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Recombinant human tumor necrosis factor-α covalently conjugated to long-circulating liposomes

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Abstract

Recombinant tumor necrosis factor-α (rHuTNF) was covalently conjugated to a phospholipid, *N*-glutaryl phosphatidylethanolamine (NGPE). The resultant rHuTNF-NGPE conjugates were incorporated into liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) with or without polyethyleneglycol conjugated to phosphatidylethanolamine (PEG3000-PE). Efficient incorporation (35–50%) of rHuTNF-NGPE conjugates into liposomes was obtained for both PC/Chol and PC/Chol/PEG3000-PE liposomes. An in vitro cytotoxicity assay showed that rHuTNF-NGPE conjugates incorporated into liposomes exhibit a reduced biological activity as compared to the free rHuTNF. Biodistribution studies using ^{125}I-labeled rHuTNF showed a significant increase in the circulation time of rHuTNF by incorporation into PC/Chol/PEG3000-PE liposomes, but not conventional PC/Chol liposomes. However, studies using a radioactive lipid as a liposome marker showed that incorporation of rHuTNF-NGPE conjugates resulted in increased clearance from the blood and accumulation in the spleen and liver of both liposomal formulations. The liposome clearance from the blood depends on the protein/lipid ratio of liposomes. The higher the protein/lipid ratio, the higher the liposome clearance from the blood and accumulation in the spleen and liver, suggesting that accumulation of rHuTNF-bound liposomes in the spleen and liver involves interactions with TNF-receptors in these organs.

Keywords: Biodistribution; Drug delivery; Long-circulating liposome; Recombinant tumor necrosis factor-α (rHuTNF)

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Abbreviations: Chol, cholesterol; DCC, 1,3-dicyclohexylcarbodiimide; DTPA-SA, diethylenetriamine pentaacetic acid distearylamide complex; NGPE, *N*-glutaryl phosphatidylethanolamine; NHS, *N*-hydroxysuccinimide; OG, octyl-β-D-glucopyranoside; PBS, phosphate buffered saline; PC, egg phosphatidylcholine; PEG, polyethyleneglycol; PEG-PE, dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine; RES, reticuloendothelial system; rHuTNF, recombinant human tumor necrosis factor-α.

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1. Introduction

Tumor necrosis factor-α (TNF[a]) is a cytokine with a wide range of biological functions (Fiers, 1991). Cytotoxic and cytostatic activities of TNF against certain tumors have attracted many investigators with a prospect that this cytokine could be used as a specific antitumor drug (Porter, 1991; Sidhu and Bollon, 1992). Recombinant human TNF (rHuTNF) has been in intense evaluations in both preclinical and clinical studies. Studies in clinical trials, however, showed that the usefulness of rHuTNF as an antitumor agent is limited by dose-limiting side effects such as fever, myalgia and hypotension (Spriggs and Yates, 1992).

Various strategies to improve the therapeutic efficacy of rHuTNF therapy have been proposed (Sidhu and Bollon, 1992). One approach is targeted to prolong the circulation time of rHuTNF in the blood. rHuTNF has been shown to be cleared rapidly from the blood with a half-life of < 20 min (Pacici et al., 1987; Ferraiolo et al., 1988; Ferraiolo et al., 1989). Studies using radiolabeled proteins showed that the liver and kidney are major sites of accumulation of rHuTNF after i.v. and i.m. administrations (Ferraiolo et al., 1988). It has been shown that the liver plays an important role in protein clearance, while the kidney is an important site of catabolism for low molecular weight proteins (< *M*r. 50 000) including rHuTNF (McMartin, 1992). Thus, it would be reasonable to hypothesize that a suitable carrier with a low affinity for the liver and kidney may provide a system to increase the circulation time of rHuTNF in the blood.

Liposomes have been used for formulations of a broad range of therapeutic agents (Lasic, 1993). Conventional liposomes have been used to target therapeutic agents to the spleen and liver owing to their high affinity for the reticuloendothelial system (RES) in these organs (Alving, 1986). Several investigators previously described liposomal formulations of rHuTNF (Debs et al., 1989; Debs et al., 1990; Nii et al., 1991). These studies showed that rHuTNF encapsulated into liposomes retains its biological activity in vitro (Debs et al., 1989; Nii et al., 1991) and exhibits reduced systemic toxicity in vivo as compared to free rHuTNF (Debs et al., 1990). However, these liposomal formulations with high affinity for the RES may not be suitable when a target is located outside the RES organs.

