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Lyophilized HER2-specific PEGylated immunoliposomes for active siRNA gene silencing

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ABSTRACT

The development of a tumor-specific immunoliposome delivering small interfering RNA (siRNA) represents a practical way in cancer gene therapy. In this study, we developed PEGylated 3β-[N-(N′, N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol)/dioleoylphosphatidyl ethanolamine (DOPE) immunoliposomes conjugated with the Fab' of recombinant humanized anti-HER2 monoclonal antibody (PIL) for siRNA delivery. The results demonstrated that the lyophilized PIL (LPIL) prepared by the lyophilization/rehydration method possessed a significantly enhanced HER1 gene, a model target, silencing ability compared with PIL in HER2-overexpressing SK-BR3 cells. Among a series of LPIL with different PEGylation degree, LPIL containing 2.5%PEG (2.5%PEG LPIL) showed the best HER1 gene silencing activity. Confocal microscope studies demonstrated that 2.5%PEG LPIL could specifically bind to SK-BR3 cells and were sequentially internalized into them. Using RhoA as a cancer therapeutic target, 2.5%PEG LPIL entrapping anti-RhoA siRNA could specifically silence RhoA expression and inhibit cell invasion in SK-BR3 cells. In conclusion, these finding demonstrated the potential use of 2.5%PEG LPIL in specifically delivering siRNA to HER2-overexpressing cancers.

1. Introduction

DC-chol liposomes were the first FDA-approved cationic liposomes (CLs) for clinical trials and their safety was also demonstrated in treating cystic fibrosis in vivo [1]. At present, CLs composed of 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidyl ethanolamine (DOPE) (DC-Chol/DOPE liposomes) have been classified as one of the most efficient gene delivery systems [1–3]. DC-chol/DOPE liposomes were biocompatible and showed low cytotoxicity [4]. However, the further development of DC-chol/DOPE liposomes was severely hampered by their low transfection efficiency, poor serum stability and short circulation lifetime [1,5,6]. If these problems were overcome, a better clinical use would be achieved for DC-chol/DOPE liposomes.

The short circulation lifetime of CLs might be overcome by attaching poly(ethylene glycol) (PEG) at the surface of CLs [7]. Nevertheless, PEGylation represents a major barrier for nucleic acid internalization and endosomal escape, resulting in much reduced transfection efficiency [3]. Therefore, the PEGylation degree of CLs should be optimized to overcome the siRNA-releasing barrier. To further enhance the transfection efficiency of PEGylated CLs, it is a practical way to develop immunoliposomes conjugated with targeting ligands [8]. One of the most usually used targeting ligands was recombinant humanized anti-HER2 monoclonal antibody (rhuMAbHER2). The Fab' of rhuMAbHER2 (anti-HER2 Fab') was often adopted owing to its reduced immunogenicity, improved pharmacokinetic profiles and better penetration into solid tumors than the whole monoclonal antibody [9,10].

Drug loading method in PEGylated CLs is also very important for siRNA transfection [9]. Direct mixing siRNA with preformed PEGylated CLs leads to the results that most of the siRNA were only bound to the outer surface of the liposomes and would immediately release and be degraded by serum nucleases [11,12]. Two strategies are often performed to entrap siRNA in the core of PEGylated CLs. The first strategy is to hydrate the lipid film (containing PEGylated and cationic lipids) directly with a concentrated solution of siRNA

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(HYDRA protocol) [11]. The second way called "post-PEGylation" method was preparing liposomes/siRNA complex using non-PEGylated liposomes with subsequent PEGylation of the preformed liposomes/siRNA complex [13]. However, if PEGylated immunoliposomes/siRNA complex is prepared using the above two strategies, the post-preparation environment should be nuclease-free after liposomes/siRNA complex was formed. Also, the preformed liposomes/siRNA complex had a risk in losing their entrapped siRNA. It is thus ideal if any technology could be developed to conquer such drawbacks. The lyophilization/rehydration method is a useful way to entrap drugs in liposomes, consisting of (i) lyophilization of prepared drug-free liposomes and (ii) drug encapsulation in the course of liposome reconstitution through rehydration in an aqueous solution of the drugs [14]. For siRNA entrapment, this method offers several prominent advantages over the traditional methods mentioned above. It avoids nuclease that may degrade the siRNA, as all the preparation steps are done in the absence of siRNA. This absence also means the risk of losing entrapped siRNA is nonexistent [14]. D. Peer et al. used this lyophilization/rehydration method to develop a targeted stabilized liposomes entrapping siRNA (β_7 I-tsNPs) and demonstrated that β_7 I-tsNPs could specifically silence Cyclin D1 expression in leukocytes, suggesting this lyophilization/rehydration method has great potential in developing liposomes entrapping siRNA [15].

