) through the aorta abdominalis, and fixative (2% glutaraldehyde) was introduced for 1 min. Then, the tumor tissue was excised, cut into small blocks in

the same fixing solution and further fixed by immersion in 2% glutaraldehyde for 2 h. The blocks were washed in buffer, postfixed in 1% OsO_4 solution in 90 mM sucrose-40 mM cacodylate buffer (pH 7.4) solution for 1 h, dehydrated in an ethanol series, and embedded in an Epon-Araldite mixture (18). For *in vitro* binding experiments, Colon 26 cells (1×10^6 cells) were suspended in 1 ml of RPMI 1640 medium without fetal bovine serum containing colloidal gold-TF-PEG-liposomes (100 μg total lipid) and the mixture was incubated for 2 h at 37°C. After incubation, cells were fixed with 2% glutaraldehyde-45 mM cacodylate buffer (pH 7.4) solution for 1 h at 4°C, and stained with 1% OsO_4 solution in 90 mM sucrose-40 mM cacodylate buffer (pH 7.4) solution. Cells were buried in 3% agarose and embedded in an Epon-Araldite mixture. Ultrathin sections were cut with an ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate and were examined under a Hitachi 7000TEM electron microscope (Tokyo).

RESULTS

Characterization of Association of TF-PEG-Liposomes to Tumor Target Cells *In Vitro*

The number of binding sites and the affinity of TF to Colon 26 cells were determined by Scatchard analysis. Scatchard analysis showed 4×10^5 TF receptors/cell and an apparent dissociation constant 42 nM. Though these binding affinities of TF to Colon 26 cells were not so high, as compared with that to human erythroleukemia K562 cells (16), they were much higher than that on normal cell (8). We first tested whether TF-PEG-liposomes specifically bound to Colon 26 cells *in vitro* under the presence of 10% FCS or 10% mouse serum. The data at 4°C in Fig. 1A indicated that TF-PEG-liposomes readily bound with Colon 26 cells in the medium containing 10% FCS or 10% mouse serum. The degree of target cell binding of TF-PEG-liposomes was greater than that of nontargeted PEG-liposomes. The presence of 50 μg of free TF decreased significantly the degree of cell binding of TF-PEG-liposomes. These results revealed that the binding of TF-PEG-liposomes to Colon 26 cells is indeed receptor specific, and free PEG (not linked to antibody) in liposomes does not interfere sterically with TF-receptor binding, since TF is covalently linked to the distal terminal of PEG chains on the external surface of PEG-liposomes. Increasing the incubation temperature from 4°C to 37°C induced a high degree of association of TF-PEG-liposomes with the target cells, as shown in Fig. 1B. These high associations at 37°C indicate endocytotic uptake of TF-PEG-liposomes by Colon 26 cells after binding. Fig. 2 shows the time course of specific association (binding + internalization) of TF-PEG-liposomes (an average of 25 TF molecules per liposome) with Colon 26 cells. For estimation of internalized TF-PEG-liposomes, cells were treated with pronase to remove surface-bound TF. The results indicate that Colon 26 cells rapidly bound TF-PEG-liposomes, and internalized them via endocytosis. Thus, TF-PEG-liposomes were bound and taken up by endocytosis at 37°C.

The endocytotic uptake pathway of TF-PEG-liposomes into Colon 26 cells was confirmed by transmission electron microscopy. The liposomes were most frequently seen on the cell surface. The photographs in Fig. 3 show liposomes and gold particles present on the plasma membrane and in the endosome (enlarged photo in Fig. 3). Thus, it was confirmed that TF-PEG-liposomes bound to specific cell surface receptors and were internalized by receptor-mediated endocytosis.

Biodistribution Studies of TF-PEG-Liposomes in Tumor-Bearing Mice

Liposomes, with an average diameter of 100–130 nm, were injected intravenously into tumor-bearing mice, and the distribution and tumor accumulation were studied. Fig. 4 shows the plasma-time curve and the biodistribution of TF-PEG-liposomes in Colon 26 tumor-bearing mice, as compared with that of TF-liposomes. TF-PEG-liposomes, bearing approximately 20 TF molecules per liposome, showed prolonged residence in the circulation and low liver uptake, as