With the development of liposomes exhibiting a reduced affinity for the RES and prolonged circulation times in the blood, the drug targeting by liposomes is no longer limited to the spleen and liver (Klibanov and Huang, 1992; Woodle and Lasic, 1992). Many studies have showed potential usefulness of long-circulating liposomes for targeting a wide variety of therapeutic agents, such as traditional antitumor drugs with a low molecular weight (Papahadjopoulos et al., 1991; Unezaki et al., 1993) and protein and peptide drugs (Maruyama et al., 1991; Woodle et al., 1992), to the non-RES organs. For example, several studies showed that long-circulating liposomes are able to deliver therapeutic agents to the solid tumor more efficiently than the conventional liposomes (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991; Unezaki et al., 1993).

In the present study, in an attempt to develop a system capable of increasing the circulation time of rHuTNF in the blood and its distribution to non-RES organs, rHuTNF was formulated in long-circulating liposomes containing polyethyleneglycol conjugated to phosphatidylethanolamine (PEG3000-PE). Since rHuTNF normally exhibits a poor encapsulation efficiency due to its low affinity for liposome membranes, rHuTNF was first modified with a phospholipid, *N*-glutaryl phosphatidylethanolamine (NGPE), and incorporated into liposomes. These rHuTNF-bound liposomes were characterized with respect to their biodistribution in mice and biological activity in an in vitro system.

2. Materials and methods

2.1. Materials

Recombinant human tumor necrosis factor-α (rHuTNF), with a specific activity of 2×10^7

units per mg protein, was produced in *Escherichia coli* as previously described (Pennica et al., 1984) and contained less than 0.025 ng endotoxin per mg protein. rHuTNF was radiolabeled with ^{125}I using the Iodo-Gen method (Pierce, Rockford, IL) to a specific activity of 4×10^8 cpm per mg protein. ^{125}I-labeled protein was purified using a spin column chromatography on Sephadex G-25. Egg phosphatidylcholine (PC) and dioleoyl phosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL), and cholesterol (Chol) was obtained from Sigma Chemical Co. (St. Louis, MO). Polyethyleneglycol (PEG, *M*r. 3000) was obtained from Nippon Oil and Fats Co. (Tsukuba, Japan). 1,3-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade. Synthesis of dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine (PEG-PE) (Klibanov et al., 1991) and diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) (Kabalka et al., 1987) has been described previously. Radiolabeling of DTPA-SA with ^{111}In was performed as described previously (Klibanov et al., 1991).

2.2. rHuTNF derivatization

NGPE dissolved in $CHCl_3$ was mixed with NHS in *N,N*-dimethyl formamide and DCC in $CHCl_3$ at the NGPE/NHS/DCC molar ratio of 1:1:3. The reaction mixture was incubated overnight at the room temperature. The activation of NGPE to its NHS ester (NGPE-OSu) was confirmed by the thin-layer chromatographic analysis of the reaction mixture and subsequently by visual identification under UV lamp before/after NH_3 exposure. The reaction mixture was placed at -20°C to facilitate precipitation of reagents which were subsequently removed by filtration. The NGPE-OSu obtained was dried with N_2 gas, vacuum desiccated, redissolved in $CHCl_3$ at 10 mg/ml, and kept in desiccator at -20°C before use. For conjugation of rHuTNF to NGPE, the solvent-free NGPE-OSu was first solubilized with octyl-β-D-glucopyranoside (OG) in HEPES buffer (pH 7.4) at the NGPE-OSu/OG molar ratio of 0.06:1. rHuTNF with a trace amount of ^{125}I-labeled rHuTNF were then added to solubilized NGPE-OSu at the rHuTNF/NGPE molar ratio of 1:10. The resultant mixture was incubated for 12 h at 4°C with gentle stirring to obtain rHuTNF-NGPE conjugates and used immediately for incorporation into liposomes.

