Accumulation of Protein-Loaded Long-Circulating Micelles and Liposomes in Subcutaneous Lewis Lung Carcinoma in Mice

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Purpose. The purpose of our work was to compare the biodistribution and tumor accumulation of a liposome- or micelle-incorporated protein in mice bearing subcutaneously-established Lewis lung carcinoma.
Methods. A model protein, soybean trypsin inhibitor (STI) was modified with a hydrophobic residue of N-glutaryl-phosphatidyl-ethanolamine (NGPE) and incorporated into both polyethyleneglycol(MW 5000)-distearoyl phosphatidyl ethanolamine (PEG-DSPE) micelles (< 20 nm) and PEG-DSPE-modified long-circulating liposomes (ca. 100 nm). The protein was labeled with ^{111}In via protein-attached diethylene triamine pentaacetic acid (DTPA), and samples of STI-containing liposomes or micelles were injected via the tail vein into mice bearing subcutaneously-established Lewis lung carcinoma. At appropriate time points, mice were sacrified and the radioactivity accumulated in the tumor and main organs was determined.
Results. STI incorporated into PEG-lipid micelles accumulates in subcutaneously established Lewis lung carcinoma in mice better than the same protein anchored in long-circulating PEG-liposomes.
Conclusions. Small-sized long-circulating delivery systems, such as PEG-lipid micelles, are more efficient in the delivery of protein to Lewis lung carcinoma than larger long-circulating liposomes.

KEY WORDS: tumor targeting; protein delivery; drug carriers; micelles; long-circulating liposomes; Lewis lung carcinoma; tumor targeting.

INTRODUCTION

Growing evidence suggests that the accumulation of macromolecules and microparticulates, such as liposomes and/or micelles, in the tumor interstitium is determined to a great extent by both their ability to penetrate through the leaky tumor vascular endothelium (1,2) and by the lack of functional lymphatic drainage in tumor tissue (3). This phenomenon, known as the Enhanced Permeability and Retention (EPR) effect, leads to increased retention of carrier-associated drugs in the tumor interstitium (4,5), as has been demonstrated for the passive targeting of peptides and proteins to solid tumors using soluble polymers (6). However, penetration through vascular pores and the extent of accumulation were recently shown to be strongly dependent on the pore cutoff size of the tumor blood vessel wall, and this cutoff size varies for different tumors (7).

In general, the biodistribution of a microparticulate carrier-associated anticancer drug depends on its circulation time in blood (summarized in (8) using liposomes as an example). It has been repeatedly demonstrated that the incorporation of polyethylene glycol (PEG)-derivatized phospholipids in the liposomal membrane substantially extends liposome circulation time, thus causing their increased accumulation in implanted tumors (9).

In contrast to these early studies, it was later found that, in some cases, even the use of long-circulating liposomes could not enable their sufficient accumulation in certain tumors. Parr and co-authors (10) recently reported that coating 100 nm liposomes with PEG did not result in an increased accumulation of liposome-encapsulated doxorubicin in a subcutaneously established murine Lewis lung carcinoma. This phenomenon may be determined by the low vascular permeability (small cutoff size) of some tumors. In those cases, drug carriers with a smaller size than liposomes may provide more efficient drug delivery into tumors.

Since long-circulating liposomes and micelles gain increasing recognition as drug carriers for intratumoral delivery (8,11,12), information on their comparative ability to penetrate through the vascular wall in a tumor should be of a great importance for the proper choice of an optimum drug formulation. Here we demonstrate that micelle-incorporated protein accumulates to a higher extent in subcutaneously-established murine Lewis lung carcinoma than the same protein incorporated into liposomes. Our results, together with the data of Parr et al. (10), may be considered as an indication that subcutaneous Lewis lung carcinoma is a tumor with a small vascular pore cutoff size. This makes passive drug delivery with very small long-circulating delivery systems, such as PEG-based micelles, more efficient than with long-circulating liposomes.