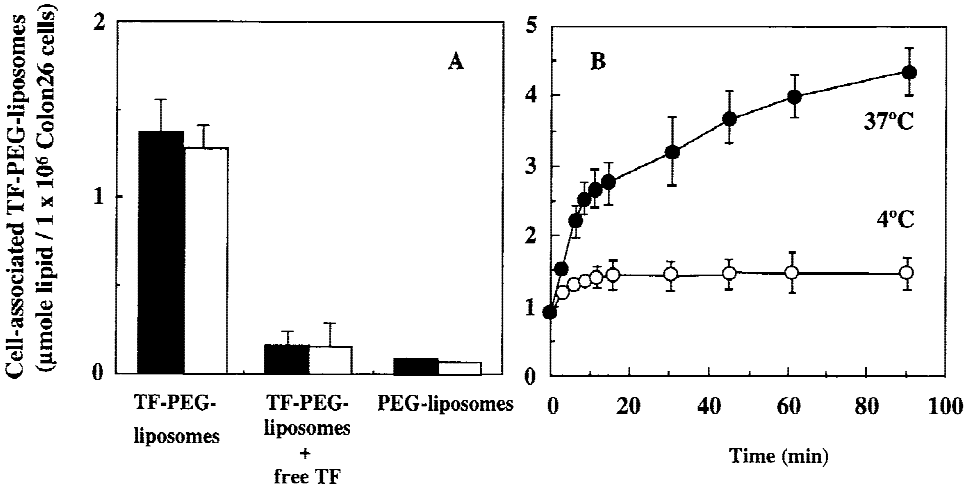

Fig. 1. Binding characterization of TF-PEG-liposomes to Colon 26 cells. Panel A: 500 μg lipid of ^{3}H-CHE-labeled TF-PEG-liposomes, with an average of 25 TF molecules per liposome, were incubated with Colon 26 cells (1×10^6 cells) in RPMI 1640 medium with 10% fetal bovine serum (■) or 10% mouse serum (□) for 1 h at 4°C. Blocking tests of TF-PEG-liposomes-cell binding were performed with excess dose (50 μg) of free TF. Panel B: Time course of binding of ^{3}H-CHE-labeled TF-PEG-liposomes (500 μg lipid), with an average of 25 TF molecules per liposome, to Colon 26 cells at 4°C (○) or 37°C (●).

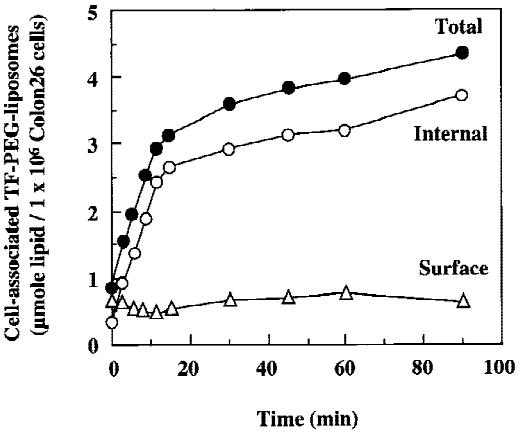

Fig. 2. Time course of cell association and internalization of TF-PEG-liposomes, with an average of 25 TF molecules per liposome, in Colon 26 cells at 37°C. Colon 26 cells (5×10^6 cells) were incubated with ^{3}H-CHE-labeled TF-PEG-liposomes (100 μg lipid) in RPMI 1640 medium with 10% fetal bovine serum. Cells were treated with pronase to remove surface-bound TF-PEG-liposomes, at the indicated times.

did PEG-liposomes without TF. In contrast, TF-liposomes, bearing approximately 20 TF molecules per liposome, were cleared much faster from the blood and were taken up more efficiently by liver than the above two types of liposomes. Thus, the conjugation of TF to the PEG terminal did not alter the RES uptake of PEG-liposomes, presumably because TF is a glycoprotein existing in blood. Furthermore, PEG chains occupying the liposome surface played a role in the prolonged circulation of TF-PEG-liposomes.

Fig. 5 shows the time course of accumulation of liposomes in Colon 26 solid tumor tissue after injection. Relatively high accumulation in the tumor tissue was obtained with PEG-liposomes and TF-PEG-liposomes. The accumulation rates of liposomes were clearly correlated to the prolonged circulation time (Fig. 4). It is noteworthy that the residence time of extravasated TF-PEG-liposomes in tumor tissues was much longer than that of PEG-liposomes, as observed at 48–120 h after injection. These results indicated that TF-PEG-liposomes extravasated and bound to the TF-receptors and were then internalized by receptor-mediated endocytosis.

