Neuroscience 339 (2016) 308–318

# C3 TRANSFERASE GENE THERAPY FOR CONTINUOUS CONDITIONAL RHOA INHIBITION

**CLAIRE-ANNE GUTEKUNST,[a*] JACK K. TUNG,[a,c] MARGARET E. MCDOUGAL[a] AND ROBERT E. GROSS[a,b,c*]**

[a] *Department of Neurosurgery, Emory University School of Medicine, Atlanta, GA, United States*

[b] *Department of Neurology, Emory University School of Medicine, Atlanta, GA, United States*

[c] *Wallace H. Coulter Department of Biomedical Engineering, Emory University School of Medicine and Georgia Institute of Technology College of Engineering, Atlanta, GA, United States*

**Abstract—Regrowth inhibitory molecules prevent axon regeneration in the adult mammalian central nervous system (CNS). RhoA, a small GTPase in the Rho family, is a key intracellular switch that mediates the effects of these extracellular regrowth inhibitors. The bacterial enzyme C3-ADP ribosyltransferase (C3) selectively and irreversibly inhibits the activation of RhoA and stimulates axon outgrowth and regeneration. However, effective intracellular delivery of the C3 protein *in vivo* is limited by poor cell permeability and a short duration of action. To address this, we have developed a gene therapy approach using viral vectors to introduce the C3 gene into neurons or neuronal progenitors. Our vectors deliver C3 in a cell-autonomous (endogenous) or a cell-nonautonomous (secretable/permeable) fashion and promote *in vitro* process outgrowth on inhibitory chondroitin sulfate proteoglycan substrate. Further conditional control of our vectors was achieved via the addition of a Tet-On system, which allows for transcriptional control with doxycycline administration. These vectors will be crucial tools for promoting continued axonal regeneration after CNS injuries or neurodegenerative diseases.** **© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: RhoA inhibition, C3 transferase, axonal regeneration, central nervous system, gene therapy.**

## INTRODUCTION

The Rho family of small GTPases comprises intracellular molecular switches that play critical roles in regulating diverse cellular processes from cell division and migration to axon outgrowth (Luo, 2000; Stankiewicz and Linseman, 2014). Three Rho GTPases – RhoA, Rac1 and Cdc42 – are central to the regulation of the actin and microtubule cytoskeleton involved in axon growth. In simplified terms, Rac1 regulates lamellipodia formation, Cdc42 regulates filipodia, and RhoA regulates axon retraction (stress fiber formation in non-neural cells). As such, RhoA is a pivotal switch in the axonal response to environmental cues that regulate axon extension versus retraction (Gross et al., 2007).

The injured central nervous system (CNS) in the adult contains several types of molecules that inhibit the outgrowth and lead to retraction of axon growth cones, thus contributing to degeneration of fiber pathways and preventing regeneration of CNS pathways after various types of injury. Overcoming inhibitory molecules associated with myelin and the glial scar could greatly improve regeneration in the nervous system (McKerracher and Rosen, 2015). RhoA mediates the effects of diverse extracellular cues present after injury, including the myelin associated inhibitors (e.g. Nogo66), chondroitin sulfate proteoglycans (CSPGs), and some semaphorins that are commonly found in glial scars. Indeed, biochemical blockade of RhoA activity promotes axon growth and increased axon regeneration in the presence of these inhibitory molecules after CNS injury (Niederost et al., 2002; Fu et al., 2007). These promising effects of RhoA blockade are currently being evaluated in human clinical trials for the treatment of spinal cord injury (Fehlings et al., 2011).

C3 transferase (C3) is a bacterial exoenzyme that specifically and irreversibly inhibits activation of RhoA by ADP ribosylation. Direct delivery of C3 to neurons has been shown to promote axon outgrowth (Niederost et al., 2002). However, C3 is not cell-permeable so modifications have been made to improve its entry into cells (Winton et al., 2002; Tan et al., 2007). *In vivo* inhibition of RhoA by direct injection of C3 promotes robust axonal regeneration in the CNS, as demonstrated in models of optic nerve crush (ONC) or spinal cord injuries (SCIs). C3 recombinant

*Corresponding authors at: Emory University, Department of Neurosurgery, 101 Woodruff Circle, WMB, Suite 6000, Atlanta, GA 30322, United States.

E-mail addresses: cguteku@emory.edu (C.-A. Gutekunst), jtung@emory.edu (J. K. Tung), mmcdoug@emory.edu (M. E. McDougal), rgross@emory.edu (R. E. Gross), rgross@emory.edu (R. E. Gross).

*Abbreviations:* AAV2, adeno-associated serotype 2 virus; CNS, central nervous system; C3, C3 transferase; CBA, chicken β-actin; CSPGs, chondroitin sulfate proteoglycans; DMEM, Dulbecco's Modified Eagle's medium; eC3, endogenous C3; EGFP, enhanced GFP; HEK, Human embryonic kidney; hUbC, Ubiquitin C; ICC, immunocytochemistry; KRAB, Kruppel-associated box; LV, lentiviral vectors; NGS, normal goat serum; NDS, normal donkey serum; ONC, optic nerve crush; PLL, poly-L-lysine; PCR, polymerase chain reaction; SCIs, spinal cord injuries; spC3, secretable/permeable C3; TATκ, trans-acting activator of transcription; Tet-on, Tet-regulated; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

http://dx.doi.org/10.1016/j.neuroscience.2016.10.022
0306-4522/© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