2.3. Liposome preparation

rHuTNF-bound large unilamellar liposomes composed of PC/Chol (10:5, mol/mol) and PC/Chol/PEG3000-PE (10:5:1, mol/mol) were prepared by the detergent-dialysis method. The solvent-free lipid mixture containing ^{111}In-labeled DTPA-SA, as a nonexchangeable and nonmetabolizable lipid marker, at 1.0 mol% of the lipid mixture was solubilized with OG (100 mM in phosphate-buffered saline (PBS), pH 7.4) at the lipid/OG molar ratio of 1:5. The resultant solution was mixed vigorously with the ^{125}I-labeled rHuTNF-NGPE conjugates normally at the protein/lipid weight ratio of 1:100. For the preparation of liposomes containing different amounts of protein conjugates, rHuTNF-NGPE conjugates were mixed with lipids at various protein/lipid ratios. The mixture was then dialyzed against PBS (pH 7.4) for 36 h at 4°C to remove the detergent. The resulting rHuTNF-bound liposomes were extruded $\sim$ 20 times through stacked 0.4- and 0.2-μm Nucleopore membranes using Liposo-Fast™ (Avestin, Inc., Ottawa, ON, Canada) to generate liposomes with homogeneous size distributions. Liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, FL) and expressed as average diameter with S.D. The extruded rHuTNF-bound liposomes were chromatographed on a BioGel A1.5M column to remove the unbound protein. The peak liposome fractions were pooled, and the amount of protein incorporated into liposomes and the protein/lipid weight ratio were calculated from the specific radioactivities of ^{125}I for the protein and ^{111}In for lipids. For the ^{125}I radioactivity, the overflow of ^{111}In into ^{125}I channel on a Beckman gamma-counter was estimated and subtracted from ^{125}I radioactivity countings.

2.4. Biodistribution study

Free ^{125}I-labeled rHuTNF or ^{125}I-labeled rHuTNF-NGPE conjugates incorporated in ^{111}In-labeled liposomes were injected i.v. into female C3H/HeJ mice (6–8 weeks old) (Charles River Laboratories, Wilmington, MA) at a dose of 0.5 μg rHuTNF or 0.1 mg lipid per mouse in 0.2 ml of PBS (pH 7.5). At specified time intervals, mice were anesthetized, bled by retroorbital puncture, sacrificed by cervical dislocation and dissected. Blood and major organs including the spleen, liver, lung, heart and kidney were collected and weighed. Biodistribution of rHuTNF and liposomes was determined by analyses of ^{125}I and ^{111}In radioactivity countings, respectively, in each organ using a Beckman gamma-counter. Data were expressed as the percentage of the total injected dose of rHuTNF and liposomes in each organ. Liposome levels in the blood were determined by assuming that the blood volume of mouse is 7.3% of the body weight (Wu et al., 1981).

2.5. Cytotoxicity assay

The cytotoxicity of free rHuTNF and rHuTNF-NGPE conjugates incorporated into liposomes was assessed with L929 mouse fibroblast according to the method described previously (Ruff and Gifford, 1981). Briefly, L929 cells, grown in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (200 units/ml) and streptomycin (100 μg/ml), were plated onto 96-well plates at 2.5×10^4 cells in 100 μl of the media per well and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2/95% air. After washing the cells, 200 μl of the medium containing actinomycin D (1 μg/ml) (Sigma) and various concentrations (0.0002–200 ng/ml) of free or liposomal rHuTNF were added. After incubation for 16 h at 37°C, the cells were washed 3 times with Hank's balanced salt solution (HBSS), fixed with methanol/acetic acid (3:1, vol/vol), and incubated for 5 min with crystal violet (Sigma) (0.5 mg/ml) dissolved in 20% ethanol. Cells were then extensively washed with PBS (pH 7.4), and lysed with the addition of 100 μl of 20% methanol/10% acetic acid. The amount of crystal violet absorbed in the cells was quantitated by measurement of the absorbance at 540 nm using a multiscan auto platereader. Data were expressed as the percentage of viable cells relative to the control cultures incubated in the medium. The IC_{50} for each rHuTNF formulation was determined graphically.

3. Results

3.1. Incorporation of rHuTNF-NGPE conjugates into liposomes

In the present method, rHuTNF was first conjugated with preactivated NGPE, and resulting rHuTNF-NGPE conjugates were mixed with lipids in the presence of detergent. Incorporation of rHuTNF-NGPE conjugates into liposomes was achieved upon removal of the detergent by dialysis. Subsequent extrusion produced liposomes with relatively narrow size ranges. Incorporation of rHuTNF-NGPE conjugates into liposomes did not cause aggregation or precipitation of lipids and/or proteins, suggesting that the conjugates are fully compatible with liposomal lipids. Fig. 1 shows the representative elusion profiles for ^{125}I-labeled protein and ^{111}In-labeled liposomes, obtained by gel permeation chromatography of extruded liposomes. rHuTNF-NGPE conjugates and liposomes were co-eluted with identical peak fractions as determined by analyses of ^{125}I and ^{111}In radioactivities, respectively, indicating incorporation of rHuTNF-NGPE conjugates into liposome membranes. The second peak for ^{125}I radioactivity represents the free unbound rHuTNF. The incorporation efficiency of rHuTNF into liposomes varied to some degrees from experiment to experiment. Normally, 30–50% of the initial amount of rHuTNF was incorporated into liposomes. Incorporation efficiencies for PC/Chol and PC/Chol/PEG3000-PE liposomes were, however, similar in parallel preparations (data not shown), indicating that the presence of PEG3000-PE does not affect incorporation of rHuTNF-NGPE conjugates. When the addition of NGPE-OSu was omitted in the deriva-