To test the effect of size on the liposomal accumulation in solid tumor tissue, TF-PEG-liposomes of different sizes were prepared and injected i.v. into Colon 26 tumor-bearing mice. The results are summarized in Fig. 6. At the same size, the accumulation rate of TF-PEG-liposomes was higher than that of PEG-liposomes. At 120 nm average diameter, each type of liposomes showed the highest accumulation in the solid tumors. Increasing the diameter resulted in lower tumor accumulation. The accumulation of the smallest TF-PEG-liposomes (60 nm) was much higher than that of PEG-liposomes with 60 nm average diameter. These results, except for the smallest TF-PEG-liposomes, confirm earlier observations by others that small (100–200 nm) liposomes with prolonged circulation time exhibit favorable tumor accumulation (17).

Extravasation and localization of TF-PEG-liposomes were examined by electron microscopy in Colon 26 tumor-bearing mice. TF-PEG-liposomes of 132 ± 38 nm mean diameter, encapsulating colloidal gold particles, were used. Liposomes and gold particles were present on the plasma membrane (Fig. 7A), in the process of endocytosis (Fig. 7B), and in the endosome (Fig. 7C).

DISCUSSION

One of the major limitations of active targeting using ligand-directed immunoliposomes has been their rapid clearance due to nonspecific uptake by the RES. However, the development of PEG-liposomes conjugated with ligands has revived interest in this field because PEG-liposomes are not rapidly cleared by the RES (19,20). We have reported previously the preparation of pendant-type PEG-immunoliposomes by coupling of the Fab′ fragment instead of intact an-

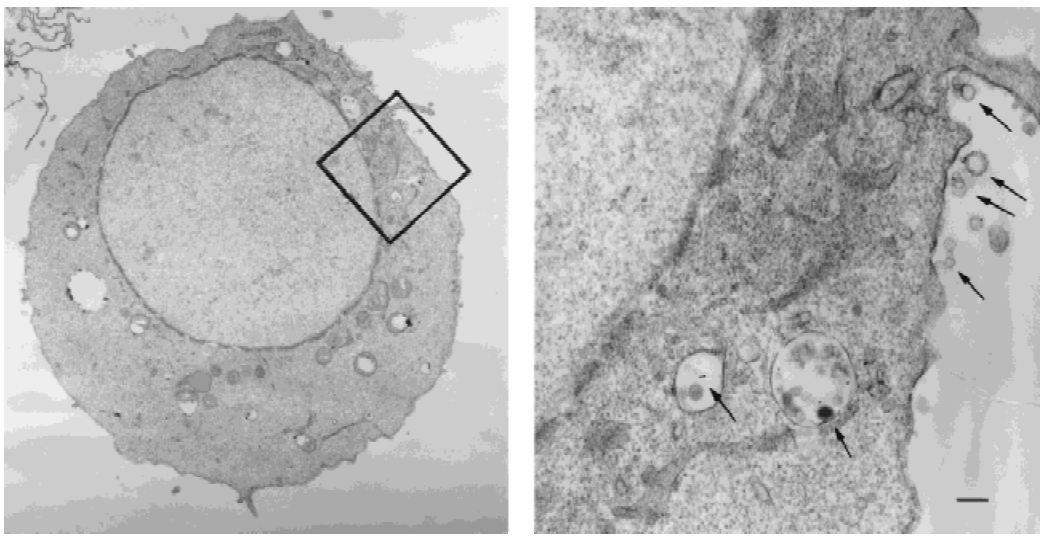

Fig. 3. Transmission electron micrographs of the localization of TF-PEG-liposomes in Colon 26 cell. Colon 26 cells were incubated with TF-PEG-liposomes entrapping colloidal gold and coupling an average of 25 TF molecules per liposome, at 37°C for 1 h. Liposomes and gold particles were observed on the plasma membrane and in the endosome of Colon 26 cell. Bars = 200 nm.