protein delivered directly to the injured optic nerve at the crush site allowed processes to extend beyond the lesion site, but was limited by the short period during which injured axon processes could take up the C3 reagent (Lehmann et al., 1999). A single application of a cell-permeable version of recombinant C3, C3-07, resulted in neuroprotection of RGCs for one week, as well as increased outgrowth of RGC axons across an ONC lesion (Bertrand et al., 2005). Additional injections resulted in improved survival and regeneration over a 2 week period over the single injection (Bertrand et al., 2007). Similarly, groups have documented axon regeneration by RhoA inhibition after SCIs. In rats, permeable C3 was delivered to a T7 dorsal -hemisection SCI model resulting in extensive axonal sprouting into the lesion site and scar. Subsequent SCI studies reconfirmed that a single injection of a cell permeable C3 (Cethrin) was detectable in cells 7 days later and blocked SCI – induced RhoA activation and apoptosis for that period (McKerracher and Higuchi, 2006). Further results following permeable C3 (Cethrin) injections into SCI have yet to be reported, but are the subject of a human clinical trial (Fehlings et al., 2011; McKerracher and Anderson, 2013). Although these modifications have increased the versatility of utilizing C3 for RhoA inhibition, these studies indicate that without a continuous source of cell-permeable C3, its cellular actions are limited to a duration of several days, which is likely insufficient for the regeneration of long axon pathways that are commonly damaged in neurodegenerative diseases.

To address these limitations, we have generated viral vectors expressing C3 transferase to achieve specific, widespread, long term, and conditional RhoA inactivation. These novel vectors express either an endogenous C3 (eC3) or a secretable/permeable C3 (spC3) fused to the green fluorescent protein (GFP). Borrowing from the genetic nomenclature, we have called these C3 variants cell autonomous eC3 for expression within infected cells, and cell nonautonomous spC3, for an effect beyond the infected cells, respectively. We hypothesize that the latter should be able to affect a greater number of neurons than those infected with the cell autonomous approach. To temporally regulate and reduce any potential risks or side effects of C3 expression, we also developed expression vectors that are regulated by doxycycline (Szulc et al., 2006). As proof of principal, we have tested our transgenes *in vitro* and shown that C3 expression from both cell autonomous and cell non-autonomous vectors inhibit RhoA activation and promote neurite growth on inhibitory substrates. C3 expression in the rat striatum could also be conditionally controlled by doxycycline treatment with no significant adverse effects on striatal integrity. This novel toolbox of C3 vectors provides a versatile means of inhibiting RhoA in the nervous system and offers a promising translatable therapy for the regeneration of injured CNS pathways.

## EXPERIMENTAL PROCEDURES

### Plasmid construction

The myc-tagged C3 gene was polymerase chain reaction (PCR) amplified from the pRK5-mycC3 vector generously provided by Dr. Alan Hall (Aktories et al., 1989) and inserted into the multiple cloning site of the pEGFP-N2 plasmid (Clontech, Mountain View, CA, USA), in frame with the amino terminus of enhanced GFP (EGFP) to generate pEGFP-N2-C3 that expresses an endogenous form of C3GFP (eC3GFP). Next, a vector that expresses a secretable and permeable form of C3GFP was generated. Overlapping primers containing the DNA sequence for the synthetically modified trans-acting activator of transcription (TATκ) permeability peptide were PCR amplified and inserted at the amino terminus of C3GFP to create pEGFP-N2-TATκC3 (Tunnemann et al., 2006). The cassette containing TATκC3GFP was then PCR amplified and inserted into the multiple cloning site of the pSecTag2 Hygro A plasmid (Invitrogen, Carlsbad, CA, USA) to add an immunoglobulin K (IgK) peptide signal sequence to the N-terminus of TATκC3GFP, thus allowing for the secretion of TATκC3GFP (spC3GFP) into the extracellular milieu. A control IgK-TATκGFP (spGFP) cassette was generated in the same fashion. The cassettes containing eGFP, eC3GFP, spGFP and spC3GFP were subsequently cloned into the FUGW lentiviral and the pAAV backbone plasmids for production of 2nd generation lentivirus and adeno-associated serotype 2 virus (AAV2), respectively.

### Cell culture

Human embryonic kidney (HEK) 293T cells and NIH3T3 cells were both maintained in a humidified 5% $CO_2$ atmosphere at 37 °C in complete medium consisting of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 international units (IU)/ml penicillin, and 100 μg/ml streptomycin.

### Western Blot (WB)

Transient transfections were carried out with the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). HEK293 cells were seeded in 6-well plates at 2 × 105 cells per well, grown for 24 h (hr) and then incubated for 16 h with 1 ml of serum-free medium containing 2.5 μg of plasmid and 5 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After incubation for 16 h, the medium was replaced by fresh medium containing 10% fetal calf serum for 24 h. Cell extracts were prepared in lysis buffer (Promega, Madison, WI, USA) and protein concentration was determined using a bicinchoninic acid protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with either rabbit anti-C3 (generously provided by Dr. Lisa McKerracher, Bioaxone, Cambridge, MA (Winton et al., 2002)) or rabbit anti-GFP antibody (Clontech, Mountain View, CA, USA) followed by a donkey anti-rabbit DyLight 800 secondary antibody (Pierce, ThermoFisher Scientific, Waltham, MA, USA). Infrared (IR) detection was performed on an Odyssey IR scanner (Li-cor Biotechnology, Lincoln, NE, USA).

Download English Version:

https://daneshyari.com/en/article/6270671

Download Persian Version:

https://daneshyari.com/article/6270671

Daneshyari.com