MATERIALS AND METHODS

Materials

Soybean trypsin inhibitor (STI, Type I-S, MW 21.5 kDa) was purchased from Sigma, all phospholipids were from Avanti Polar Lipids, Inc. Molecular porous membrane tubing was obtained from Spectrum^R. All other reagents, salts, and components of buffer solutions were of analytical grade.

Female C57BL/6J mice (18–20 g body weight, 8 weeks old) were used for all animal experiments.

Methods

Protein Modification and Radiolabeling

To incorporate water-soluble STI into the lipid phase of micelles or liposomes, the protein must be modified with hydrophobic group(s). For protein hydrophobization with the phospholipid (PE) residue, 0.3 mg of NGPE (13) were solubilized with 0.5 ml of 0.016 M octylglucoside (OG) in 50 mM MES, pH 4.5, followed by the addition of 12 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 15 mg of N-hydroxysulfosuccinimide (HSSI). After 5 minutes, the NGPE solution was mixed with 2 ml of 1 mg STI/ml in 0.1 M HEPES, pH 7.6; pH was then adjusted to 8.0 and the mixture was

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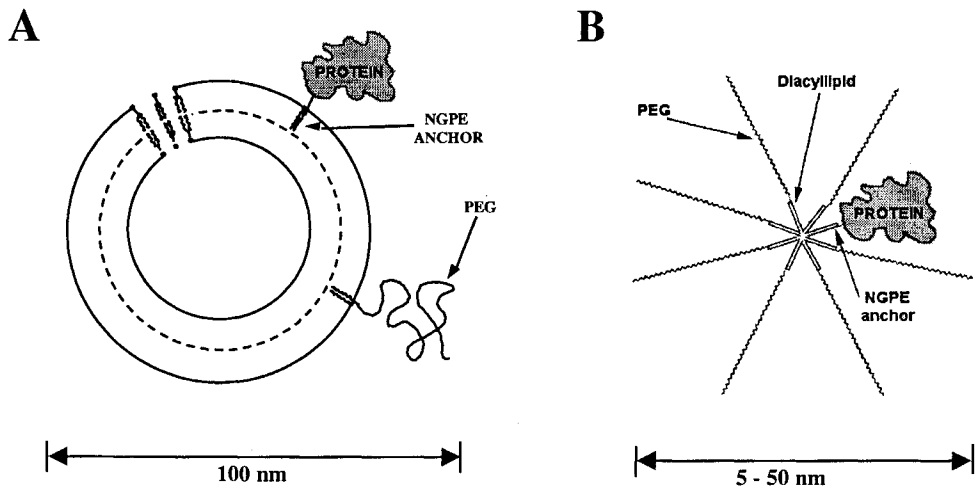


Fig. 1. Principal structure of a long-circulating liposome (panel A) and a polyethyleneglycol-diacyllipid micelle (panel B) with incorporated hydrophobized protein

incubated for at least 2 hours followed by dialysis against 0.1 M carbonate buffer, pH 8.0, containing 1 mM OG (14). The approximate molar ratio of NGPE to all lysine residues was 0.15:1 to keep the number of NGPE molecules attached per protein molecule low (15).

To attach radioactive ^{111}In to STI, chelating residues were first introduced into the STI molecule. For this purpose, 4 × 25 μl diethylene triamine pentaacetic acid anhydride (DTPA, strong chelating agent) solution (40 mg/ml, in DMSO) were slowly added to the protein solution (0.8 mg/ml) while vortexing. The reaction was performed during 30 min at room temperature in carbonate buffer containing 1 mM OG. Unbound DTPA was removed by dialysis against carbonate buffer.

