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RhoA knockdown by cationic amphiphilic copolymer/siRhoA polyplexes enhances axonal regeneration in rat spinal cord injury model

So-Jung Gwak [a], Christian Macks [a], Da Un Jeong [a], Mark Kindy [b], Michael Lynn [c], Ken Webb [a], Jeoung Soo Lee [a, *]

[a] Drug Design, Development, and Delivery (4D) Laboratory, Department of Bioengineering, Clemson University, Clemson, SC 29634, USA
[b] Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, FL 33612, USA
[c] Department of Neurosurgery, Greenville Health System, Greenville, SC 29615, USA

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ABSTRACT

Spinal cord injury (SCI) results in permanent loss of motor and sensory function due to developmentally-related and injured-induced changes in the extrinsic microenvironment and intrinsic neuronal biochemistry that limit plasticity and axonal regeneration. Our long term goal is to develop cationic, amphiphilic copolymers (poly (lactide-co-glycolide)-g-polyethylenimine, PgP) for combinatorial delivery of therapeutic nucleic acids (TNAs) and drugs targeting these different barriers. In this study, we evaluated the ability of PgP to deliver siRNA targeting RhoA, a critical signaling pathway activated by multiple extracellular inhibitors of axonal regeneration. After generation of rat compression SCI model, PgP/siRhoA polyplexes were locally injected into the lesion site. Relative to untreated injury only, PgP/siRhoA polyplexes significantly reduced RhoA mRNA and protein expression for up to 4 weeks post-injury. Histological analysis at 4 weeks post-injury showed that RhoA knockdown was accompanied by reduced apoptosis, cavity size, and astrogliosis and increased axonal regeneration within the lesion site. These studies demonstrate that PgP is an efficient non-viral delivery carrier for therapeutic siRhoA to the injured spinal cord and may be a promising platform for the development of combinatorial TNA/drug therapy.

1. Introduction

Functional recovery following spinal cord injury (SCI) is limited by multiple developmentally-related and injury-induced mechanisms that restrict plasticity and axonal regeneration in the adult central nervous system (CNS). Damaged axons that survive the initial insult and secondary neuronal cell death are confronted with degenerating myelin and glial scarring. Three myelin-associated inhibitors (MAIs) have been identified (Nogo-A, myelin associated glycoprotein, and oligodendrocyte myelin glycoprotein) that bind to neuronal NgR1 and PirB receptors [1–5]. In addition, reactive astrocytes in the glial scar up-regulate expression of chondroitin sulfate proteoglycans (CSPGs) that bind to PTPsigma, leukocyte common antigen-related (LAR) phosphatase, and NgR1/NgR3 [6–8]. The signaling pathways of both classes of inhibitors as well as several axon guidance molecules converge on the activation of RhoA/Rho kinase (ROCK) [9–12] Subsequent effects on downstream targets including myosin light chain, LIM kinase/cofilin, and collapsin response mediator protein 2 interfere with cytoskeletal dynamics necessary for axonal growth [13–15]. A wide range of therapeutic strategies targeting growth inhibitory ligands, their receptors, and Rho/ROCK signaling have been shown to increase axonal regeneration and improve functional recovery, including preclinical primate models and initial human clinical trials [16–18]. However, the incomplete and variable regenerative response achieved by these approaches suggests the existence of additional barriers that restrict regeneration.

Recently, analyses of embryonic CNS neurons, the dorsal root ganglion conditioning lesion model, and transcriptomic/proteomic comparisons of PNS/CNS injury response have highlighted the importance of intrinsic neuronal biochemistry in determining

* Corresponding author. Clemson University, Department of Bioengineering, 301 Rhodes Research Center, Clemson, SC 29634-0905, USA.
E-mail address: ljspia@clemson.edu (J.S. Lee).

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regenerative capacity [19–21]. Relative to adult CNS neurons, these models have identified substantial differences in retrograde injury signaling [22], axonal transport [23], microtubule stability/organization [24], mTOR activation [25,26], cAMP levels [27], and transcription factor expression [26,28,29]. One of the most promising intrinsic targets is phosphatase and tensin homolog (PTEN) that negatively regulates the Akt and mTOR pathways involved in cell survival and metabolism, respectively [30]. However, PTEN deletion alone does not elicit a maximal regenerative response and can be significantly enhanced by co-deletion of Nogo or suppressor of cytokine signaling 3 (SOCS3), a negative regulator of the Jak/STAT signaling pathway activated by some neurotrophic factors [31,32]. Similarly, improved anatomical and functional outcomes have been achieved in several preclinical models using two or more treatments to simultaneously activate intrinsic growth capacity and neutralize extrinsic growth inhibition [33–35]. Collectively, these studies demonstrate the importance of combination therapies in overcoming the complex barriers to regeneration in the adult CNS [36–38].

Our long-term goal is to develop neuron-specific, micellar nanotherapeutics for combinatorial delivery of siRNA and hydrophobic drugs to the injured CNS. Toward this end, we have previously synthesized and characterized a cationic, amphiphilic block co-polymer, poly (lactide-co-glycolide)-graft-polyethylenimine (PgP) [39]. PgP micelles offer a hydrophobic core for solubilization of neuroprotective or neurogenic drugs, while the cationic shell can form polyelectrolyte complexes with therapeutic nucleic acids. siRNA offers several advantages for neural regeneration applications, including the large number of CNS targets therapeutically responsive to knockdown (RhoA, PTEN, SOCS3, etc.), specificity, and the ability to design sequences for different targets with minimal change in overall physicochemical properties that might affect carrier interactions and delivery properties. Previously, we have shown that PgP can efficiently transfect a variety of neural cell lines *in vitro* in the presence of 10% serum as well as deliver pDNA to the normal rat spinal cord [39]. Using RhoA as a well-established therapeutic target, here we investigate the ability of PgP to deliver siRNA (siRhoA) in B35 cells and in a rat compression spinal cord injury model. We show that PgP/siRhoA exhibits increased tissue retention time relative to naked siRNA after local injection and maintains RhoA knockdown for up to 4 weeks, resulting in reduced apoptosis and cavitation/astrogliosis and increased axonal regeneration relative to untreated SCI animal groups.

