Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Antibody-modified lipid nanoparticles for selective delivery of siRNA to tumors expressing membrane-anchored form of HB-EGF

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ARTICLE INFO

Article history:
Received 2 May 2014
Available online xxxx

Keywords:
siRNA delivery
Lipid nanoparticles
Antibody
HB-EGF
Breast cancer

ABSTRACT

An Fab' antibody against heparin-binding epidermal growth factor-like growth factor (HB-EGF) was applied to achieve advanced tumor-targeted delivery of siRNA. Lipid nanoparticles (LNP) encapsulating siRNA (LNP-siRNA) were prepared, pegylated, and surface modified with Fab' fragments of anti-HB-EGF antibody (αHB-EGF LNP-siRNA). αHB-EGF LNP-siRNA showed high-binding affinity to recombinant human HB-EGF in a Biacore assay. In addition, αHB-EGF LNP-siRNA selectively associated with cells expressing HB-EGF *in vitro*. Confocal microscopic images showed that siRNA formulated in αHB-EGF LNP-siRNA was efficiently internalized into MDA-MB-231 human breast cancer cells, on which HB-EGF is highly expressed. In addition, siRNA encapsulated in αHB-EGF LNP induced obvious suppression of both target mRNA and protein levels in MDA-MB-231 cells. These results indicate that αHB-EGF LNP have excellent potential to deliver siRNA to target cancer cells, resulting in effective gene silencing.

1. Introduction

For clinical application of small interfering RNA (siRNA), many studies have been carried out in both basic and clinical fields [1–3]. Since siRNA is unstable in serum and hardly penetrates the cell membrane, an appropriate siRNA delivery system is necessary for the establishment of siRNA therapies. For this purpose, nanoparticle-mediated delivery of siRNA has been studied to obtain efficient gene silencing [4–6]. Previously, we developed a non-viral vector, named dicetyl phosphate tetraethylenepentamine (DCP-TEPA)-based polycation liposomes (TEPA-PCL), for delivery of double-stranded small RNAs [7]. Our data showed that siRNA or microRNA complexed with TEPA-PCL was highly taken up into cells and had remarkable gene silencing effects [8–10]. Surface modification of TEPA-PCL-based lipoplexes with polyethyleneglycol and peptide ligands, such as Arg-Gly-Asp (RGD)-peptide for targeting integrin $\alpha_v\beta_3$ or Ala-Pro-Arg-Pro-Gly (APRPG)-peptide for targeting vascular endothelial growth factor receptor 1 (VEGFR1), enabled targeted delivery of siRNA and subsequent gene silencing after systemic administration [7,10–12]. On the other hand, we previously designed another type of lipid-based vector for small RNA delivery [13]. We prepared cationic cores carrying siRNA by using palmitoyl RRRRRRGGRRRRG and wrapped them with lipids. lipid nanoparticles (LNP) encapsulating siRNA (LNP-siRNA) thus obtained showed significant gene silencing when they were modified with cell-penetrating peptides on their surface [13].

In the present study, we modified LNP with specific antibody against heparin-binding epidermal growth factor-like growth factor (HB-EGF) to induce gene silencing in target cancer cells in a selective manner. Active targeting of nanoparticles to tumors by antibody conjugation is a promising approach, since tumor cells often express characteristic molecules on their surface that are not found on normal cells [14,15]. HB-EGF is known to be highly expressed on the cell surface of various cancers, such as breast, ovarian, and liver cancers [16,17]. The precursor of HB-EGF is expressed on the cell surface as a membrane-anchored form (proHB-EGF) and then processed to a soluble form (HB-EGF), which mediates the intracellular signaling. Thus, we expected HB-EGF to be a useful target molecule for delivering siRNA to tumors. In fact, our previous study showed that anti-HB-EGF antibody-modified liposomes can efficiently deliver an anticancer agent to cancer cells

Abbreviations: LNP, lipid nanoparticles; LNP-siRNA, LNP encapsulating siRNA; αHB-EGF-LNP, anti-HB-EGF antibody-modified LNP.