DTPA-modified protein was loaded with ^{111}In after its incorporation into liposomes or micelles. The labeling was performed by a transchelation mechanism. For this purpose, the required quantity of radioactive ^{111}In as a citrate complex was mixed with DTPA-STI-liposomes or -micelles in HBS, pH 7.4, and the mixture was incubated with stirring for 30 min at room temperature for the transchelation of the radiometal from the weak citrate complex into a strong complex with DTPA. Remaining ^{111}In-citrate was removed by overnight dialysis against HBS.

Preparation of STI-Containing Micelles

For micelle preparation, 66 mg of PEG(5000)-DSPE was solubilized in chloroform. After removal of the organic solvent on a rotary evaporator, 2 ml of OG solution (50 mg/ml HBS) and 0.7 ml of NGPE-DTPA-STI solution from the hydrophobization step (0.56 mg STI) were added to the dried micelle-forming components. Sonication for 5 minutes using a bath sonicator yielded a clear solution. The detergent was removed by dialysis for 3 days against HBS (several buffer changes) using membrane tubing with a MWCO of 3,500.

Preparation of STI-Containing PEG-Liposomes

Sterically protected long-circulating liposomes (egg phosphatidylcholine:cholesterol:PEG-DSPE = 8:2:1, molar ratio) were prepared by drying the lipid solution in chloroform with a rotary evaporator, followed by addition of 2 ml of OG solution (50 mg/ml HBS) and 0.7 ml of NGPE-DTPA-STI solution from the hydrophobization step (0.56 mg STI). Final lipid concentration was 16 mg/ml. Liposomes obtained by overnight dialysis against HBS were sized by repeated extrusion through 0.1 nm polycarbonate filters (Poretics, Livermore, CA) until 95% of the liposome population had an average diameter between 95 and 110 nm.

Particle Size Measurements

The size and size distribution of liposome and micelle samples were determined by photon correlation spectroscopy with the Coulter™ Model N4+ MD sub-micron particle analyzer with size distribution analysis processor and multiple angle detection (Coulter Electronics, Hialeah, Florida, USA).

Animal Experiments

C57BL/6J mice were inoculated s.c. into rear flank with 300,000 Lewis lung carcinoma cells in 200 μl of HBS. After tumors reached ca. 5 mm in size (usually after 18–25 days), mice were injected i.v. via the tail vein with 0.1 ml of liposome or micelle sample (2 mg of lipid/polymer and ca. 20 μCi of ^{111}In per mouse). The concentration of PEG(5000)-DSPE in the injected micelle samples was about 5 mM in all experiments. Mice were sacrificed in groups of 5 after 1, 4 and 23 hours, organs were harvested and the radioactivity was determined in a gamma-counter.

RESULTS AND DISCUSSION

The incorporation of lipid-modified protein into liposomes and micelles is schematically shown in Fig. 1. While long-circulating liposomes are more frequently used as drug carriers (8), the utilization of micelle forming block-copolymers as long-circulating drug carriers for sparingly soluble or amphiphilic drugs has also recently been reviewed (11,12). An important property of micelles as drug carriers is their size, which normally varies between 5 and 50 nm, thus filling the gap between individual molecules (ca. 1 nm or less in size) and nanoparticu-