tization reaction of rHuTNF, only a small fraction (6.1%) of rHuTNF was co-eluted with liposomes. Thus, efficient incorporation of rHuTNF into liposomes was due to its conjugation with NGPE. rHuTNF-bound liposomes were stable with respect to size for at least several weeks as assessed using dynamic laser light scattering. In addition, gel permeation chromatographic analyses of rHuTNF-bound liposomes showed that modified rHuTNF did not dissociate from liposomes at 2 weeks after the preparation.

3.2. Disposition of rHuTNF-NGPE conjugates

Since rHuTNF-NGPE conjugates are incorporated into liposome membranes, it is expected that the disposition of rHuTNF-NGPE conjugates after systemic administration would be altered depending on liposomes used for the formulation. To examine this, ^{125}I-labeled rHuTNF-NGPE conjugates were incorporated into liposomes with the lipid compositions of PC/Chol and PC/Chol/PEG3000-PE at similar protein/lipid weight ratios

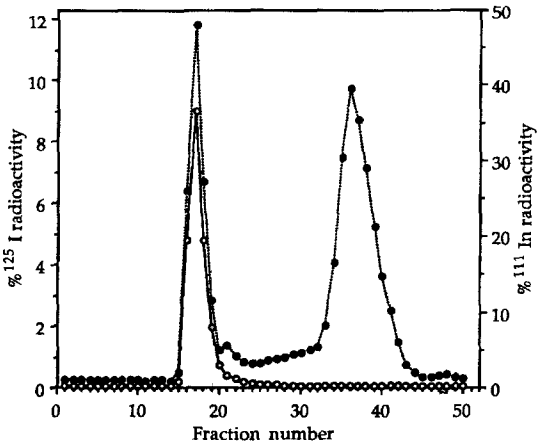

Fig. 1. Incorporation of rHuTNF-NGPE conjugates into liposomes. ^{125}I-labeled rHuTNF were conjugated with NGPE, and incorporated into ^{111}In-labeled liposomes with a lipid composition of PC/Chol as described in Materials and methods. rHuTNF-bound liposomes were analyzed with gel permeation chromatography on a BioGel A1.5M column, and fractions were analyzed for (●) rHuTNF and (○) liposomes by ^{125}I and ^{111}In radioactivity counting, respectively. Data were plotted using the percentage of rHuTNF and liposomes in each fraction.

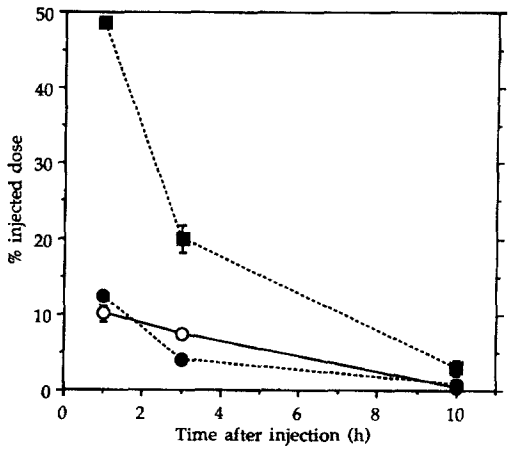

Fig. 2. Blood levels of i.v. injected free and liposomal rHuTNF. ^{125}I-labeled rHuTNF-bound liposomes were prepared by the detergent-dialysis method and injected i.v. into mice at a dose of 0.5 μg rHuTNF or 0.1 mg lipid per mouse. Levels of rHuTNF in the blood were examined at indicated time intervals after injections. Bars represent S.D.; n = 3. (○) free rHuTNF; (●) rHuTNF-bound PC/Chol liposomes, protein/lipid ratio = 1:195 (w/w), average diameter = 156 (32) nm; (■) rHuTNF-bound PC/Chol/PEG3000-PE liposomes, protein/lipid ratio = 1:193, average diameter = 127 (narrow) nm.

