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Effective anti-tumor activity of oxaliplatin encapsulated in transferrin–PEG-liposome

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Abstract

Oxaliplatin (*trans*-L-diaminocyclohexane oxalatoplatinum, L-OHP) is a novel cisplatin derivative that can improve the side effects of cisplatin such as toxicity to the kidneys and peripheral nerve system. However, L-OHP is effective only when combined with 5-Fluorouracil (5-FU) and Leucovorin. The relatively low anti-tumor index of L-OHP alone is because low levels accumulate in tumor tissues due to high partitioning to erythrocytes *in vivo*. A successful outcome of cancer therapy using L-OHP requires the selective delivery of a relatively high concentration of the drug to tumors. The present study examines tumor-selective delivery of L-OHP using liposomes modified with transferrin-conjugated polyethyleneglycol (TF–PEG-liposomes). Delivery using these liposomes significantly reduced L-OHP partitioning to erythrocytes and improved the circulation time of L-OHP *in vivo*, resulting in enhanced extravasation of liposomes into tumors. The TF–PEG-liposomes maintained a high L-OHP concentration in tumors for over 72 h after intravenous injection, which was longer than that of the liposomes modified with PEG (PEG-liposomes). Intravenously administered L-OHP encapsulated within TF–PEG-liposomes (L-OHP: 5 mg/kg) suppressed tumor growth more effectively than PEG-liposomes, Bare-liposomes and free L-OHP. Although L-OHP is usually combined with 5-FU and Leucovorin, our results suggest that L-OHP encapsulated within TF–PEG-liposomes has potential for cancer therapy.

Keywords: Liposomes; Transferrin; Oxaliplatin; Targeting; Cancer therapy; Polyethyleneglycol (PEG)

1. Introduction

Surgery, radiotherapy and chemotherapy comprise the current choice of strategies used to fight cancer. However, the present range of anti-cancer drugs does not deliver satisfactory therapeutic effects due to many undesirable side effects.

Cisplatin (*cis*-diamminedichloroplatinum(II)) is one of the most effective agents against testicular, ovarian, head, neck and lung cancers. However, side effects include kidney toxicity, nausea, hearing impairment and irreversible peripheral nerve damage (Durant, 1980). To resolve these issues, considerable effort has been directed towards the development of cisplatin derivatives among which, oxaliplatin (*trans*-L-diaminocyclohexane oxalatoplatinum, L-OHP) has an anti-tumor effect against cisplatin-resistant murine leukemia cells (L1210 cells) (Kidani et al., 1980). L-OHP inhibits DNA synthesis by forming DNA adducts like cisplatin. In addition, it can also inhibit RNA synthesis unlike cisplatin (Tashiro et al., 1989). However, although L-OHP has no renal toxicity (Mathe

Abbreviations: CH, cholesterol; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; EPR, enhanced permeability and retention; L-OHP, *trans*-L-diaminocyclohexane oxalatoplatinum (oxaliplatin); MIP-MS, microwave-induced plasma mass spectrometer; PEG, polyethylene glycol; RES, reticuloendothelial system; TF, transferrin; TF–PEG-liposomes, transferrin-coupling pendant-type PEG-liposomes

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et al., 1989; Extra et al., 1990; Machover et al., 1996), its high incidence of adverse drug reactions including peripheral sensory neuropathy and thrombocytopenia remain clinically problematic (Extra et al., 1990). In the FOLFOX4 treatment together with 5-FU and Leucovorin (Rothenberg et al., 2003; Goldberg et al., 2004), L-OHP is a pivotal first line chemotherapeutic agent for treating colon cancer in over 60 countries.

A significant portion of L-OHP in whole blood is sequestered into erythrocytes (Pendyala and Creaven, 1993). The $T_{1/2}$ of L-OHP is 2.3 min (α-phase) and 49 min (β-phase) in mice, and 26 min (α-phase) and 38.7 h (β-phase) in humans. Free L-OHP in the blood is rapidly excreted by the kidneys resulting in the short α-phase, but the high partitioning of L-OHP to erythrocytes is reflected in the long β-phase. To induce the powerful anti-tumor effect of L-OHP, interaction with erythrocytes must be reduced, so that more L-OHP is delivered to tumors for internalization.