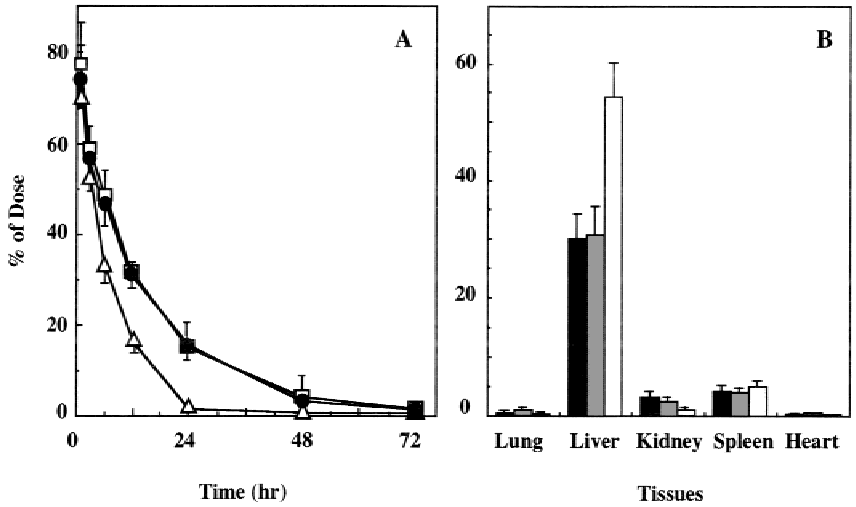

Fig. 4. Blood clearance (A) and biodistribution at 24 h (B) of TF-PEG-liposomes, with an average of 25 TF molecules per liposome, after intravenous injection. They were compared with PEG-liposomes and TF-liposomes with an average of 20 TF molecules per liposome. Liposomes (100–130 nm average diameter) labeled with ^{3}H-CHE were injected into Colon 26 tumor-bearing mice at a dose of 500 μg lipid. Data are expressed as mean ± SD (n = 3–5). ● and ■, TF-PEG-liposomes; □ and ■, PEG-liposomes; △ and □, TF-liposomes.

tibody, 21B2, specific for human CEA, to the extremities of surface-grafted PEG chains. These pendant-type PEG-immunoliposomes evaded RES uptake and remained in the circulation for a long time, resulting in enhanced accumulation of the liposomes in the solid tumor (7). The absence of the Fc portion and the presence of free PEG (not linked to Fab′ fragment) play a role in the prolonged circulation of the liposomes.

In the present experiments, we prepared TF-coupled pendant-type PEG-liposomes for intracellular drug delivery to solid tumors. TF receptors are abundant in cancerous tissues and reflect the tumor growth potential. It is therefore reasonable to assume that the transferrin receptor might be available as a target molecule for therapy. TF-pendant-type PEG-liposomes exhibited interesting properties in biodistribution, tumor accumulation, and internalization *in vivo*. Covalent binding of TF molecules to the PEG terminus did not appear to cause any interference with TF binding to its receptors; furthermore, the PEG coating of the liposome surface apparently continues to retard the opsonization of the liposomes, leading to an extended circulation half-life. Coupling of TF molecules did not cause enhanced RES uptake of liposomes, presumably because TF is an abundant serum glycoprotein, which transports ferric ion in the body. In contrast, TF-liposomes were cleared much faster from the blood and were taken up more efficiently by the liver, as compared with TF-PEG-liposomes, because the association of serum protein with PEG-free liposomes mediated liposomal uptake by macrophages in the RES (21).

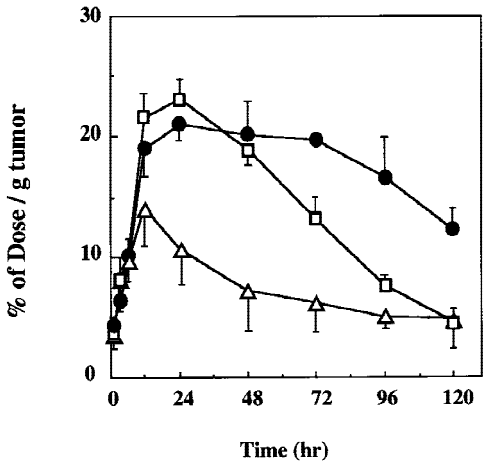

Fig. 5. Time course of accumulation of TF-PEG-liposomes in solid tumors in Colon 26 tumor-bearing mice after injection, as compared with PEG-liposomes and TF-liposomes. Conditions were as described in the legend to Fig. 4. ●, TF-PEG-liposomes; □, PEG-liposomes; △, TF-liposomes.