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http://dx.doi.org/10.1016/j.bbrc.2014.05.043
0006-291X/

overexpressing HB-EGF both *in vitro* and *in vivo* [18]. Here, we developed LNP-siRNA modified with Fab' fragments of anti-HB-EGF antibody (αHB-EGF LNP-siRNA) and evaluated their potential as a siRNA vector *in vitro*.

2. Materials and methods

2.1. Materials

siRNA was purchased from Hokkaido System Science Co. (Hokkaido, Japan). In this study, siRNA for the luciferase2 gene was used unless otherwise stated. The nucleotide sequences for enhanced luciferase 2 (siLuc2) were 5′-GCUAUGGGCUGAAUACAA-ATT-3′ (sense) and 5′-UUUGUAUUCAGCCCAUAGCTT-3′ (antisense); and for Lamin A/C (siLamin) with a 2-nucleotide overhang (underline) as 5′-GGUGGUGACGAUCUGGGCUTT-3′ (sense) and 5′-AGCCCAGAUCGUCACCACCTT-3′ (antisense). For the use of fluorescein isothiocyanate (FITC)-labeled siRNA, FITC was conjugated to siLuc2 at the 3′ end of the antisense strand.

A palmitoyl derivative of RRRRRRGGRRRRG peptide was purchased from Operon Biotechnologies (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE), dimyristoylphosphoglycerol (DMPG), distearoylphosphatidylethanolamine (DSPE)-polyethyleneglycol (PEG) 5000 (DSPE-PEG), and maleimide-conjugated DSPE-PEG5000 (DSPE-PEG-maleimide) were purchased from NOF Co. (Tokyo, Japan). Cholesterol was kindly provided by Nippon Fine Chemical Co. (Hyogo, Japan). Monoclonal antibody clone 3E9 specific for HB-EGF was obtained by the method described previously [19]. The 3E9 clone recognizes the EGF-like domain of human proHB-EGF, but not that of mouse proHB-EGF. Recombinant human HB-EGF (rhHB-EGF) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of anti-HB-EGF-modified LNP-siRNA

LNP-siRNA were prepared as described previously [13]. siRNA and palmitoyl RRRRRRGGRRRRG (1/16.8 as a molar ratio, containing 1 nmol of siRNA) dissolved in RNase-free water (1 mL, Invitrogen, Rockville, MD) were mixed and incubated for 30 min at room temperature to obtain the cationic cores. On the other hand, DOPE, cholesterol, and DMPG (6/5/2 as a molar ratio, total lipids: 5 μmol) dissolved in chloroform were evaporated under reduced pressure, and stored *in vacuo* for at least 1 h. LNP-siRNA were prepared by hydration of the thin lipid film with 1 mL of the cationic core solution and sized by use of mild sonication for 3 min at room temperature.

Fab' fragments of anti-HB-EGF monoclonal antibody were prepared as described previously [18]. For the modification of LNP-siRNA with Fab' fragments of anti-HB-EGF antibody, 1 mL of the LNP-siRNA solution was incubated with 45 μL of 5 mM DSPE-PEG and 5 μL of 5 mM DSPE-PEG-maleimide dissolved in RNase free water at 37 °C for 2 h, forming PEG/PEG-maleimide-inserted LNP-siRNA (PEG-mal-LNP-siRNA). The coupling reaction of Fab' fragments with the maleimide moiety of PEG-mal-LNP-siRNA was performed according to the method described earlier [20]. Fab' fragments and PEG-mal-LNP-siRNA (1/1 as a molar ratio of Fab' and maleimide moiety) were mixed, and the coupling reaction was carried out at 4 °C for 16 h. Excess Fab' fragments were separated from the Fab'-coupled PEG-mal-LNP-siRNA by gel-filtration chromatography (Sepharose™ 4 Fast Flow column, GE healthcare, Piscataway, NJ), and the LNP-siRNA fractions were collected. After ultracentrifugation (453,000×*g*, 4 °C, 15 min), anti-HB-EGF Fab'-modified LNP (αHB-EGF LNP-siRNA) were resuspended with 1 mL of RNase- free water. Similarly, the surface of LNP-siRNA was decorated with Fab' fragments of control mouse IgG (MGG-0500, MBL, Nagoya, Japan; Control LNP-siRNA). The particle size and ζ-potential of the particles were measured by using a Zetasizer Nano ZS (Malvern, Worcs, UK).