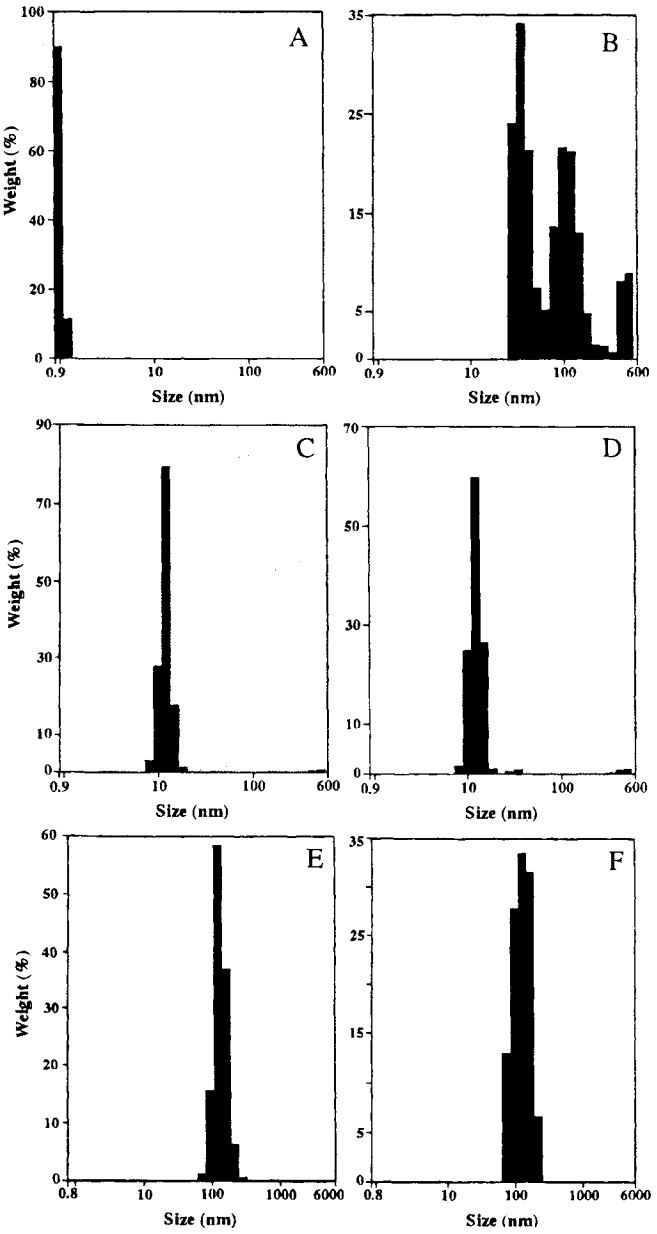


Fig. 2. Size distribution of native STI (A), NGPE-DTPA-STI (B), PEG(5000)-DSPE micelles (C), micellar incorporated NGPE-DTPA-STI (D), liposomes (E) and liposomes with incorporated NGPE-DTPA-STI (F). For details see MATERIALS AND METHODS.

lates such as liposomes (ca. 100 nm or greater in size) as drug carriers. The use of PEG-lipid conjugates as structural amphiphilic polymers for the formulation of micelles extends the micelle's blood circulation time upon intraveneous administration (11).

As follows from our data, we could easily incorporate hydrophobized protein into both PEG-liposomes and PEG(5000)-DSPE micelles. As a model protein for our experiments we have chosen soybean trypsin inhibitor (STI) with a molecular weight of 21.5 kDa. After derivatization of this protein with NGPE (13) according to (14), the modified protein was simply added to the starting mixtures for preparing liposomes and micelles, providing quantitative incorporation of STI-PE into both carriers.

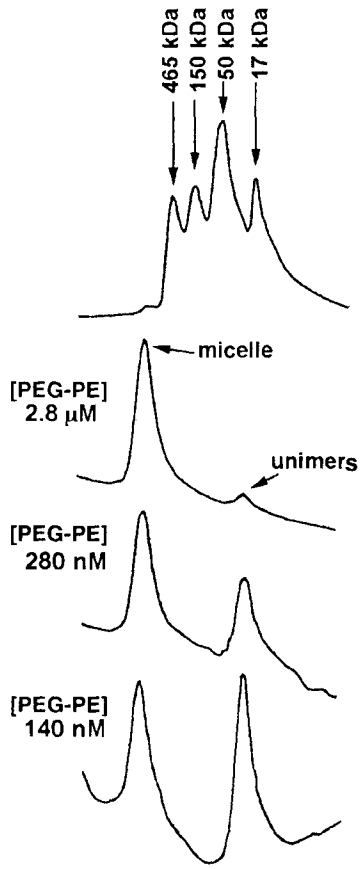


Fig. 3. HPLC-based gel permeation chromatography of serial dilutions of PEG(5000)-DSPE micelles with incorporated STI. Sample volume 20 μl. Column: Shodex Protein KW-804. Solvent HBS, pH 7.4. Detection at 215 nm.

