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RPM peptide conjugated bioreducible polyethylenimine targeting invasive colon cancer

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ABSTRACT

CPIEDRPMC (RPM) peptide is a peptide that specifically targets invasive colorectal cancer, which is one of the leading causes of cancer-related deaths worldwide. In this study, we exploited RPM peptide as a targeting ligand to produce a novel and efficient gene delivery system that could potentially be used to treat invasive colon cancer. In order to achieve enhanced specificity to colon cancer cells, the RPM peptide was conjugated to a bioreducible gene carrier consisting of a reducible moiety of disulfide-crosslinked low molecular weight polyethylenimine, IR820 dye, and polyethylene glycol. Here, we examined the physiochemical properties, cytotoxicity, in vitro transfection efficiency, and in vivo biodistribution of the RPM-conjugated polyplex. Our results showed that the RPM-conjugated gene carrier formed a compact polyplex with pDNA that had low toxicity. Furthermore, the RPM-conjugated polymer not only had higher cellular uptake in invasive colon cancer than the non-targeted polymer, but also showed enhanced transfection efficiency in invasive colon cancer cells in vitro and in vivo.

1. Introduction

Despite significant improvement in early diagnosis and available treatments, colorectal cancer (CRC) remains one of the leading causes of cancer-related deaths worldwide. Incidence of CRC is increasing annually in both sexes, ranking second and third in 2011 for cancers in men and women, respectively [1]. Treatments for invasive CRC require a combination of surgery, radiation therapy, and chemotherapy; because CRC generally remains undiagnosed until symptoms become apparent, is strongly invasive in the progressive stage, and has a high post-operative recurrence rate, achieving a radical cure is difficult [2,3]. In addition, drug resistance and systemic toxic effects limit the effectiveness of chemotherapy [2]. Consequently, there is an increasing demand for the development of more efficient CRC treatment systems.

Gene therapy is a potential novel treatment modality for treatments of tumors. In particular, gene delivery systems that use cationic polymers have attracted considerable attention because they confer several advantages such as facile synthesis and modification, less-immunogenic, a large capacity for genes, low cytotoxicity, and the spontaneous formation of a nano-sized complex (polyplex) with negatively-charged DNA by electrostatic interaction which leads to efficient cell internalization and easier endosomal escape [4–6]. Branched polyethylenimines (bPEI) are effective cationic polymers that are widely used for gene delivery. bPEIs with a high molecular weight (HMW) (>25 kDa) show high transfection efficiency with significant cytotoxicity, while bPEIs with a low MW (LMW) (<1.8 kDa) show low transfection efficiency with low cytotoxicity, which limits their use in gene delivery systems [7–15]. Previously, in order to achieve high transfection efficiency while maintaining low cytotoxicity, we introduced disulfide (–S–S–) linkage to form bioreducible bPEI (SS-bPEI) containing a multiple amine backbone [9,11,16]. Introducing disulfide linkage not only increased gene transfection, but also provided biodegradable capabilities via endogenous enzymes such as glutathione reductase [16,17].

The cancer-targeting moiety of a delivery vehicle can also play an important role in efficient treatment by reducing unwanted systemic toxicity and overcoming dose limitation [18,19]. First discovered in 2003, the CPIEDRPMC (RPM) peptide has an RPM conserved motif and binds specifically to integrin $\alpha5\beta1$ in human invasive colon cancer cells [20]. RPM was derived from a peptide library via phage display and, in 2004, introduced as a molecular probe for early detection of colon cancer and in vivo imaging [21]. However, RPM peptide is yet to be tested as a potential vector for targeting therapy or for use in other RPM-conjugated applications. Therefore, in this study, we selected reducible PEI systems, which were previously developed in our group, and modified them by using RPM peptides to increase the localization of the polyplex in a tumor microenvironment produced by invasive

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colon cancer (Fig. 1). In order to achieve hydrophilic shielding following complexation with plasmid DNA (pDNA), we also incorporated polyethylene glycol (PEG) into the system, thus resulting in neutral zeta potential, low cytotoxicity, and colloidal stability [22–29]. Thus, we synthesized an RPM-conjugated bioreducible gene carrier (SS-bPEI-PEG-RPM) and evaluated its physicochemical properties, in vitro cytotoxicity, in vitro transfection efficiency, cellular uptake efficiency, and in vivo biodistributions.

2. Materials and methods

2.1. Polymers and reagents

IR820 dye and HMW bPEI (MW: 25,000, bPEI25K), 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide hydrochloride (EDC), fluorescein isothiocyanate (FITC), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent were all purchased from Sigma-Aldrich (St. Louis, USA). LMW bPEI (MW: 1200, bPEI1.2K) was purchased from Polyscience, Inc. (Warrington, PA), TOTO-3 iodide from Invitrogen (Eugene, Oregon, USA), and mounting medium for fluorescence with DAPI from VECTOR (Burlingame, USA). *N,N*-Dimethylformamide (DMF) was purchased from Acros Organics (Geel, Belgium) and dimethyl sulfoxide (DMSO) from Burdick & Jackson (Honeywell International Inc., USA). Heterobifunctional polyethylene glycol, and α-maleimide-ω-*N*-hydroxysuccinimide ester polyethylene glycol (MAL-PEG-NHS, MW: 5000), was purchased from NOF Corporation (White Plains, NY, USA) and RPM peptide (Sequence: FITC-aca-CPEIDRPM) from AnyGen, Inc. (Korea). Agarose powder and Tris-acetate-EDTA (TAE) buffer were purchased from Bioneer Corp (Daejeon, Korea), while RPMI 1640, penicillin-streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate buffered saline (DPBS) were all purchased from Corning (Manassas, VA, USA). A luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI, USA), Bradford protein assay reagent from Pierce Chemical Co. (Rockford, IL, USA), and D-luciferin from Caliper Life Sciences (Hopkinton, MA). HCT-15 and HCT-116 (human differentiated CRC lines), HCT-8 (human differentiated drug resistance CRC line), HT-29 (human invasive and undifferentiated CRC line), and CT-26 were obtained from the Seoul National University Cell Bank (Seoul, Korea). FHC (human normal colon cell line, CRL1831) was obtained from ATCC.