2.3. Transmission electron microscopy (TEM)

Ten microliters of 5 mM αHB-EGF LNP-siRNA was added onto a grid (Nisshin EM, Tokyo, Japan) and dried-out by warm air. After that, the sample was negatively stained with 10 μL of 1 w/v% ammonium molybdate for 1 min, and imaged with an HT7700 TEM System (Hitachi High-Technologies, Tokyo, Japan). TEM images were recorded with a CCD camera at 1024 × 1024 pixels (Advanced Microscopy Techniques, Woburn, MA).

2.4. Assay to detect binding of αHB-EGF LNP to rhHB-EGF

A Biacore sensor chip was activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/HCl (EDC) and *N*-hydroxysuccinimide (NHS), and then it was coated with rhHB-EGF dissolved in acetate buffer (pH 4.5). Ethanolamine was used as a blank. PEG-mal-LNP-siRNA, Control LNP-siRNA, or αHB-EGF LNP-siRNA were applied to the sensor chip for binding analysis using the Biacore 2000 system (GE healthcare, Tokyo, Japan) (injection time: 10 min, flow rate: 15 μL/min).

2.5. Cell culture

African green monkey kidney-derived Vero cells overexpressing HB-EGF (Vero-H) [21] were cultured in MEM medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, AusGeneX, Oxenford, Australia), 100 units/mL penicillin G (MP Biomedicals, Irvine, CA), 100 μg/mL streptomycin (MP Biomedicals), and 1 μg/mL G418 (SIGMA-Aldrich, St. Louis, MO) in a CO_2 incubator. MDA-MB-231 human breast cancer cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 100 units/mL penicillin G, and 100 μg/mL streptomycin in a CO_2 incubator.

2.6. siRNA Transfection

Cells were seeded onto a culture plate and pre-cultured overnight. Before transfection, the medium was changed to a fresh one containing FBS but not antibiotics. Control LNP-siRNA or αHB-EGF LNP-siRNA was added to the culture medium at a final concentration of 60 nM (as siRNA), and the cells were then incubated for 24 h at 37 °C in a 5% CO_2 incubator. After a medium change, the cells were incubated for the desired time as described for each experimental procedure.

2.7. Association of αHB-EGF LNP-siRNA with cells overexpressing HB-EGF

Vero-H cells (2×10^4 cells/0.5 mL/well) or MDA-MB-231 cells (4×10^4 cells/0.5 mL/well) were seeded onto 24-well plates (BD Bioscience, San Jose, CA). These cells were incubated for 6, 12 or 24 h with FITC-labeled siRNA (60 nM, Hokkaido System Science Co.) formulated in Control LNP or αHB-EGF LNP. Naked FITC-siRNA was also incubated with the cells as a control. The cells were washed 3 times with PBS and lysed with 1 w/v% n-octyl-β-D-glucoside (Dojindo, Kumamoto, Japan) containing protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma–Aldrich), 2 μg/mL leupeptin (Sigma–Aldrich), 2 μg/mL aprotinin (Sigma–Aldrich), and 2 μg/mL pepstatin A (Sigma–Aldrich). The fluorescence intensity of FITC was determined with a Tecan Infinite M200 microplate reader (Salzburg, Austria) according to the manufacturer's instructions (ex. 485 nm, em. 535 nm) and corrected by total protein content measured with a BCA Protein Assay Reagent

Please cite this article in press as: A. Okamoto et al., Antibody-modified lipid nanoparticles for selective delivery of siRNA to tumors expressing membrane-anchored form of HB-EGF, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.043