We recently described novel target-sensitive liposomes bearing polyethylene glycol (PEG), called pendant-type PEG immunoliposomes, in which antibodies or specific ligands are coupled to the extremities of surface-grafted PEG chains (Maruyama et al., 1995, 1997, 2004; Ishida et al., 2001; Iinuma et al., 2002; Hatakeyama et al., 2004; Kakudo et al., 2004; Miyajima et al., 2006). Functionalized PEG derivatives couple antibodies directly to the distal terminal of PEG chains incorporated in liposomes. We demonstrated that transferrin-coupling pendant-type PEG-liposomes (TF–PEG-liposomes) are effectively extravasated into solid Colon 26 tumors in mice, and internalized into tumor cells (Ishida et al., 2001). The residence time of TF–PEG-liposomes in the circulation is prolonged and reticulo-endothelial system (RES) uptake is low in tumor-bearing mice, resulting in enhanced extravasation of the liposomes into solid tumors. This phenomenon has been characterized as the tumor-selective enhanced permeability and retention (EPR) effect of macromolecules and lipidic particles including liposomes (Matsumura and Maeda, 1986; Ishida et al., 1999). Accumulation in solid tumors is due to the unique vascular characteristics of tumors such as hypervasculature and enhanced vascular permeability, as well as the absence of a lymphatic recovery system (Jain and Gerlowski, 1986; Dvorak et al., 1988). After reaching a tumor site, TF–PEG-liposomes are internalized by receptor-mediated endocytosis and are absorbed into endosome-like intracellular vesicles. The transferrin (TF) receptor concentration on tumor cells is considerably higher than that on normal cells (Wagner et al., 1994). TF receptor-mediated endocytosis is a normal physiological process through which TF delivers iron into cells (Huebers and Finch, 1987; Aisen, 1994). Therefore, the clearance of TF–PEG-liposomes from tumor tissue is so impaired that they remain in the tumor interstitium for prolonged periods (Ishida et al., 2001).

Since selective delivery and cell-entry mechanisms are features of TF–PEG-liposomes, the delivered species of liposome does not need natural affinity for the targeted tumor cells, thus rendering this system potentially applicable to a wide variety of effector molecules, including L-OHP. Here, we examined the potential of liposomes to selectively deliver therapeutic quantities of L-OHP to tumors. We prepared TF–PEG and PEG-liposomes encapsulating L-OHP and compared their tissue distribution in Colon 26 tumor-bearing mice with those of Bare-liposomes and free L-OHP. In addition, we examined the anti-tumor activities of TF–PEG-liposomes encapsulating L-OHP in mice bearing Colon 26 tumors.

2. Materials and methods

2.1. Animals and tumor cells

Six-week-old male BALB/c mice (Tokyo Experimental Animals, Inc., Tokyo, Japan) were maintained at the animal care facility of Teikyo University (Kanagawa, Japan) under a regulated period of light and provided with water and food ad libitum. Colon 26 cells, which are derived from a mouse colon carcinoma, were maintained in RPMI 1640 medium (Sigma–Aldrich Japan, Tokyo) containing 10% fetal calf serum (Gibco, Gaithersburg, MD) under a 5% CO_2 atmosphere at 37 °C.

2.2. Lipids and chemicals

Nippon Oil and Fats Co. (Tokyo, Japan) donated distearoylphosphatidylcholine (DSPC) (COATSOME MC-8080), distearoylphosphatidylethanolamine (DSPE) (COATSOME ME-8080), monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu) and polyethyleneglycol bis(succinimidyl succinate) (PEG-2OSu). The number-average molecular weight of PEG(2K)-OSu and of PEG(3K)-2OSu was 2219 and 3230, respectively, and their polydispersity was 1.03 and 1.04, respectively, as measured using gel permeation chromatography. DSPE-PEG(2K) and DSPE-PEG(3K)-COOH were synthesized as described (Maruyama et al., 1995). Cholesterol (CH) and triethylamine were purchased from Wako Pure Chemicals (Osaka, Japan). Human iron-saturated TF was purchased from Sigma (St. Louis, MI) and L-OHP was donated by its developer, Dr. Y. Kidani (Kidani et al., 1980).

2.3. Liposome preparation

Bare-liposomes and PEG-liposomes were prepared from DSPC and CH (2:1, molar ratio) and DSPC, CH, DSPE-PEG(2K) (2:1:0.192, molar ratio), respectively. Small unilamellar vesicles (SUV) of the two types of liposomes were prepared using reverse-phase evaporation (REV). Lipids (300 mg) were dissolved in 4 ml of chloroform/diethyl ether (1:1, v/v) and then 2 ml of 8 mg/ml L-OHP in 9% (w/v) sucrose was dropped into the lipid mixture to form a w/o emulsion. The volume ratio of the aqueous to the organic phase was maintained at 1:2. The emulsion was sonicated for 1 min and then the organic phase was removed to form liposomes by evaporation in a rotary evaporator at 30 °C under vacuum for 1 h. The resulting liposome was extruded through a polycarbonate membrane (100 nm pore size) using an extruder device (Lipex Biomembranes Inc., Canada) maintained at 60 °C to obtain liposomes of a homogeneous size. Unencapsulated free L-OHP was removed by ultracentrifugation at 200,000 × g for 20 min at 4 °C (Hitachi CS120, S100AT5 rotor), and the pellets were resuspended in 9% sucrose.