Figure 2 shows the size distribution of the native STI, hydrophobized STI and both free and STI-loaded liposomes and micelles. STI incorporation does not noticeably influence liposome or micelle size, which is in the range around 100 nm and 15 nm, respectively.

While liposome stability in diluted aqueous solutions does not differ from that in more concentrated solutions (17), micelles are known to dissociate upon dilution, the extent of dissociation being dependent on the critical micellation concentration (CMC). However, as it was proven (11), PEG-lipid-based micelles are remarkably stable upon dilution. Figure 3 presents an HPLC-based gel permeation chromatogram of serial dilutions of PEG(5000)-DSPE micelles with incorporated STI. It is evident from the data presented, that micelles are still present at a concentration of 140 nM, together with unimers. This indicates an extremely low, nanomolar CMC value, which is in the millimolar range for many other low-molecular-weight surfactants. This led us to expect, that STI-loaded PEG-lipid micelles will remain intact even upon dilution in a large volume of blood.

Animal experiments revealed different biodistribution patterns of all tested preparations - free STI, STI-liposomes and STI-micelles (Fig. 4). As one can expect, the native protein demonstrates the fastest disappearance from the blood and the maximum accumulation in the liver and spleen. Both micelles and liposomes demonstrate clear longevity in the blood, however, liposomes remain in the circulation significantly longer

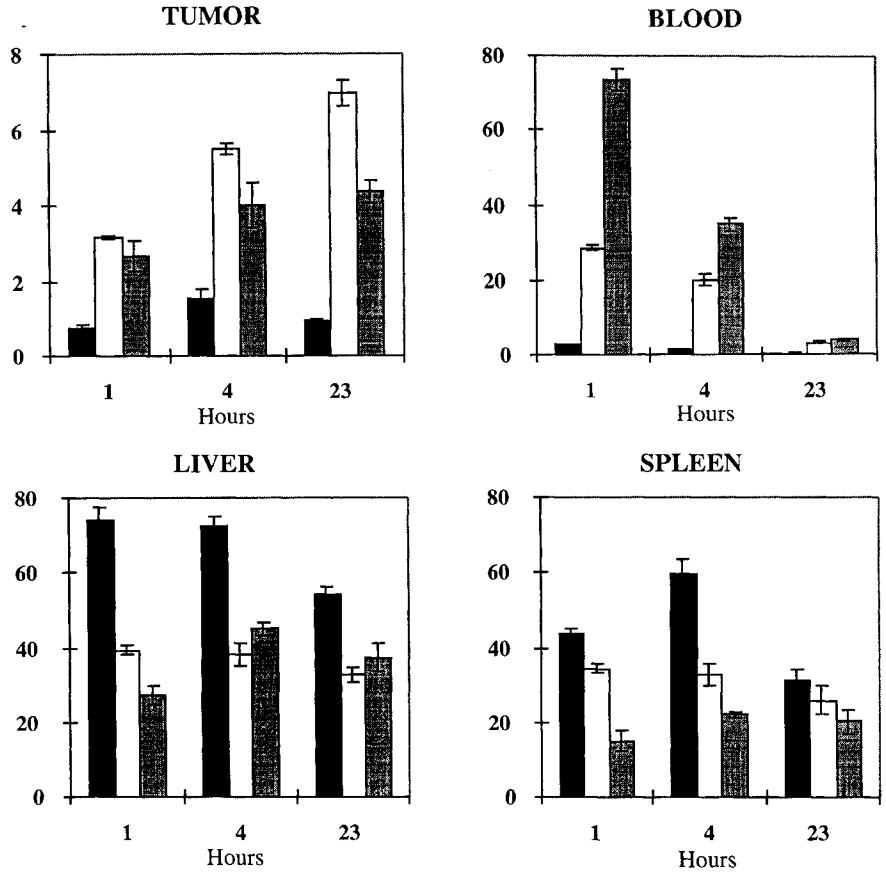
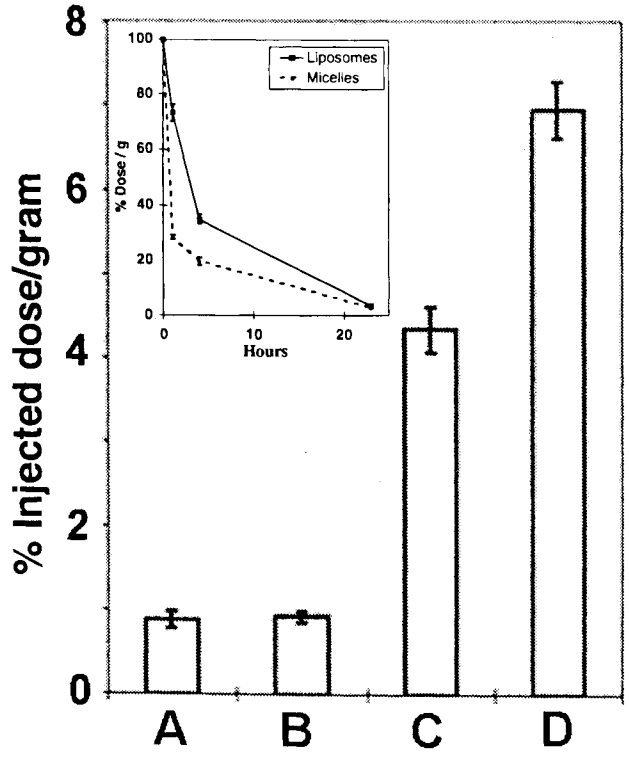


