Journal of Controlled Release 144 (2010) 251–258

Contents lists available at ScienceDirect

Journal of Controlled Release

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

NANOMEDICINE

Targeted delivery of SiRNA to CD33-positive tumor cells with liposomal carrier systems

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ARTICLE INFO

Article history:
Received 23 October 2009
Accepted 16 February 2010
Available online 22 February 2010

Keywords:
siRNA
AML
t(8;21) translocation
Liposomes
Immunoliposomes
Silencing

ABSTRACT

SiRNA molecules represent promising therapeutic molecules, e.g. for cancer therapy. However, efficient delivery into tumor cells remains a major obstacle for treatment. Here, we describe a liposomal siRNA carrier system for targeted delivery of siRNA to CD33-positive acute myeloid leukemia cells. The siRNA is directed against the t(8;21) translocation resulting in the AML1/MTG8 fusion protein. The siRNA was encapsulated in free or polyethylene imine (PEI)-complexed form into PEGylated liposomes endowed subsequently with an anti-CD33 single-chain Fv fragment (scFv) for targeted delivery. The resulting siRNA-loaded immunoliposomes (IL) and immunolipoplexes (ILP) showed specific binding and internalization by CD33-expressing myeloid leukemia cell lines (SKNO-1, Kasumi-1). Targeted delivery of AML1/MTG8 siRNA, but not of mismatch control siRNA, reduced AML1/MTG8 mRNA and protein levels and decreased leukemic clonogenicity, a hallmark of leukemic self-renewal. Although this study revealed that further modifications are necessary to increase efficacy of siRNA delivery and silencing, we were able to establish a targeted liposomal siRNA delivery system combining recombinant antibody fragments for targeted delivery with tumor cell-specific siRNA molecules as therapeutic agents.

1. Introduction

Oncogenes such as leukemic fusion genes, which arise from chromosomal translocations, are promising targets for therapeutic approaches as they are exclusively expressed in premalignant and malignant tissues. Frequently, tumor cells are absolutely dependent on, i.e. "addicted" to such oncogenes. However, the majority of these translocations affect transcription factors, which are difficult to target by conventional small molecule-based approaches, consequently limiting their attractiveness as cancer-specific targets [1]. In this situation, short interfering RNAs (siRNAs) provide a promising option [2]. These small, double-stranded RNAs of 21 to 23 nucleotides in length exploit the RNA interference (RNAi) pathway by guiding the RNA-induced silencing complex (RISC) to complementary target sequences leading to the cleavage and subsequent degradation of the targeted transcript [3,4]. Since the design of siRNAs primarily requires the sequence of the target transcript, virtually any gene of interest including "undruggable" transcription factor genes can be suppressed. Thus, siRNA approaches are commonly used in drug development programs for target validation. RNAi libraries are also employed for target identification. Finally, combinations of siRNA-mediated knockdown and small molecular drug screening have been successfully used to identify new treatment options in cancer [5]. However, the therapeutic potential of direct siRNA applications has not been realized yet because of their unfavorable pharmacokinetic properties such as low stability in body fluids and rapid renal clearance. Several promising approaches using siRNA-packaging particles with and without ligands have been described [6,7]. Advanced delivery systems, e.g. liposomal carrier systems, employ siRNA complexed to polycationic substances encapsulated into a sterically stabilized lipid bilayer. Furthermore, these particle surfaces have been modified to faciliate endosomal release, e.g. through incorporation of fusogenic peptides [8]. However, in the majority of the cases, siRNA accumulation was restricted to liver, lung, spleen and kidney, with only marginal amounts found in other organs such as brain and bone marrow.

The translocation t(8;21) is the most frequent chromosomal rearrangement found in AML [9]. It replaces the C-terminal transactivation domain of AML1 (also known as RUNX1), a transcription factor vital for definitive hematopoiesis, with the almost complete open reading frame of MTG8 (also known as RUNX1T1 or ETO) [10,11]. The resultant AML1/MTG8 represents a chimeric transcription factor,

Abbreviations: AML, acute myeloid leukemia; scFv, single-chain fragment variable; PEG, polyethylene glycol; siRNA, short interfering RNA; RNAi, RNA interference; IL, immunoliposome; ILP, immunopolyplex.