(1:195 and 1:193, respectively). These liposomes also had similar average diameters (156 ± 42 and 127 ± 34 nm, respectively). Fig. 2 shows the percentage of injected proteins in the blood at different time intervals after injections. Free ^{125}I-labeled rHuTNF was cleared rapidly from the blood. Incorporation of rHuTNF-NGPE conjugates into PC/Chol/PEG3000-PE liposomes resulted in significantly lower protein clearance from the blood than free rHuTNF. On the other hand, rHuTNF-NGPE conjugates incorporated into PC/Chol liposomes did not show a prolonged circulation time.

Table 1 shows the tissue disposition of free and liposomal rHuTNF at 1 h after injection. Free rHuTNF accumulated primarily in the liver (6.1%) and secondarily in the kidney (3.1%). Only a small fraction (21%) of injected free rHuTNF was recovered from major organs, while 78% and 86% of rHuTNF-NGPE conjugates were recovered when incorporated in PC/Chol and PC/Chol/PEG3000-PE liposomes, respectively. Substantial amounts of rHuTNF-NGPE conjugates accumu-

Table 1
Disposition of free and liposomal rHuTNF[a]

rHuTNF formulation[b]	% injected dose[c]						Total recovery (%)
	Blood	Spleen	Liver	Lung	Heart	Kidney	
Free	10.1 (1.1)	0.4 (0.1)	6.1 (0.7)	1.2 (0.2)	0.3 (0.1)	3.1 (0.5)	21.1 (1.3)
PC/Chol	12.3 (0.6)	24.7 (1.6)	38.2 (3.8)	1.0 (0.2)	0.5 (0.2)	1.4 (0.1)	78.1 (1.9)
PC/Chol/PEG3000-PE	48.6 (0.7)	2.4 (0.1)	28.8 (2.2)	2.6 (0.5)	0.8 (0.2)	2.8 (0.3)	86.1 (3.3)

[a]^{125}I-labeled free and liposomal rHuTNF was injected i.v. into mice at 0.5 μg rHuTNF per mouse. Disposition of rHuTNF in indicated organs was examined at 1 h after injection.[b]Molar ratios of the lipid composition are as described in Materials and methods. The protein/lipid weight ratio and average diameter of liposomes were: PC/Chol liposomes, 1:195 and 156(32) nm; PC/Chol/PEG3000-PE liposomes, 1:193 and 127 (narrow) nm.[c]Data are expressed as mean (S.D.), $n = 3$.

lated in the spleen and liver when incorporated into either liposome formulation. However, the levels of accumulation of rHuTNF-NGPE conjugates in the spleen and liver were lower when incorporated into PC/Chol/PEG3000-PE liposomes than PC/Chol liposomes.

3.3. Biodistribution of rHuTNF-bound liposomes

The effect of incorporation of rHuTNF-NGPE conjugates on liposome biodistribution was then examined by determination of ^{111}In-labeled liposomes in organs in the parallel experiment shown in Fig. 2. Fig. 3 shows the percentage of injected liposomes in the blood, spleen and liver at different time intervals after injections. Conventional PC/Chol liposomes were cleared rapidly from the blood and accumulated exclusively in the spleen and liver. PC/Chol/PEG3000-PE liposomes showed higher levels in the blood and lower accumulation in the spleen and liver than PC/Chol liposomes. For example, approximately 62% of injected PC/Chol/PEG3000-PE liposomes still remained in the blood at 3 h after injection as compared to only 9.7% for PC/Chol liposomes. Incorporation of rHuTNF-NGPE conjugates into liposomes resulted in their increased clearance from the blood. The fraction of rHuTNF-bound liposomes remaining in the blood at 3 h after injection decreased to 4.1% and 31% for PC/Chol and PC/Chol/PEG3000-PE liposomes, respectively, as compared to respective protein-free liposomes. The high clearances of rHuTNF-bound liposomes from the blood were accompanied with their high accumulation in the spleen and liver. These results indicate that rHuTNF-bound liposomes exhibit a higher affinity for the spleen and liver than protein-free liposomes, irrespective of the lipid composition. Nevertheless, rHuTNF-bound PC/Chol/PEG3000-PE liposomes showed longer circulation times than rHuTNF-bound PC/Chol liposomes.