We prepared TF–PEG-liposomes by coupling TF to PEG-liposomes as described (Ishida et al., 2001). Briefly, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (S-NHS) were mixed (DSPE-PEG(3K)-COOH:EDC:S-NHS = 0.067:2.5:6.3, mole ratio) with 1 ml of PEG-liposomes comprising DSPC, CH, DSPE-PEG(2K), DSPE-PEG(3K)-COOH (2:1:0.16:0.032, molar ratio) in MES buffer (10 mM MES in 150 mM NaCl, pH 5.5), and incubated for 10 min at room temperature. The mixture was eluted through a Sephadex G-25 column equilibrated with MES buffer (pH 5.5) and liposome fractions were collected. Various amounts of TF were added to the liposome fractions and gently stirred for 3 h at room temperature. The mixture was centrifuged at 200,000 × *g* for 20 min at 4 °C, and then the pellet resuspended in 9% sucrose was mixed with $FeCl_3$–nitrilotriacetic acid to yield diferric TF. This suspension was centrifuged at 200,000 × *g* for 20 min at 4 °C, and the precipitated TF–PEG-liposomes were resuspended in 9% sucrose. The lipid concentration was estimated using a phosphorus assay. Size of liposomes was measured using an electrophoretic light scattering spectrophotometer (ELS-700, Otsuka Electronics, Tokyo) and the L-OHP content was determined using a microwave-induced plasma mass spectrometer (MIP-MS, P-7000, Hitachi, Tokyo) as described below.

2.4. In vitro cytotoxicity

Colon 26 cells (5×10^3 cells/well) were seeded into 96-well microplates and cultured overnight. Samples were added to each well and the plates incubated for 4 h at 37 °C. The medium was removed and fresh medium was added to each well. After incubation for a further 2 days, cell viability was measured using the WST-1 assay (Cell counting kit, Wako Pure Chemicals, Osaka, Japan).

2.5. Biodistribution in tumor-bearing mice

Tumor-bearing mice were prepared by inoculating a suspension of Colon 26 cells (2×10^6 cells) s.c. into the backs of BALB/c mice. Biodistribution was investigated when the tumors ranged from 6 to 8 mm in diameter. L-OHP solution or L-OHP encapsulated within Bare-, PEG- or TF–PEG-liposomes was injected into the mice via the tail vein. At selected intervals thereafter, the mice were lightly anesthetized, bled via the retro-orbital sinus, then killed by cervical dislocation and dissected. The organs were excised and their L-OHP content was determined using a MIP-MS as described below. Total blood was assumed to comprise 7.3% of the body weight. Contamination with blood in the organs was corrected by examining the distribution of ^{51}Cr-labeled erythrocytes (Maruyama et al., 1993).

2.6. Partitioning of L-OHP into erythrocytes

Samples of blood collected in the presence of anticoagulant (sodium citrate) were centrifuged to obtain plasma and the precipitate containing erythrocytes (erythrocytes fraction). And concentrations of L-OHP were measured at various time points in whole blood, plasma and erythrocyte fraction using a MIP-MS as described below. Data were presented as the percentage of the total injected dose for each sample.

2.7. Quantification of L-OHP

Liposomes, whole blood, plasma, erythrocyte or tissue samples were digested using a programmed microwave procedure. Diluted liposome samples, 20–100 mg of wet tissue or 100 μl of plasma were weighed and placed in PFA vials (Taf-tainer vial, GL Science, Tokyo). Thereafter, 400 μl of concentrated nitric acid and 200 μl of hydrogen peroxide (Tama Pure AA 100, Tama Chemicals, Tokyo) were added and digestion proceeded in a microwave oven (MLS-1200 MEGA; Milestone s.r.l., Italy) as follows; 250 W for 5 min, 0 W for 1 min, 250 W for 5 min, 400 W for 5 min, and 600 W for 5 min. The digested samples were brought to 1.0 ml with milli Q water, and then diluted. The content of L-OHP was measured using a MIP-MS. Europium (Eu) was added to the assay mixture and calibration standards at 0.1% and 20 ng/ml (final concentrations), respectively. The L-OHP concentrations were calculated from ion counts at platinum (Pt) using the calibration method with internal standard correction. The L-OHP concentrations in tissues were expressed as micrograms of L-OHP per gram tissue.