In this study, we developed PEGylated DC-Chol/DOPE immunoliposomes conjugated anti-HER2 Fab' (PIL) and lyophilized PIL (LPIL), and investigated the influence factors of the siRNA gene silencing efficiency of PIL and LPIL. The specific binding/internalization and potential anticancer ability were also evaluated in 2.5%PEG LPIL, which was demonstrated to possess the best gene silencing ability, among a series of LPIL with different PEGylation degree.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidyl ethanolamine (DOPE), 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine [methoxy(polyethyleneglycol)-2000] (mPEG–DSPE), maleimide derivatized PEG2000-DSPE (Mal-PEG–DSPE) and 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-Carboxyfluorescein(CFPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other organic reagents and the cryoprotectant sucrose were of analytical grade and purchased from Sinopharm (Shanghai, China). RhuMAbHER2 and C225 (human mouse chimeric anti-HER1 monoclonal antibody) were kindly provided by National engineering research center for antibody medicine (Shanghai, China). Anti-HER2 Fab' (1 mg/ml) was prepared as we described previously [16]. FITC-goat anti-human IgG (H + L) was purchased from Zymed (San Francisco, CA). 2-iminothiolane (Traut's Reagent) was obtained from Pierce (Oud Beijerland, NL). The siRNA targeting human HER1 or RhoA, negative control (NC) siRNA and FAM-labeled NC siRNA were synthesized by GenePharma (Shanghai, China). Silencer® Cy™3-labeled NC siRNA (Cy3-siRNA) was obtained from Ambion (Austin, USA). All the primers were synthesized by Invitrogen (Shanghai, China). See Supplementary Table 1 for detailed sequence of the siRNA and primers. Amicon® Ultra-15 centrifugal filter devices (100,000 NMWL) were purchased from Millipore (Massachusetts, USA). Lipofectamine™ 2000 (lipo2000), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbag, CA, USA).

2.2. Cell lines

The human breast cancer cell lines SK-BR3 and MCF-7 were purchased from ATCC (American Type Culture Collection, VA, USA). The cells were maintained in DMEM supplemented with 10% FBS, 25 mM HEPES buffer, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Liposome preparation

PEGylated DC-Chol/DOPE immunoliposomes (PIL) were prepared as described before [17,18]. Briefly, multilamellar liposomes (MLL) composed of DC-chol, DOPE, Mal-PEG–DSPE and mPEG–DSPE at molar ratios of (49.5–0.5X):(49.5–0.5X): 1:X (X% represent the molar ratio of mPEG–DSPE in total lipid) were prepared by a lipid film method. For fluorescent liposome preparation, 0.1% CFPE (molar ratio) was added in the lipid film. The lipid film was hydrated with 10 mM phosphate buffer saline (PBS, pH 7.4) to create MLL. The resulting MLL were extruded into unilamellar nano-scale liposomes (ULL) with a hand held extruder (Avestin, Ottawa) at ambient temperature. The extrusion was performed in a stepwise manner using progressively decreasing pore sized membranes (from 200, 100 and 80 nm) (Nucleopore, Whatman), with 10 cycles per pore size [18]. In addition, anti-HER2 Fab' was thiolated by 2-iminothiolane at a molar ratio of 100:1 (2-iminothiolane: Fab') [18]. The sulfhydryl groups of anti-HER2 Fab' were determined using Ellman's assay [19]. Then, the thiolated anti-HER2 Fab' was mixed with the prepared liposomes containing maleimide-terminated linker at the anti-HER2 Fab'/Mal-PEG–DSPE molar ratio of 1/10 and the mixture was incubated for 2 h at ambient temperature under N_2. Subsequently, unconjugated anti-HER2 Fab' was removed by gel chromatography on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using PBS as the eluant. Resulting PIL were collected in the void volume fraction, sterilized by passage through a 0.22 μm sterile filter, and stored at 4 °C. Nontargeted PEGylated DC-chol/DOPE liposomes (PL) were prepared in the same way as PIL except that Mal-PEG–DSPE was replaced by mPEG–DSPE. The phospholipids concentration was determined with ammonium ferrothiocyanate method [20]. The DC-chol concentration was calculated from the phospholipids concentration correspondingly. Lyophilized PIL (LPIL) or lyophilized PL (LPL) was obtained as follows. Briefly, PIL or PL, of which DC-chol concentration was diluted to 0.8 μg/μl, was mixed with 5.4 μg/μl sucrose (the final concentration of sucrose was 9%, weight/volume ratio) and lyophilized for 16–18 h using VirTis® AdVantage™ Benchtop Freezer.