To examine further the effect of the protein incorporation on liposome biodistribution, PC/Chol/PEG3000-PE liposomes containing varying amounts of rHuTNF (protein/lipid weight ratios of 1:193–1:1288) were prepared by altering the initial protein/lipid ratio in the reaction mixture. These preparations with different initial protein/lipid ratios had similar incorporation efficiencies for the protein (47% ~ 52%). Table 2 shows the percentage of injected liposomes in the blood, spleen and liver at 3 h after injection, together with a respective RES (spleen + liver)/blood ratio. All rHuTNF-bound liposomes showed lower levels in the blood and higher levels of accumulation in the spleen and liver than protein-free liposomes. Among various rHuTNF-bound liposomes, liposomes with the highest protein/lipid weight ratio (1:193) showed the lowest level (31%) in the blood with the RES/blood ratio of 1.9. With decreasing the protein/lipid weight ratios to 1:600 and 1:1288, higher levels of liposomes (44% and 46%, respectively) remained in the blood with the RES/blood ratios of 0.7 and 0.8, respectively. These results indicate that the affinity of rHuTNF-bound liposomes for the spleen and liver, and thus their level of clearance

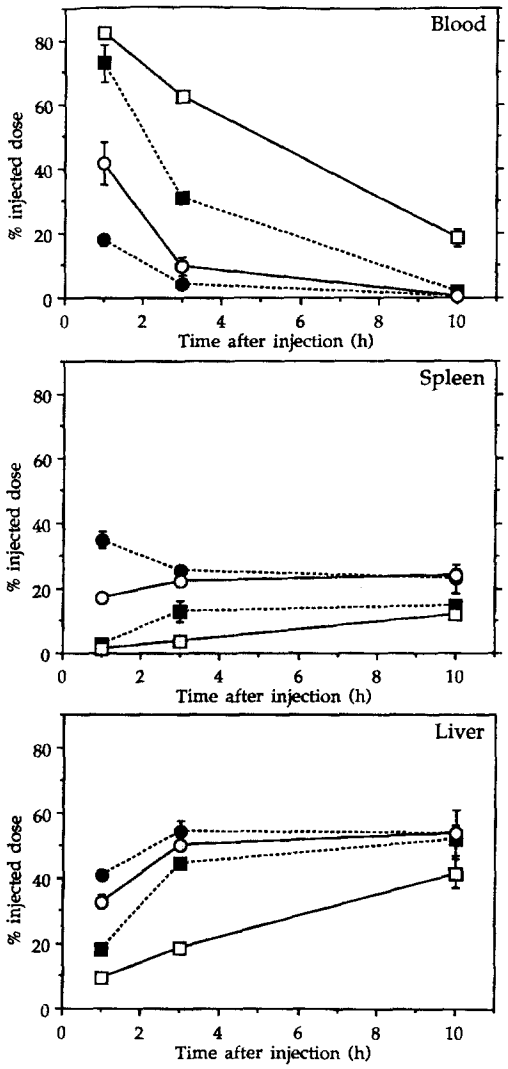

Fig. 3. Biodistribution of rHuTNF-bound liposomes. ^{111}In-labeled liposomes with or without rHuTNF-NGPE conjugates were prepared by the detergent-dialysis method and injected i.v. into mice at a dose of 0.5 μg rHuTNF or 0.1 mg lipid per mouse. Liposome levels in the blood, spleen and liver were examined at indicated time intervals after injections. Bars represent S.D.; n = 3. (○) PC/Chol liposomes, average diameter = 160 (35) nm; (●) rHuTNF-bound PC/Chol liposomes, protein/lipid ratio = 1:195 (w/w), average diameter = 156 (32) nm; (□) PC/Chol/PEG3000-PE liposomes, average diameter = 134 (39) nm; (■) rHuTNF-bound PC/Chol/PEG3000-PE liposomes, protein/lipid ratio = 1:193, average diameter = 127 (narrow) nm.

from the blood, is determined by the protein/lipid ratio as well as the lipid composition of liposomes.

3.4. Biological activity of rHuTNF-NGPE conjugates incorporated into liposomes

The biological activity of rHuTNF-NGPE conjugates incorporated into liposomes were assessed in vitro using the L929 cell cytotoxicity assay (Ruff and Gifford, 1981). In this study, rHuTNF-bound liposomes with the lipid compositions of PC/Chol and PC/Chol/PEG3000-PE were prepared by the detergent-dialysis method to similar protein/lipid ratios and average diameters. The actinomycin D-treated L929 cells were incubated with different concentrations (0.002–200 ng/ml) of liposomal rHuTNF for 16 h. The fractions of viable cells were quantitated by incorporation of crystal violet and were plotted as a function of protein concentration (Fig. 4). Free rHuTNF showed the concentration-dependent cytotoxicity with the IC_{50} value of approximately 0.1 ng/ml. Incorporation of rHuTNF-NGPE conjugates into PC/Chol and PC/Chol/PEG3000-PE liposomes resulted in the decreased cytotoxicity of rHuTNF with IC_{50} values of approximately 3.2 and 33 ng/ml, respectively.