2.8. Measurement of serum albumin, total protein, glutamic–oxaloacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT) and blood urea nitrogen (BUN)

A solution of L-OHP or L-OHP encapsulated within Bare-, PEG- or TF–PEG-liposomes was injected into tumor-bearing mice via the tail vein at a dose of 5 mg L-OHP/kg. Three days later, the mice were anesthetized and blood samples were collected using glass capillaries from the vein of the fundus oculi. Serum obtained from blood samples by centrifugation was tested for albumin, total protein, GOT, GPT and BUN using respective kits (A/G B test Wako for albumin and total protein, GOT-UV Test Wako for GOT, GPT-UV Test Wako for GPT and Urea Nitrogen B Test Wako for BUN, Wako Pure Chemicals, Osaka, Japan).

2.9. Therapeutic effects of L-OHP encapsulated liposomes

A suspension of Colon 26 cells (2×10^6 cells) was inoculated s.c. into the backs of BALB/c mice. Therapeutic effects were examined when the tumors ranged from 8 to 10 mm in diameter. A solution of L-OHP or L-OHP encapsulated within Bare-, PEG- or TF–PEG-liposomes was injected twice into the tumor-bearing mice via the tail vein at a dose of 5 mg L-OHP/kg on days 9 and 12 after tumor cell inoculation. The two perpendicular diameters of tumors were obtained at intervals of a few days using a slide caliper and then tumor volumes were calculated using the formula 0.5 ($A \times B^2$), where A and B are the longest and shortest dimensions (mm) of the tumor, respectively. Tumor growth ratio was represented as the ratio for the tumor

volume on days 9 (before L-OHP treatment) after tumor cell inoculation.

2.10. Statistical analysis

Differences between PEG-liposomes and TF–PEG-liposomes were compared with unpaired Student's *t*-test.

3. Results

3.1. Cytotoxicity of L-OHP encapsulated TF–PEG-liposomes

L-OHP was encapsulated within Bare-, PEG- or TF–PEG-liposomes, all measuring about 180 nm in diameter. The amounts of encapsulated L-OHP within the Bare-, PEG- and TF–PEG-liposomes measured by MIP-MS were 12.3, 19.2 and 14.8 μg/mg liposomal lipid, respectively. We initially assessed the cytotoxicity of these liposomes against Colon 26 cells, which overexpress TF receptors, *in vitro*. Both L-OHP in solution and encapsulated within all three types of liposomes were cytotoxic against Colon 26 cells in a dose-dependent manner (Fig. 1). The ED_{50} values of L-OHP in solution and encapsulated within Bare-, PEG- and TF–PEG-liposomes were 2, 60, 18, and 8 μg/ml for Colon 26 cells, respectively. The L-OHP encapsulated within TF–PEG-liposomes was the most cytotoxic among the three types of the liposomes. In addition, we examined whether the cytotoxicity of L-OHP encapsulated within TF–PEG-liposomes was due to uptake of the liposomes via TF receptor into Colon 26 cells. When liposome uptake via TF receptors was inhibited by adding an excess of TF into medium, the cytotoxicity of L-OHP encapsulated within TF–PEG-liposomes was decreased (Fig. 1). On the other hand, blocking TF receptors did not influence the cytotoxicity of L-OHP encapsulated within PEG-liposomes. These results indicated that TF–PEG-liposomes were internalized into Colon 26 cells via TF receptor-mediated endocytosis and delivered L-OHP into the cytoplasm.

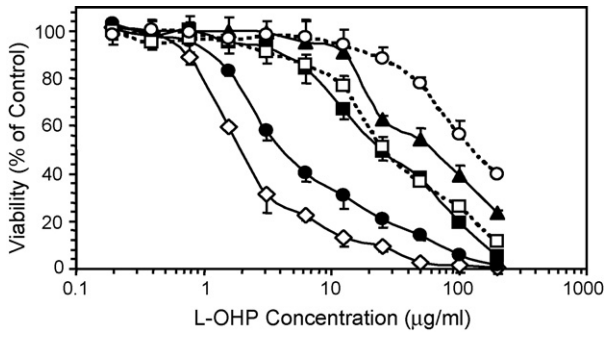

Fig. 1. Cytotoxicity of L-OHP in solution and liposomal L-OHP against Colon 26 cells. Cells were incubated with L-OHP in solution (open diamonds) or encapsulated within Bare- (solid triangles), PEG- (solid squares) or TF–PEG-liposomes (solid circles) without TF, or with PEG- (open squares) or TF–PEG-liposomes (open circles) with TF for 4 h 37 °C under 5% CO_2. Thereafter, cells were washed and incubated with fresh medium for 2 days at 37 °C in 5% CO_2. Cell growth was assayed using WST-1 assay. Maximal cell growth (100%) was obtained by incubating cells without L-OHP. Data are shown as means and standard deviation ($n = 5$).