2.4. siRNA entrapment in liposomes

siRNA was dissolved in DEPC-treated water at a final concentration of 20 μM. Then siRNA solution (9 μl) was diluted with DEPC-treated water to a certain volume (9–90 μl). For entrapment of lyophilized liposomes, LPL or LPIL (containing 7.2–72 μg DC-chol) were rehydrated with a certain amount of diluted siRNA solution (9–90 μl) and were incubated at ambient temperature for 20 min. For entrapment of unlyophilized liposomes, PL or PIL (9–90 μl, containing 7.2–72 μg DC-chol) were mixed with the diluted siRNA solution of equal volume (9–90 μl) and were incubated at ambient temperature for 20 min. The entrapment procedure was performed immediately before use.

2.5. Characterization of liposomes

2.5.1. Particle size and zeta potential

After the liposomes and their siRNA complex were dispersed in deionized water, their particle size and zeta potential were analyzed using Zeta sizer Nano S (Malvern instruments, UK).

2.5.2. Evaluation of siRNA encapsulation efficiency (EE)

siRNA EE was determined by a Quant-iT™ RiboGreen® RNA assay (Molecular Probes, Invitrogen) as described before [15,21]. Briefly, a number of siRNA solutions with known concentrations were prepared to construct a calibration line. RiboGreen fluorescence was measured with a spectrofluorometer (Synergy™ 2, Bio-Tek, WI, USA) using excitation and emission wavelengths of 495 and 525 nm, respectively. The siRNA concentration of prepared liposomes/siRNA complex was then determined by comparing the fluorescence before or after addition of 0.5% Triton X-100 to the siRNA calibration line. The siRNA EE was determined by the following formula: $(C_T - C_N)/C_T \times 100\%$. C_T and C_N were denoted as siRNA concentration after and before addition of 0.5% Triton X-100, respectively.

2.5.3. Determination of anti-HER2 Fab' on the surface of liposomes

The presentation and integrity of anti-HER2 Fab' after conjugated to the liposome surface were detected by SDS-PAGE. The gels were run under non-reducing conditions at a constant voltage of 160 V in a Tris/glycine/SDS buffer. The gels were stained with Coomassie brilliant blue to reveal protein, destained and dried.

2.6. siRNA serum stability

Serum stability of siRNA in aqueous solution versus in liposomes preparations was characterized using agarose gel electrophoresis. Samples of siRNA either in aqueous solution or as a liposomes/siRNA complex were mixed in a 1:1 volume ratio with fresh serum to give 50% serum concentration and incubated at 37 °C. At different incubation times, aliquots containing 0.25 μg siRNA of each sample were loaded onto a gel and electrophoresis was performed to visualize intact siRNA.

2.7. In vitro transfection

For transfection efficiency analysis, SK-BR3 cells were seeded in 48-well plates with a density of 6×10^4 cells per well overnight. The above prepared liposomes entrapping FAM-siRNA were incubated with the cells (0.4 μg siRNA per well, the final siRNA concentration was 100 nM) for 6 h. Then the cells were trypsinized, washed and analyzed by flow cytometry analysis (FCM). FCM was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

For gene silencing analysis, SK-BR3 cells were seeded in 48-well plates with a density of 6×10^4 cells per well overnight. The above prepared liposomes