4. Discussion

The use of modified rHuTNF exhibiting a high affinity for the liposome membrane offers several potential advantages over the unmodified protein in liposomal formulations. Unmodified rHuTNF normally shows a poor encapsulation efficiency in liposomes due to its hydrophilic nature. In addition, a leakage of unmodified rHuTNF from the aqueous space of liposomes may be problematic. On the other hand, modified rHuTNF conjugated with an appropriate hydrophobic anchor shows efficient and stable incorporation in the liposome membrane, which is especially advantageous from the pharmaceutical view point. Utsumi and coworkers previously reported that rHuTNF modified with various fatty acids exhibits efficient incorporation into liposomes (Utsumi et al., 1991). In our method, rHuTNF was first modified

Table 2
Effect of protein density on biodistribution of rHuTNF-bound liposomes[a]

Lipid composition[b]	TNF/L (w/w)	Average diameter (nm)	% injected dose[c]			RES/blood
			Blood	Spleen	Liver	
PC/Chol	None	160 (35)	9.7 (2.7)	22.1 (1.4)	50.2 (0.6)	7.5
PC/Chol/PEG3000-PE	None	134 (39)	62.2 (0.4)	3.5 (0.7)	18.4 (0.9)	0.35
	1:1288	142 (51)	43.9 (1.3)	4.4 (0.4)	29.4 (1.5)	0.77
	1:600	156 (55)	46.4 (8.3)	4.4 (0.3)	27.0 (1.2)	0.68
	1:193	127 (34)	30.9 (1.8)	12.9 (3.2)	44.6 (1.3)	1.9

[a]rHuTNF-bound ^{111}In labeled liposomes with the indicated lipid composition, protein/lipid ratio (w/w), and average diameter were injected i.v. into mice at a dose of 0.5 μg rHuTNF per mouse. Biodistribution of liposomes was examined at 3 h after injection.[b]Molar ratios are as described in Materials and methods.[c]Data are expressed as mean (S.D.), $n = 3$.

with a phospholipid, NGPE. The resultant rHuTNF-NGPE conjugates showed efficient incorporation into liposomes (Fig. 1). In the present study, modified rHuTNF was incorporated in either conventional or long-circulating liposomes, and their biodistribution and biological activity were examined.

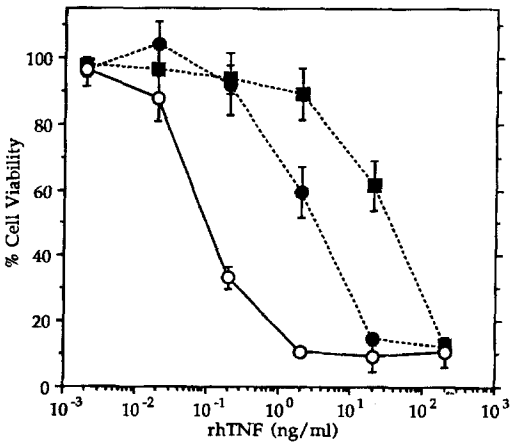

Fig. 4. Biological activity of rHuTNF-NGPE conjugates incorporated into liposomes. rHuTNF-bound liposomes were prepared by the detergent-dialysis method. The biological activity of (○) free rHuTNF and rHuTNF-NGPE conjugates incorporated into (●) PC/Chol and (■) PC/Chol/PEG3000-PE liposomes were examined using the L929 cell cytotoxicity assay as described in Materials and methods. Data were expressed as the percentage of viable cells relative to the control culture and were plotted as a function of a protein concentration. Bars represent S.D.; $n = 5$. The protein/lipid ratio and average diameter of liposomes were: PC/Chol liposomes, 1:197 and 169 (56) nm; PC/Chol/PEG3000-PE liposomes, 1:200 and 171 (51) nm, respectively.