Fig. 4. Biodistribution of NGPE-DTPA-STI (black bars), NGPE-DTPA-STI incorporated into PEG(5000)-DSPE micelles (empty bars) and liposomal NGPE-DTPA-STI (shaded bars).



than micelles. Thus, after 1 hour, the blood concentration of liposomes is still ca. 75% dose/g, but only ca. 30% dose/g for micelles. Micelles also accumulate somewhat faster in the spleen, while the liver accumulation pattern is similar for both carriers.

The longevity of PEG-micelles and PEG-liposomes, together with their slow uptake in the liver and spleen, naturally result in their much improved accumulation in the tumor compared with the native STI. Less expected is the fact that despite their faster blood clearance, micelles accumulate in the tumor significantly better than liposomes, delivering more STI (Fig. 5).

This result confirms that the efficacy of passive delivery to solid tumors is not only controlled by the exposure level around sites of extravasation but also by the more complex relationship between the tumor's microvascular permeability and the size of a drug carrier. Although the permeability of

Fig. 5. Tumor accumulation 23 hours after i.v. injection of native STI (A), NGPE-DTPA-STI (B), NGPE-DTPA-STI anchored in 100 nm long-circulating liposomes (C) and micellar-bound NGPE-DTPA-STI (D). Insert: Blood clearance of NGPE-DTPA-STI anchored in long circulating liposomes and PEG(5000)-DSPE micelles, respectively. For details see Materials and Methods.

tumor vesssels is, in general, higher than that of normal vessels, some tumors lack this hyper-permeability or demonstrate it to a lesser extent (summarized in (2)). Contrary to Parr's findings (10), we have observed noticeable accumulation of radiolabeled liposome-associated STI in subcutaneous Lewis lung carcinoma, which might be explained by variability in liposome size and/or composition. However, this accumulation was enhanced even further for PEG-lipid micelles.

Our results suggest that in certain tumors (such as subcutaneously-established murine Lewis lung carcinoma), small micelles may provide better delivery of a drug (protein) than larger long-circulating liposomes.

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