3.2. Biodistribution of L-OHP encapsulated within TF–PEG-liposomes in mice bearing tumors

Liposomes with encapsulated L-OHP were injected i.v. into mice bearing Colon 26 tumors and L-OHP distribution was evaluated. Fig. 2A shows the time course of plasma clearance after the i.v. injection of L-OHP in solution and liposomal L-OHP. The L-OHP in solution was rapidly cleared from the blood circulation whereas the circulation of L-OHP encapsulated within liposomes was increased. The blood concentrations of L-OHP encapsulated within PEG- and TF–PEG-liposomes were much higher than that of L-OHP encapsulated within Bare-liposomes. We also assessed the biodistribution of L-OHP in solution and of L-OHP encapsulated within various liposomes at 6 h after i.v. injection (Fig. 2B). The results showed that very little L-OHP was distributed to the major tissues in mice. In contrast, the distribution of L-OHP encapsulated in PEG- and TF–PEG-liposomes to the liver and spleen differed, but far less of both was distributed to these tissues compared with Bare-liposomes. These results indicated that the PEG layer prolonged the systemic circulation of liposomes after i.v. injection. Thus, the conjugation of TF to the PEG terminal did not alter the RES uptake of PEG-liposomes, presumably because TF is a blood glycoprotein. Furthermore, PEG chains occupying the liposome surface played a role in the prolonged circulation of TF–PEG-liposomes.

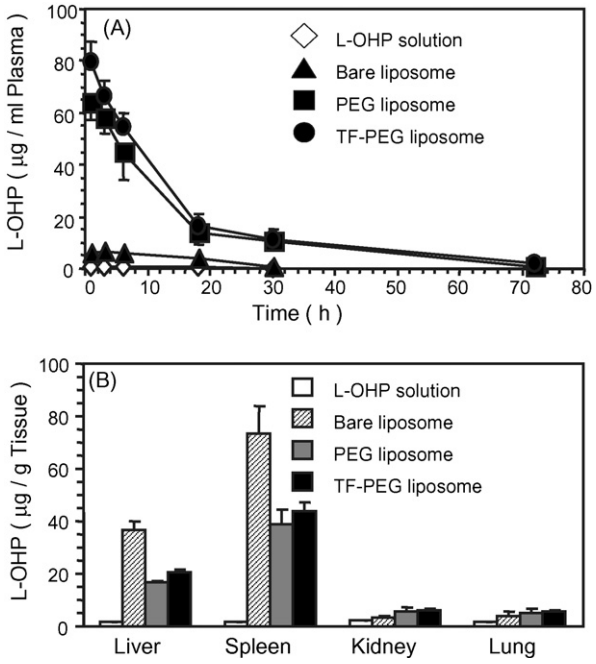

Fig. 2. Plasma clearance (A) and biodistribution (B) of L-OHP solution or liposomal L-OHP in Colon 26-bearing mice. (A) L-OHP in solution or encapsulated within Bare-, PEG- or TF–PEG-liposomes (L-OHP: 5 mg/kg) was injected via tail veins of Colon 26-bearing mice. At various times thereafter, blood samples were collected using glass capillaries from veins of fundus oculi. Plasma L-OHP levels were measured by MIP-MS. (B) L-OHP in solution or encapsulated within Bare-, PEG- or TF–PEG-liposomes (L-OHP: 5 mg/kg) was injected via tail veins of Colon 26-bearing mice. Six hours later, mice were sacrificed and liver, spleen, kidneys and lungs were collected. Concentrations of L-OHP in tissue samples were measured by MIP-MS. Data are shown as means and standard deviation ($n = 3$).

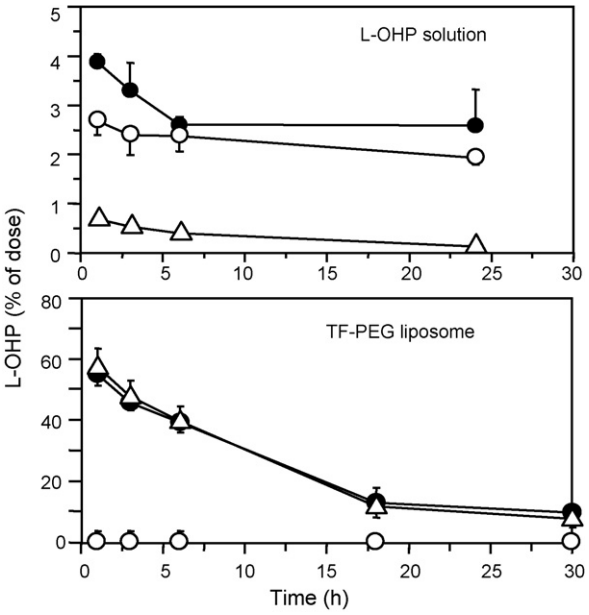

Fig. 3. Partitioning of L-OHP into the erythrocytes in whole blood as a function of time after injecting L-OHP in solution or in TF–PEG-liposomes into tail veins of Colon 26-bearing mice. Concentrations of L-OHP were measured at various time points in whole blood (solid circle), plasma (open triangle) and erythrocyte fraction (open circle). Data are presented as the percentage of the total injected dose for each sample. Data are shown as means and standard deviation ($n = 3$).