2. Materials and methods

2.1. Materials

Poly (lactide-co-glycolide) (PLGA 4 kDa, 50:50) with a carboxylic end group was purchased from Durect Corporation (Pelham, AL). Branched polyethylenimine (bPEI, 25 kDa), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and heparin sodium salt from porcine intestinal mucosa were purchased from Sigma-Aldrich (St. Louis, MO). Carbon-coated grid and 0.5% Ruthenium tetroxide (RuO_4) solution were obtained from Electron Microscopy Sciences (Electron Microscopy Sciences, PA). The silencer® Pre-designed siRNA targeting RhoA (ras homolog family member A, NCBI Reference Sequence: NM_057132.3, siRhoA) and Silencer Negative Control siRNA (NT-siRNA) were purchased from Ambion (Austin, TX). RNeasy plus mini kit and QuantiTect®SYBR Green PCR Kit were purchased from Qiagen (Valencia, CA). Protein marker and a molecular weight ladder (1 kb DNA Ladder) were from Bio-Rad (Hercules, CA). BCA protein assay kit, N-PER™ neuronal protein extraction reagent and albumin standard were obtained from Thermo Fisher (Rockford, IL). Label IT® siRNA Tracker Intracellular Localization Kit was purchased from Mirus (Madison, WI) and ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit from EMD Millipore (Billerica, MA). Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 mix with L-glutamine (DMEM/F12), 100X stock solution of penicillin/streptomycin, and 0.05% trypsin/0.53 mM EDTA in Hank's Balanced Salt Solution were obtained from Mediatech Inc (Manassas, VA) and fetal bovine serum (FBS) from Hyclone (Logan, UT). Other reagents were commercial special-grade, used without further purification.

2.2. Stability of PgP/siRNA polyplexes by gel retardation assay

Cationic, amphiphilic copolymer PgP (poly(lactide-co-glycolide)–g-polyethylenimine) was synthesized using PLGA (4 kDa, 50:50) with a carboxylic end group and branched polyethylenimine (bPEI, 25 kDa) as previously described [39]. The formation of stable polyplexes was evaluated by gel retardation assay. PgP/siRhoA polyplexes were prepared at various N/P ratios in deionized water and incubated for 30 min at 37 °C. bPEI/siRhoA polyplexes at N/P ratio 5/1 and RNAiMAX/siRhoA prepared according to the manufacturer's instructions were included as controls. The polyplexes were loaded on a 2% (w/v) agarose gel and electrophoresed for 90 min at 80 V. The gel was stained with ethidium bromide (0.5 μg/ml) for 30 min and imaged on a UV illuminator (Alpha Innotech FluorChem SP imager) to visualize the migration of polyplexes and naked siRhoA.

2.3. Knockdown efficiency and cytotoxicity of PgP/siRhoA polyplexes in serum condition in vitro

B35 neuroblastoma cells (CRL-2754, ATCC, Manassas, VA) were seeded in 24-well plates at a density of 8×10^4 cells/well in 10% serum-supplemented medium. After overnight incubation, the cells were washed twice with fresh medium. PgP/siRhoA polyplexes (1 μg of siRhoA) at N/P ratios ranging from 5/1 to 30/1 were prepared. PgP/NT-siRNA at N/P ratio of 30/1, bPEI/siRhoA polyplex at N/P ratio of 5/1, and RNAiMAX/siRhoA prepared according to manufacturer's protocol were also included for comparison. Non-transfected cells were used as a control. The cells were transfected with polyplexes in medium containing 10% FBS, incubated for 24 h and then the media containing polyplexes were removed and replaced by fresh medium containing 10% FBS. The cells were incubated an additional 48 h. At 72 h post-transfection, the cells were lysed and total RNA was isolated using RNeasy mini kit. The isolated RNA quality and quantity were evaluated by Take 3 using a BioTek synergy microplate reader (BioTek, Synergy HT). Complementary DNA (cDNA) was synthesized by reverse transcription reactions with isolated total RNA (0.5 μg) using moloney murine leukemia virus (MMLV) reverse transcriptase with oligo (dT) primers (RetroScript Kit; Ambion). Real-time PCR was performed using target-specific primers (final concentration: 0.5 μM) using SYBR Green PCR kit in a Rotorgene Q thermal cycler (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Primers for Rho A: forward primer: 5′-TTC GGA GTC GTC GTC TTG AG-3′, reverse primer: 5′-CCA CAA GCT CCA TCA CCA AC -3'. Primers for GAPDH: forward primer: 5′- ATG GCC TTC CGT GTT CCT AC-3'; reverse primer: 5′-AAC TTT GGC ATC GTG GAA GG -3'. The cycle number at which the amplification plot crosses the threshold was calculated (CT). Relative mRNA expression levels of RhoA were calculated using the $2^{-\Delta\Delta}$Ct method [40]. The minus RT (reverse transcriptase) reactions performed on a representative subset of samples demonstrated that genomic DNA contamination was not significant (data not shown). Reaction specificities were routinely verified by melting curve analysis.

Cytotoxicity of polyplexes in B35 cells was analyzed by MTT