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GENE DELIVERY

Erythropoietin gene delivery using an arginine-grafted bioreducible polymer system

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ABSTRACT

Erythropoietin (EPO) plays a key regulatory role in the formation of new red blood cells (RBCs). Erythropoietin may also have a role as a therapeutic agent to counteract ischemic injury in neural, cardiac and endothelial cells. One of the limitations preventing the therapeutic application of EPO is its short half-life. The goal of this study was to develop a gene delivery system for the prolonged and controlled release of EPO. The arginine grafted bioreducible polymer (ABP) and its PEGylated version, ABP-PEG10, were utilized to study the expression efficiency and therapeutic effectiveness of this erythropoietin gene delivery system *in vitro*. Poly (ethylene glycol) (PEG) modification of the ABP was employed to inhibit the particle aggregation resulting from the interactions between cationic polyplexes and the negatively charged proteins typically present in serum. Both the ABP and the ABP-PEG10 carriers demonstrated efficient transfection and long-term production of EPO in a variety of cell types. The expressed EPO protein stimulated hematopoietic progenitor cells to form significant numbers of cell colonies *in vitro*. These data confirm that this EPO gene delivery system using a bioreducible polymeric carrier, either ABP or ABP-PEG 10, merits further testing as a potential therapeutic modality for a variety of clinically important disease states.

1. Introduction

Erythropoietin (EPO) plays a key regulatory role in the formation of new red blood cells (RBCs). Erythropoietin may also have a role as a therapeutic agent to counteract ischemic injury in neural, cardiac and endothelial cells [1]. Several reports have demonstrated the capacity of EPO to protect and revascularize the myocardium following ischemic injury [2–7]. One of the limitations preventing the therapeutic application of EPO is its short half-life. The development of a more sustained form of EPO with a longer half life would remove a significant barrier to its development as a therapeutic agent. Recently, several researchers have focused on the development of an EPO plasmid DNA delivered using either viral or non-viral carriers to promote the prolonged and controlled release of EPO *in vivo* [8–11].

Compared to viral vectors, polymer carriers offer several advantages for gene delivery *in vivo* including stability, non-immunogenicity, high loading capacity of plasmid DNA, and relative ease of large-scale production [12–14]. Cationic polymers such as poly(ethylenimine) (PEI), poly(L-lysine) (PLL), and poly(amidoamine)s dendrimers, however, are limited by toxicity related to their poor biocompatibility and biodegradability. To overcome these limitations, biocompatible and biodegradable polymers have been developed which are significantly less toxic [15,16]. We have designed and synthesized several types of these biodegradable polymers [17–19]. In particular, we have developed an arginine-grafted bioreducible polymer (ABP) with enhanced transfection efficiency and low cytotoxicity, due to the localizing ability of the arginine residues and the biodegradability of a reducible disulfide bond. We have previously reported on the use of this ABP for the delivery of siRNAs and plasmid DNA *in vitro* [17,28].

Another important limitation of cationic polyplexes is their positive surface charge. This positive surface charge may interact with the negatively charged proteins present in serum [20,21], resulting in particle aggregation and a reduction in transfection efficiency *in vivo*. To overcome this limitation, conjugation of poly(ethylene glycol) (PEG) to polycationic polymers has been employed. Several studies have demonstrated that PEGylation enhances carrier function in the presence of serum *in vivo*. To enhance the efficacy of our ABP carrier *in vivo*, we designed a PEGylated form of the ABP carrier, i.e. PEG_{5K}-ABP, and then studied the influence of various formulations of PEG_{5K}-ABP on polyplex formation, size, surface charge, serum stability, induction of hemolysis and transfection efficiency. We then tested the functionality of our PEG_{5K}-ABP carrier using the gene for

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erythropoietin in plasmid form (pEPO) to determine if this PEGylated ABP would result in enhanced gene delivery and the sustained release of erythropoietin. We carried out the transfection assays of the pEPO polyplex in a variety of cell types and analyzed the time-course release of EPO *in vitro*. A colony forming cell (CFC) assay was carried out to determine the biological activity of the expressed EPO protein. The cardio-protective effects of the released EPO were investigated by an apoptosis assay in rat cardiomyocyte H9C2 cells under hypoxic conditions using flow cytometry.

2. Materials and methods

2.1. Materials

N,N′-Cystaminebisacrylamide (CBA) was purchased from Poly-Sciences, Inc. (Warrington, PA). Hyperbranched poly(ethylenimine) (bPEI25k), *tert*-Butyl-*N*-(6-aminohexyl) carbamate (*N*-Boc-1,6-diaminohexane, *N*-Boc-DAH), trifluoroacetic acid (TFA), triisobutylsilane (TIS), N,N-diisopropylethylamine (DIPEA), N,N,N′,N′-tetramethylazodicarboxamide (TMAD) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM), and Annexin-V-fluorescein isothiocyanate (FITC) apoptotic kit were purchased from Invitrogen (Carlsbad, CA). Spectrapor dialysis membrane was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Plasmid pCMV-Luc, containing a firefly luciferase reporter gene DNA (gWiz-Luc) was purchased from Aldevron, Inc. (Fargo, ND). Luciferase assay system and reporter lysis buffer were purchased from Promega (Madison, WI). Recombinant human EPO protein (rhEPO, Aropotin) was kindly provided by TS Corporation & Bioplant, Korea.