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doi:10.1016/j.jconrel.2010.02.020

which interferes with myeloid differentiation by recruiting histone deacetylases to AML1-regulated promoters as well as by sequestering transcription factors driving differentiation, thus converting an important transcriptional modulator to a constitutive repressor of gene expression [12]. Suppression of AML1/MTG8 with siRNAs relieves the differentiation block in leukemic cells and impairs leukemic growth both in cell culture and in animal models [13–17].

Tumor therapy involving antibodies or antibody-derived molecules offers the possibility for tumor targeting by directly reaching the tumor cells via surface antigen binding. The high internalization rate of CD33 [18], its restriction to the myeloid lineage and its expression on AML cells and leukemic stem cells make it a suitable antigen for targeted drug delivery in AML. CD33 is down regulated in mature granulocytes but retained in monocytes [19]. CD33 has already been used for clinical approaches of targeted drug delivery [20,21]. Experience with Gemtuzumab-ozogamycin (GO, Mylotarg), an anti-CD33 monoclonal antibody-drug conjugate, has shown clinical relevance [22]. In another study, a CD33-specific immunotoxin was highly effective in killing human myeloid leukemia cells [23]. Thus, CD33 is an interesting surface antigen for targeted delivery. For instance, immunoliposomes, generated by coupling of antibodies to a liposomal surface, allow for an active tumor targeting, e.g. through binding to tumor cell-specific receptors, and thus present a promising approach for targeted drug delivery [24].

Here, we describe the generation of liposomal carrier systems for targeted delivery of anti-leukemic siRNA into CD33-positive myeloid tumor cells. As a targeting ligand we used an anti-CD33 single-chain Fv fragment [23], which was further modified with a C-terminal cysteine residue to allow for a site-directed conjugated to PEGylated lipids. The siRNA was encapsulated into liposomes either in free form (immunoliposomes; IL) or complexed with polyethylenimine (PEI) (immunolipoplexes, ILP). Specific binding and uptake into tumor cells was observed, which led to silencing of the AMG1/MTG8 fusion protein and a reduced growth in colony formation assays as compared to a control siRNA.

2. Materials and methods

2.1. Materials

The human CD33-expressing acute myeloid leukemic cell lines SKNO-1 [25] and Kasumi-1 [26] (DSMZ No. ACC 220) as well as the acute T-cell leukemia line Jurkat [27] (DSMZ No. ACC 282) were grown in RPMI1640 containing 10% FCS, 2 mM glutamine or in case of SKNO-1 20% FCS + 7 ng/mL GM-CSF (ImmunoTools, Friesoythe, Germany). FITC-labeled anti-CD33 antibody was purchased from ImmunoTools (Friesoythe, Germany). Anti His-Tag unconjugated mAb IgG1 mouse DIA 900 from Dianova (Hamburg, Germany), goat anti-mouse IgG-R-Phycoerythrin (PE), P9287 and rabbit anti-mouse IgG-FITC, F9137 from Sigma-Aldrich (St. Louis, USA). Egg phosphatidylcholine (EPC) was purchased from Lipoid (Ludwigshafen, Germany) and cholesterol was purchased from Calbiochem (Merck, Darmstadt, Germany). All other lipids were purchased from Avanti Polar Lipids (USA). Human plasma (stabilized with citrate-phosphate-dextrose solution) was kindly provided by the blood donation center of the Katharinenhospital (Stuttgart, Germany). His-probe (H-3) Horseradish Peroxidase (HRP)-conjugated mouse monoclonal antibody was purchased from Santa Cruz Biotechnology (SC8036, Santa Cruz, USA). AML1/RHD antibody was purchased from Calbiochem (Ab-2, PC285, Merck Chemicals, Nottingham, UK), goat anti-rabbit IgG peroxidase conjugate, A0545 from Sigma-Aldrich (St. Louis, USA). SiRNA was purchased from Eurofins MWG Operon (Ebersberg, Germany): siAGF1 5′- CCU CGA AAU CGU ACU GAG AAG -3′, siAGF6 5′- CCU CGA AUU CGU UCU GAG AAG -3′, siMA6 5′-AAG AAA AGC AGA CCU ACU CCA-3′. Cy3-labeled siRNA: siMAX Cy3-AML1/MTG8 5′-(Cy3) CCU CGA AAU CGU ACU GAG A(dTdT) -3′. Polyethylenimine (PEI 25) was purchased from BASF (Ludwigshafen, Germany). Cell tracker green-CMFDA was purchased from Invitrogen (San Diego, USA). Methylcellulose was purchased from Fluka (Buchs, Switzerland) and XTT assay from Sigma-Aldrich (St. Louis, USA).