rHuTNF has been previously shown to be cleared from the blood with two phases: rapid α- and subsequent slow β-phases (Pacici et al., 1987; Ferraiolo et al., 1988; Ferraiolo et al., 1989). Although the blood levels of rHuTNF in the α-phase (0–1 h after injection) was not determined in the present study (Fig. 2), the level of free rHuTNF remaining in the blood at 1 h after injection (10%) appeared to be consistent with the previously reported half-life (< 20 min) of rHuTNF in the blood (Fig. 2). Also shown in Fig. 2 is that the blood levels of rHuTNF-NGPE conjugates are dramatically increased only when incorporated into long-circulating PC/Chol/PEG3000-PE liposomes, but not the conventional PC/Chol liposomes. Increased levels of rHuTNF-conjugates formulated in PC/Chol/PEG3000-PE liposomes in the blood were accompanied by their decreased accumulation in the spleen and liver as compared with those formulated in PC/Chol liposomes (Table 1). These results clearly showed the effectiveness of PEG3000-PE-containing liposomes in prolonging the circulation time of modified rHuTNF. Based on the previous observations that long-circulating liposomes are able to accumulated in the solid tumor efficiently (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991; Unezaki et al., 1993), it is expected that an increasing amount of modified rHuTNF can be delivered in the tumor by formulating in PEG3000-PE-containing liposomes. This hypothesis is yet to be tested in the tumor-bearing animals.

Data in Fig. 3 showed that incorporation of rHuTNF-NGPE conjugates into liposomes resulted in their high clearance from the blood and enhanced accumulation in the spleen and liver as compared to the protein-free liposomes. The level of liposome clearance from the blood depends on the amount of rHuTNF-NGPE conjugates incorporated in liposomes: the higher the protein/lipid ratio, the higher the liposome clearance from the blood (Table 2). This observation indicates that rHuTNF-bound liposomes show a higher affinity for the spleen and liver than rHuTNF-free liposomes. Since a fraction of rHuTNF incorporated into liposomes is directly exposed to the outer surface of liposomes, they should be free to interact with rHuTNF receptors. Presumably, such rHuTNF/receptor interactions in the spleen and liver results in accelerated accumulation of liposomes in the spleen and liver, thus resulting in their high clearance from the blood. Although the ability of PC/Chol/PEG3000-PE liposomes to remain in the blood was compromised to some extent in the presence of rHuTNF-NGPE conjugates on the liposome membrane, it is nevertheless much greater than that of the conventional PC/Chol liposomes. The observed low clearance of rHuTNF-NGPE conjugates incorporated into PC/Chol/PEG3000-PE liposomes (Fig. 2) is thus directly attributed to the ability of PEG3000-PE-containing liposomes to remain in the blood for a prolonged period of time.

It should be noted that levels of ^{125}I-labeled rHuTNF in the blood (Fig. 2) were much lower than those of ^{111}In-labeled liposomes used for formulations (Fig. 3). For example, approximately 49% of injected ^{125}I-labeled rHuTNF formulated in PC/Chol/PEG3000-PE liposomes was detected in the blood at 1 h after injection, while approximately 73% of these ^{111}In-labeled liposomes remained in the blood at this time interval. One possible explanation for this discrepancy in radioactivity countings is that ^{125}I-labeled rHuTNF might undergo rapid dehalogenation in the blood, resulting in the release and clearance of the label. Thus, there might be a potential of underestimation in the levels of rHuTNF in the blood in the present study.

Data in Fig. 4 showed that the biological activity of rHuTNF-NGPE conjugates, assessed using the L929 cell cytotoxicity assay, was significantly reduced when incorporated into liposomes. One potential mechanism of reduced biological activity of liposomal rHuTNF is that chemical modifications of the protein molecule results in alterations of the proper conformation necessary for the biological activity. This was supported by the observation that increasing the NGPE-OSu/rHuTNF ratio in the conjugation reaction resulted in a progressive decrease in the biological activity of rHuTNF (data not shown). In view of the fact that biologically active rHuTNF exists as a trimer (Fiers, 1991), it is also possible that such a protein complex might be dissociated into monomers by the use of detergent during the liposome preparation, thus resulting in the loss of the biological activity. It is also shown in Fig. 4 that the biological activity of rHuTNF is further reduced when PEG3000-PE is included in the lipid composition. Possibly, interactions of rHuTNF with its receptor may be partially inhibited by the presence of PEG-PE polymer on the liposome membrane due to steric hindrance (Mori et al., 1991). However, this mechanism is not consistent with the observation that rHuTNF-bound liposomes show higher affinity for the spleen and liver, presumably due to ligand-receptor interactions in these organs, than the protein-free liposomes (Fig. 3). More experiments are needed to test these possibilities.

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