2.2. Synthesis and characterization of polymers

The arginine-modified bioreducible polymer, ABP, was synthesized by arginine modification into the primary amines of poly(CBA-DAH) as previously described. Briefly, synthesis of the poly (CBA-DAH) backbone was conducted *via* Michael reaction of equivalent moles of N-*Boc*-DAH and CBA in MeOH/H_2O solution (9:1, v/v), and the polymerization reaction was maintained under a dark nitrogen atmosphere at 60 °C for 5 days. Then, 0.1 equivalent of N-*Boc*-DAH was added to terminate the polymerization by masking unreacted acrylamide groups and the reaction mixture was further stirred for 2 days at the same temperature. After the resulting product was precipitated with cold ether, *Boc* protecting groups of the product were removed by TFA 95% solution for 30 min in an ice bath. After de-protection, the reaction mixture was precipitated with diethyl ether, dialyzed using a dialysis membrane and then lyophilized. The synthesis of poly(CBA-DAH) was confirmed with proton NMR. To modify poly(CBA-DAH) in DMF with arginine residues, Fmoc-Arg(pbf)-OH (4 eq.), HBTU (4 eq.), and DIPEA (8 eq.) were added and the mixture was reacted overnight. The reaction was monitored with a Ninhydrin test. After the completion of the arginine modification, the crude mixture was precipitated to remove the unreacted and excess reagents with ethyl ether. The reactant was deprotected with 30% piperidine solution (DMF, V/V) for Fmoc and 95% TFA for pbf groups. After precipitation with cold ether, the crude product was dialyzed against water with the dialysis membrane (MWCO 1000) followed by freeze drying. Arginine modification was confirmed with ^{1}H NMR, and an average molecular weight was determined by size exclusion chromatography (SEC). The average molecular weight was found to be approximately ~5 K.

For the PEG conjugation of ABP, 0.1 equivalent of Methoxy PEG 5K–NHS was added to the solution (pH 7.4, 0.1 M PBS (0.15 M NaCl, 2 mM EDTA)) of ABP having about 10 residues of arginine. The reaction was carried out at room temperature for 2 h. After reaction, the resulting crude product was precipitated to remove the unreacted and excess reagents with cold ethyl ether. The collected sample was purified using a dialysis membrane and then lyophilized. The conjugation of PEG was confirmed with proton NMR and size-exclusion chromatography (SEC, Superdex 75 column, calibrated with standard poly[N-(2-hydroxypropyl)-methacrylamide] (pHPMA)) using an AKTA FPLC system.

2.3. Preparation of plasmid hEPO DNA

The human erythropoietin (hEPO) cDNA was amplified by polymerase chain reaction using pDrive-hEPO (Open Biosystems, Huntsville, AL) as a template. The PCR primer sequences were as follows: forward primer, 5′-CCGGAATTCATGGGGGTGCACGAATGTC-3′; reverse primer, 5′-GCTCTAGATCATCTGTCCCCTGTCCTGCAG-3′. The EcoRI and XbaI sites were introduced to the PCR primers for cloning. The amplified hEPO cDNA was purified by agarose gel electrophoresis and elution. The hEPO cDNA was inserted into pCI (Promega, Madison, WI) at the EcoRI and XbaI sites, resulting in construction of pCMV-hEPO. The proper construction of the pCMV-hEPO was confirmed by direct sequencing. The constructed pCMV-hEPO (pEPO) was amplified in *E. coli* DH5α and purified using the Maxi plasmid purification kit (Qiagen, Valencia, CA). Purity and concentration of the purified plasmid dissolved in TE buffer were measured using a Nanodrop 1000 spectrophotometer, and the purities at A260/A280 were 1.8–1.9.

2.4. Gel retardation assay

The pDNA condensation ability of ABP and ABP-PEG was assessed by an agarose gel electrophoresis assay. Agarose gel (0.8%, wt./vol.) containing SYBR Safe DNA gel stain solution was prepared in TAE buffer. The solutions were prepared with pDNA (0.5 μg) and a corresponding amount of polymer in Hepes buffered saline, HBS (10 mM Hepes, 1 mM NaCl, pH 7.4). The two solutions were combined at various weight ratios (polymer/pDNA), slightly vortexed, and incubated for 30 min. After the loading dye was added, polyplex samples were loaded for electrophoresis and run for 15 min at 120 V. In order to examine the DNase protection, samples of polyplexes were incubated with FBS (50%) for a designated period of time. After incubation, 1.5 μL of 10% sodium dodecyl sulfate (SDS) and 1.5 μL of loading dye were added into the aliquots of samples (10 μL). The treated samples were electrophoresed on an agarose gel (0.6%) containing SYBR Safe DNA gel stain. The bands of pDNA were detected by a UV Illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA).

2.5. Particle size and Zeta-potential measurements

The particle size and Zeta-potential values of the polyplexes were measured using a Nano ZS (ZEN3600, Malvern Instruments) with a He–Ne ion laser (633 nm). 50 μL of polyplex solutions (0.5 μg of pDNA) were prepared at various weight ratios (polymer/pDNA) ranging from 1 to 40. After a 30 min incubation, polyplex solutions were diluted in filtered water to a final volume of 600 μL before measurement.

2.6. In vitro transfection experiments

For the transfection experiments, cells were plated at a density of 5×10^4 cells/well in 24-well plates in 500 μL media containing 10% FBS. After the cells were grown to 70–80% confluence, polyplexes were prepared using 0.5 μg pDNA at different weight ratios in HBS. After 30 min incubation, polyplexes (1 μg pDNA in 1 mL) were added to the cells in the presence or absence of serum for 4 h at 37 °C. The media was then replaced with fresh DMEM containing 10% FBS. The treated cells remained in the 37 °C incubator for 1 to 6 days. For the EPO protein assay, the culture medium containing hEPO protein was collected and centrifuged. The amount of EPO