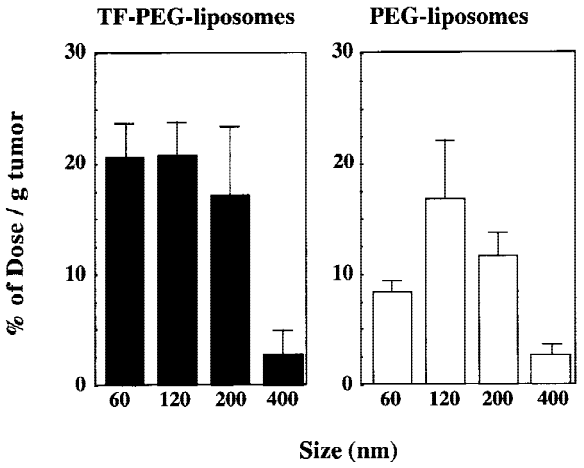

Fig. 6. Effect of liposomal size on the accumulation of TF-PEG-liposomes into solid tumors in Colon 26 tumor-bearing mice, as compared with TF-liposomes. Liposomes labeled with ^{3}H-CHE were injected into tumor-bearing mice *via* the tail vein, and the biodistribution was estimated at 48 h after administration. Data are expressed as mean ± SD (n = 3–5).

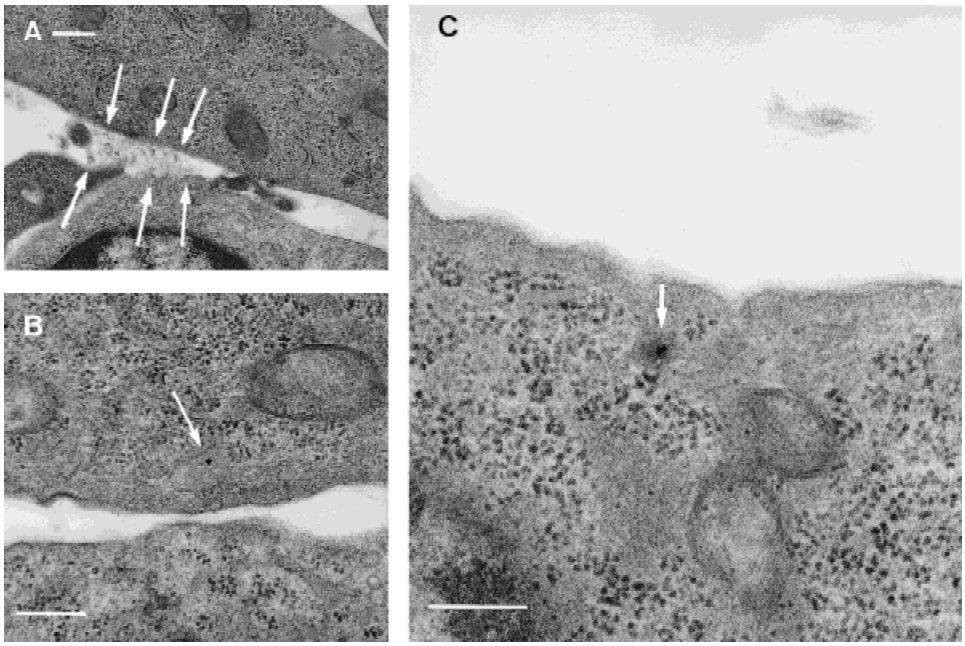

Fig. 7. Transmission electron micrographs of the localization of TF-PEG-liposomes in Colon 26 solid tumor tissue *in vivo*. TF-PEG-liposomes of 132 ± 38 nm mean diameter, entrapping colloidal gold and coupling an average of 25 TF molecules per liposome, were injected into mice *via* the tail vein, and ultrathin sections of tumor tissue were prepared at 24 h after administration. Liposomes were located on the plasma membrane (A), probably in the process of endocytosis (B), and inside the endosome-like structure (C). Bars = 250 nm.

In tumor-bearing mice, TF-PEG-liposomes were extravasated effectively into solid tumor tissue, and internalized by receptor-mediated endocytosis after binding to TF-receptors on the tumor cells. TF-PEG-liposomes were taken up into endosomal-like intracellular vesicles, as visualized by transmission electron microscopy (Fig. 3). In general, and as shown in our previous paper (17), the endothelial barriers in newly vascularized tumors are inherently leaky, due to the wide interendothelial junctions, large numbers of fenestration and transendothelial channels, and discontinuous or absent basement membrane. Their permeability is increased compared with that in normal tissues (22). These characteristics of solid tumors are believed to be the basis of the so-called EPR effect (enhanced permeability and retention effect) (23). Normal tissues outside the RES generally have continuous and nonfenestrated vascular endothelia, and extravasation of liposomes is very limited.