2.2. Preparation of pDNA

pDNA was propagated in a chemically competent *DH5a* strain (GibcoBRL, Rockville, USA) and prepared from overnight bacterial cultures by using alkaline lysis and column purification with a Qiagen plasmid Maxi kit (Qiagen, Valencia, CA). The concentration of pDNA solution was determined by measuring the absorbance at 260 nm, and we found optical density at 260 to 280 nm to be in the range 1.8–1.9.

2.3. Synthesis of gene carriers

Synthesis of SS-bPEI was performed using methods with some modification [9,10]. Briefly, for the synthesis of thiolated bPEI (SH-bPEI), the pH of a bPEI1.2K (1 g) aqueous solution was adjusted to 7.2 by dropwise addition of 0.5 or 0.1 N HCl. Subsequently, water was removed by freeze drying (EYELA, FD-1000, Japan) for 2 day. Next, the resultant yellow solid was dissolved in methanol (30 mL) in a 100 mL-capacity bottleneck flask and purged with nitrogen for 10 min before a specific amount of propylene sulfide (7 equiv. to bPEI) was added by syringe. This solution was stirred at 60 °C for 24 h. The reaction mixture was evaporated under reduced pressure, dissolved in methanol, and then precipitated twice in cold diethyl ether. For the synthesis of SS-bPEI, SH-bPEI (0.5 g) was dissolved in DMSO (50 mL) and the solution was stirred for 48 h at room temperature (RT). The product was purified by dialysis against deionized (DI) water (MWCO 10,000) and lyophilized. The chemical structure was confirmed by ^{1}H NMR (in D_2O, Bruker 300 MHz). The degree of thiolation was measured using ^{1}H NMR. The synthesis of SS-bPEI modified with IR820 was performed following the method [30]. Specifically, in IR820-SS-bPEI synthesis, the *meso*chlorine atom in IR820 was replaced with a less hindered carboxy-terminal amino linker (6-aminohexanoic acid), and this activated IR820 was conjugated to the remaining amine group of SH-BPEI by using EDC chemistry. The synthesis of IR820-SS-bPEI-PEG-RPM and IR820-SS-bPEI-PEG was performed following the previously reported method (Supplementary data, Fig. S1) [9]. RPM peptide (1.2 equiv.) and NHS-PEG-MAL (1 equiv.) were dissolved in anhydrous DMSO and stirred for 2 h at RT before SS-bPEI-PEG in DMSO was added to the solution. The reaction mixture was stirred for another 48 h at RT to produce IR820-SS-bPEI-PEG-RPM. All other experimental and characterization

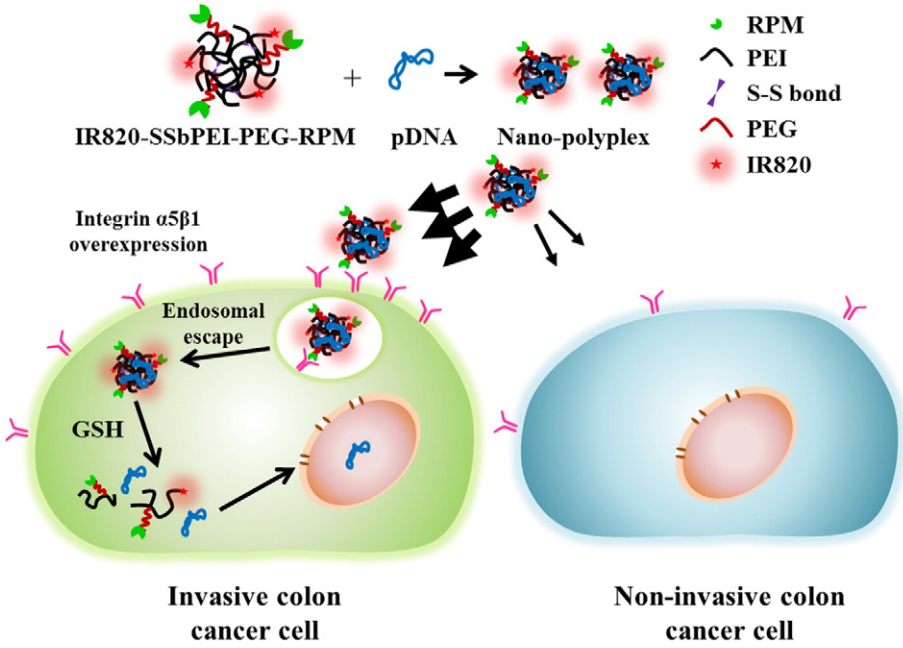

Fig. 1. Schematic diagram of gene delivery using RPM-conjugated polymers targeting invasive colon cancer cells.