2.2. Antibody production and characterization

Antibody fragments were expressed in *E. coli* TG1 and purified by immobilized metal ion affinity chromatography (IMAC) as described [28]. Protein concentration was determined by measuring the absorbance at 280 nm. ScFv′ were analyzed by SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie Brilliant Blue R250. The melting point of the scFv′ CD33 variants was determined with a ZetaSizer Nano ZS (Malvern, Herrenberg, Germany). Purified scFv′ (100 µg) was diluted in PBS to a total volume of 1 ml and sterile filtered into a quartz cuvette. Dynamic laser light scattering intensity was measured while the temperature was increased in 1 °C intervals from 30 to 70 °C with 2 min equilibration for each temperature step. The melting point was defined as the temperature at which the measured size dramatically increased.

2.3. Flow cytometry

Approximately 250,000 cells were incubated with 10 µg/ml scFv′ in 100 µl PBS containing 2% FCS, 0.02% sodium azide (PBA) for 2 h at 4 °C. After washing cells three times with PBA buffer (4 °C), cells were incubated with anti His-Tag unconjugated mAb anti-mouse IgG1 for 1 h at 4 °C and goat anti-mouse IgG-PE for 30 min at 4 °C. Cells were resuspended in 500 µl PBA buffer and analyzed by flow cytometry (Cytomics FC 500, Beckmann-Coulter). Data were evaluated with WinMDI, version 2.9. For the detection of cellular antigen expression levels FITC-conjugated anti-CD33 mAb was used at a dilution of 1:20.

2.4. Preparation of liposomes

A lipid composition of EPC : cholesterol : $mPEG_{2000}$-DSPE in a molar ratio of 13:6:1 was used for the preparation of liposomes. The lipid formulation contained DiI as a fluorescent lipid marker at a molar concentration of 0.3 mol%. A thin lipid film was formed in a round bottom flask by dissolving the lipids in chloroform and removing the solvent in a rotary evaporator for 10 min at 42 °C. Subsequently the lipid film was dried completely in a vacuum drying oven for at least 1 h at room temperature. The lipid film was hydrated in 10 mM HEPES buffer, pH 6.7 and vortexed until all components were dissolved. The final lipid concentration was 10 mM. The lipid solution was then extruded 21 times through 50 nm pore size polycarbonate filter membrane using a LiposoFast extruder to obtain small unilamellar vesicles.

2.5. Preparation of immunoliposomes

100 µg scFv′ were reduced by adding 5 µl tris(2-carboxyethyl) phosphine (TCEP) (625 nmol TCEP per 1 nmol scFv′) (Pierce, Rockford, USA) and incubated under nitrogen atmosphere for 2 h at room temperature. TCEP was then removed by dialysis against deoxygenated coupling buffer (10 mM Na_2HPO_4/NaH_2PO_4 buffer, 0.2 mM EDTA, 30 mM NaCl, pH 6.7) overnight at 4 °C. For the postinsertion method [29], scFv′ were coupled to $malPEG_{2000}$-DSPE micelles. For this purpose, 50 µl of mal-PEG_{2000}-DSPE stock solution were transferred into a 1.5 ml test tube for preparation of maleimide-functionalized micelles. The solvent was evaporated in the open tube at room temperature until a lipid film became visible. The lipid film was dissolved in H_2O to a final concentration of 4.2 mM for the formation of micelles and incubated for 5 min at 65 °C. Micellar lipid and reduced scFv′ were mixed at a molar ratio of 4.67:1, overlaid with nitrogen and incubated for 30 min at room temperature. To saturate