When L-OHP in solution was injected, the total L-OHP concentration in whole blood was much lower than that of L-OHP in TF–PEG-liposomes, and most of L-OHP in whole blood was taken up by erythrocytes (Fig. 3). In contrast, little L-OHP was distributed to erythrocytes in whole blood when L-OHP encapsulated in TF–PEG-liposomes was injected (Fig. 3). The degree of L-OHP partitioning to erythrocytes remarkably differed between the solution and TF–PEG-liposomes, since the latter were stable in the blood circulation and little L-OHP was released from the liposomes.

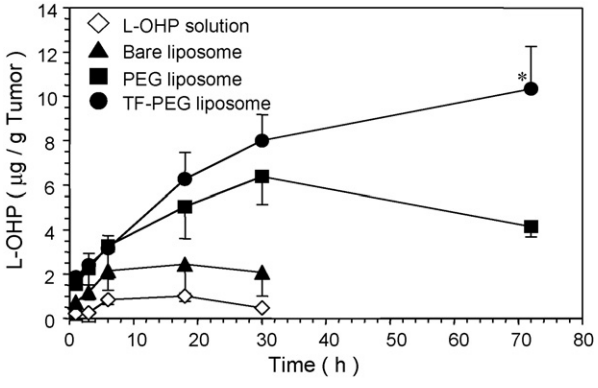

Fig. 4. Time course of tumor accumulation of L-OHP in Colon 26-bearing mice. L-OHP in solution or encapsulated within Bare-, PEG- or TF–PEG-liposomes (L-OHP: 5 mg/kg weight) was injected via tail veins of Colon 26-bearing mice. At various times thereafter, tumor tissue was collected from the mice. Concentrations of L-OHP in the tumor tissues were measured by MIP-MS. Data are shown as means and standard deviation ($n = 3$). *$P < 0.05$ (PEG-liposomes vs. TF–PEG-liposomes).

Fig. 4 shows the time course of L-OHP in Colon 26 solid tumor tissue after i.v. injection of L-OHP in solution and encapsulated within Bare-, PEG- or TF–PEG-liposomes. The concentrations of L-OHP in tumor tissue at 18 h after i.v. injection of L-OHP in solution and in Bare-liposomes were 0.98 and 2.1 μg/g tumor, respectively, and did not increase thereafter. The concentrations of L-OHP encapsulated within PEG- and TF–PEG-liposome in tumor tissue were even higher than that of L-OHP in solution and in Bare-liposomes. The L-OHP concentration in tumor tissue decreased 30 h after i.v. injection of L-OHP encapsulated within PEG-liposomes. Interestingly, the profiles of L-OHP encapsulated within TF–PEG- and PEG-liposomes in tumor tissues differed. The concentration of L-OHP encapsulated within TF–PEG-liposomes continued to increase until 72 h after i.v. injection and a high L-OHP concentration was maintained in the tumor for a longer period.

3.3. Assessment of side effects for L-OHP encapsulated within TF–PEG-liposomes

The circulation of L-OHP in the blood was prolonged when encapsulated within PEG- and TF–PEG-liposomes and the biodistribution of L-OHP encapsulated within liposomes differed from that of free L-OHP. We confirmed that liposomal L-OHP was distributed to major tissues. Thus unexpected side effects might arise when liposomal L-OHP is injected. To determine the toxicity of liposomal L-OHPs *in vivo*, we measured levels of serum albumin, total protein, GOT, GPT and BUN (Table 1). GOT, GPT and BUN are the markers of toxicity for liver. In addition, GOT is utilized as the marker of toxicity for heart. Moreover, BUN is mainly the marker of kidney. The BUN levels of mice injected with liposomal L-OHP did not differ from those of control mice injected with saline. We therefore considered that liposomal L-OHPs did not cause significant toxicity for liver and kidney. Furthermore, the other biochemical parameters tested in mice injected with liposomal L-OHPs did not significantly differ as compared with non-treated and saline-injected mice.

3.4. Therapeutic effect of L-OHP encapsulated within TF–PEG-liposomes

Mice bearing Colon 26 tumors were injected with L-OHP in solution or encapsulated within various liposomes and the therapeutic effects were examined by measuring the suppression of tumor growth (Fig. 5). Tumor growth was somewhat suppressed to a similar extent by L-OHP in solution and encapsulated within Bare- or PEG-liposomes, but obviously suppressed by L-OHP encapsulated within TF–PEG-liposomes.