We and Liu *et al.* have demonstrated previously that a relatively high level of tumor accumulation of PEG-liposomes correlated very well with both a small size (less than 200 nm in diameter) and a relatively high concentration of liposomes in the blood (17,24,25). Though there were no marked differences in accumulation in the solid tumors between TF-PEG-liposomes and PEG-liposomes of 120 nm average diameter, only TF-PEG-liposomes can bind specifically to tumor cells (Fig. 5). As shown in Fig. 6, the size limitation of TF-PEG-liposomes for tumor accumulation is of practical significance. The smallest TF-PEG-liposomes (60 nm) exhibited interesting properties of tumor accumulation as compared with PEG-liposomes with the same average diameter. Liposomes of 60 nm average diameter may pass freely through the gaps between adjacent endothelial cells. However, TF-PEG-liposomes of 60 nm average diameter are recognized and internalized by the TF-receptors at the interstitial space after extravasation from tumor vessels, resulting in accumulation of these liposomes in tumor tissue. These processes were confirmed by transmission electron microscopy (Fig. 7).

It is noteworthy that the residence time of extravasated TF-PEG-liposomes in tumor tissues was much longer than that of PEG-liposomes (Fig. 5). TF-PEG-liposomes escaped from leaky endothelial barriers and extravasated into the extravascular and interstitial space among tumor cells. As confirmed by electron microscopy (Fig. 7), this longer residence time of TF-PEG-liposomes was due to the internalization by receptor mediated endocytosis. In less flow condition in interstitial space among tumor cells, TF-PEG-liposomes have greater chances to bind to the receptors on surface of tumor cells. *In vitro* binding data showing that TF-PEG-liposomes can indeed bind to Colon 26 in the presence of 10% mouse serum support these *in vivo* results.

TF-PEG-liposomes offer several advantages for the cytoplasmic delivery of drugs and genes. Since TF is an isologous protein, problems of immunological incompatibility are avoided. Though many ligand/receptor pairs after endocytosis are targeted to lysosomes and degraded by hydrolytic enzymes, the receptor-mediated endocytosis process of TF differs from them in several respects. After binding of TF to the receptors on the cell surface, the TF-receptor complexes are internalized to form endosomes through clathrin-coated vesicles. After internalization, iron-loaded TF releases its iron at low endosomal pH, whereas iron-free TF remains bound to the receptor. These complexes are sorted into exocytic vesicles for delivery back to the cell surface and iron-free TF is released. The entire TF cycle takes only 4–5 min with a

mean transit time of about 10 min, and avoids the lysosomal compartment (8,16,26). It would be valuable to know the *in vivo* lifetime of TF-PEG-liposomes on target cells. However, the mode of intracellular delivery and the fate of the liposome contents are still obscure. Evidence exists that the entrapped molecules (gold particles in this study) enter the cell via endocytosis (Fig. 3 and 7).

A question that is assuming increasing importance is how to manipulate the rate and extent of drug release from liposomes once they reach in endosome. Many studies have shown effectiveness of pH sensitive liposomes as a carrier, which destabilize and/or fuse with endosomal membrane and then release their contents into the cytoplasm in the acidic pH of the endosome without degradation by the lysosomal enzymes (27). The combination of TF-PEG-liposomes and pH-sensitive liposomes may have potential usefulness as a cytoplasmic delivery system. Research into various ways to trigger release of liposomal drugs is currently underway, and may result in further improvements in the utilization of TF-PEG-liposomes.

In conclusion, we designed TF-coupling pendant-type PEG-liposomes that can be extravasated to and accumulated in solid tumors *in vivo*. The formulation retains the capabilities of specific receptor binding and receptor-mediated endocytosis to target cells after extravasation. Such liposomes should be useful for endocytotic internalization of drugs or plasmid DNA into target cells. Applications of TF-PEG-liposomes to the boron neutron capture therapy (BNCT) for cancer are currently under investigation in our laboratory. For successful BNCT, ^{10}B compounds should be selectively localized in the tumor cells while a low concentration is maintained in normal cells and blood. The results of these experiments will be presented in a separate report.

ACKNOWLEDGMENTS

A part of this work was supported by Grants-in-Aid for Scientific Research (no. 08672568 to Kazuo Maruyama, no. 10470254 to Hironobu Yanagie, no. 10771272 to Osamu Ishida) from the Ministry of Education, Science and Culture, Japan and a Grant-in-Aid for Cancer Research (no. 9, specified to Kazuo Maruyama) from the Ministry of Health and Welfare, Japan. The advice of Dr. T. Tagawa and Dr. K. Nagaike (The Research Center, Mitsubishi-Tokyo Pharm., Inc.) is gratefully acknowledged.

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