4. Discussion

L-OHP is a cisplatin derivative that was designed to improve side effects such as toxicity to the kidney and peripheral nerve system. However, most L-OHP in the whole blood of mice injected with L-OHP in solution was taken up by erythrocytes, so that the levels of free L-OHP in plasma were very

Table 1
Serum biochemistry of mice injected with L-OHP in solution and encapsulated within Bare-, PEG- or TF–PEG-liposomes

	Time after injection (days)	Albumin (g/dL)	Total protein (g/dL)	GOT (IU/L)	GPT (IU/L)	BUN (mg/dL)
Normal		3.1 (0.1)	4.3 (0.3)	51.3 (6.4)	29.6 (3.9)	15.9 (2.3)
Saline	3	2.8 (0.3)	4.3 (0.3)	43.0 (4.2)	29.0 (7.5)	22.9 (0.2)
Solution	3	2.8 (0.2)	4.4 (0.1)	45.5 (7.7)	27.2 (1.4)	16.7 (4.7)
Bare	3	2.8 (0.1)	4.4 (0.1)	44.8 (11.7)	22.8 (3.1)	20.8 (2.8)
PEG	3	2.9 (0.1)	4.4 (0.0)	41.6 (4.4)	27.3 (3.3)	18.3 (1.1)
TF–PEG	3	2.8 (0.1)	4.5 (0.2)	41.2 (3.3)	23.3 (3.4)	19.3 (0.7)

L-OHP (5 mg/kg) was injected via tail veins of Colon 26-bearing mice. Blood samples were collected from veins of fundus oculi on 3 days after injection of L-OHP, and serum samples were isolated from blood samples by centrifugation. Serum albumin, total protein, GOT, GPT and BUN were measured using respective kits. Data are shown as means and standard deviation ($n = 3$).

low. Pendyala and Creaven revealed that erythrocytes do not serve as reservoirs of partitioned drugs (Pendyala and Creaven, 1993). Thus, to maintain a therapeutic concentration of free L-OHP *in vivo* after injecting L-OHP in solution is difficult. Thus, L-OHP should be delivered directly to tumor cells to exert a powerful anti-tumor effect. The TF–PEG-liposomes encapsulating L-OHP that we prepared here appear to satisfy these requirements and should be very useful for cancer chemotherapy. We found that injected TF–PEG-liposomes resulted in a high concentration of L-OHP being maintained in mouse tumor tissue, and that tumor growth was more suppressed than with PEG-liposomes, Bare-liposomes and free L-OHP in solution. We previously reported that TF–PEG-liposomes are useful as a carrier targeting the cytoplasm of tumor cells *in vitro* and *in vivo* (Ishida et al., 1999, 2001; Iinuma et al., 2002; Hatakeyama et al., 2004; Kakudo et al., 2004; Maruyama et al., 2004; Miyajima et al., 2006). Tumor growth potential is reflected by abundant TF receptors in cancerous cells (Huebers and Finch, 1987). Thus, TF receptors might be a viable target molecule for therapy. We found between 2.5 and 5×10^5 surface TF receptors per Colon 26 cell (data not shown) (Ishida et al., 2001). Indeed, L-OHP encapsulated within TF–PEG-liposomes suppressed tumor cell growth *in vitro* and the suppression was abrogated by adding an excess of free TF into the medium. TF–PEG-liposome had about TF 20–25 molecules per liposome (Ishida et al., 2001). In this study, we showed that TF–PEG-liposomes were effectively internalized via TF receptor-mediated endocytosis, so that a large amount of L-OHP was introduced into cytoplasm of tumor cells.

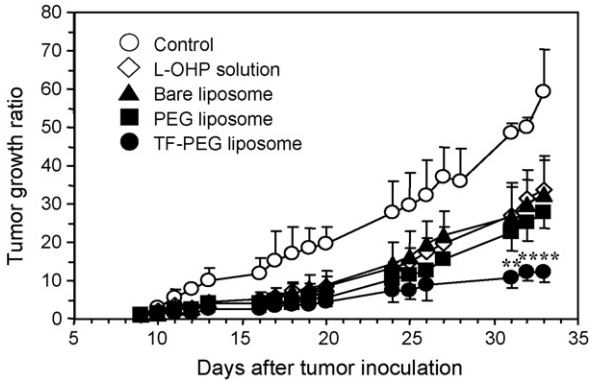

Fig. 5. Comparison of tumor growth suppression with L-OHP in solution and in liposomes in Colon 26-bearing mice. L-OHP solution or L-OHP encapsulated within Bare-, PEG- or TF–PEG-liposomes (L-OHP: 5 mg/kg) was injected via tail veins of Colon 26-bearing mice on days 9 and 12 after tumor cell inoculation. Tumor growth ratio was calculated as described in Section 2. Data are shown as means and standard deviation ($n = 4$). $^{**}P < 0.01$ (PEG-liposomes vs. TF–PEG-liposomes).

Intravenous injection of liposomal L-OHP into tumor-bearing mice resulted in increased L-OHP accumulation in solid tumors compared with free L-OHP. This phenomenon is referred to as the EPR effect of long circulating liposomes in solid tumor tissues. The most likely mechanism is the increased amount of time that the liposomes circulate in the blood and the leaky nature of the microvasculature in solid tumor tissues. We previously found that the size of the liposomes is an important factor for extravasation (Ishida et al., 1999). The circulation of TF–PEG- and PEG-liposomes with an average diameter of 100–200 nm was the most prolonged and the tumor accumulation was the highest. Thus, the accumulation of TF–PEG- and PEG-liposomes in tumor tissues is directly associated with the AUC_{blood} of the plasma clearance (Ishida et al., 2001; Maruyama et al., 2004). In this regard, a low AUC_{blood} due to high uptake of Bare-liposomes by the RES would lead to low tumor accumulation (Maruyama et al., 2004), even if the tumor vasculature were leakier than normal tissue. In other words, only small liposomes (about 100–200 nm mean diameter) with prolonged blood circulation would reach extravascular target sites (Ishida et al., 1999). Effective anti-tumor therapy by L-OHP requires its internalization into the cytoplasm of tumor cells because this drug works via the inhibition of DNA synthesis and transcription by forming DNA adducts (Pendyala et al., 1995). The therapeutic effects of PEG-liposomes and of L-OHP in solution were similar. We therefore considered that PEG-liposomes could deliver L-OHP to tumor tissue through the EPR effect, but were not effectively internalized into the cytoplasm. In contrast, TF–PEG-liposomes appeared to deliver L-OHP into the cytoplasm of tumor cells via TF receptor-mediated endocytosis after extravasation by the EPR effect (Ishida et al., 2001). Actually, the amount of time that PEG- and TF–PEG-liposomes remained in tumor tissues differed despite prolonged long circulation in the blood. These results supported the notion that TF–PEG-liposomes deliver L-OHP to the surface and the interior of tumor cells. Prolonged circulation of TF–PEG-liposomes in the blood increased the amount of time that L-OHP was exposed to various tissues including tumors. In addition, it was reported that TF receptors were also expressed normal cells such as the

hepatocyte and endothelial cells in the brain (Jefferies et al., 1984; Bomford and Munro, 1985). We considered the possibility of a concomitant increase in the incidence of acute toxicity after L-OHP single i.v. injection in Table 1. However, blood biochemistry tests revealed that the tested parameters remained within the normal range. These findings indicated that L-OHP encapsulated within TF–PEG-liposomes did not induce any significant acute toxicity in the presence of increased distribution to the liver compared with L-OHP in solution. We also examined about the biodistribution of L-OHP encapsulated within TF–PEG-liposomes in other experiment, the concentration of L-OHP in the brain was very low (about 0.06 μg/g tissue) at 72 h after i.v. injection of the liposomes. Actually, abnormal behaviors were not observed in the mice. Therefore, it was thought that significant side effects were not induced in the brain. In addition, we did not observe the remarkable decreasing of body weight after i.v. injection two times of L-OHP using the liposomes in Fig. 5. These results indicated that the frequency of serious side effects was minimal.

Although L-OHP is a useful anti-tumor drug, it is rapidly cleared from the blood. We addressed this issue by using TF–PEG-liposomes as a carrier of L-OHP. In addition, 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 (Ishida et al., 2001). Considering these results of electron microscopic studies, it was thought that TF–PEG-liposomes effectively delivered L-OHP into the cytoplasm of tumor cells bearing TF receptors. To our knowledge, we are the first to show a therapeutic effect of L-OHP against solid tumors using TF–PEG-liposomes as the carrier.

Kono et al. developed a polymer with fusogenic ability at low pH (Kono et al., 1997). In addition, another group reported that dioleoyl phosphatidylethanolamine (DOPE) can also destabilize endosomal membranes depending on endosomal pH. Thus, if the polymer and/or DOPE are applied to TF–PEG-liposomes with encapsulated L-OHP, the efficiency of L-OHP delivery into cytoplasm might increase. Further optimization of TF–PEG-liposomes encapsulating L-OHP should eventually result in the development of a highly effective cancer therapy.

In conclusion, TF–PEG-liposomes, which allow both passive and active targeting, might be a potential carrier for *in vivo* cytoplasmic targeting of L-OHP in mice with solid tumors overexpressing surface TF receptors. We believe that TF–PEG-liposomes show promise in the clinical environment as an ideal carrier for chemotherapy against various types of tumors that overexpress TF receptors.

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