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Research Paper

RhoA activation in axotomy-induced neuronal death

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ABSTRACT

After spinal cord injury (SCI) in mammals, severed axons fail to regenerate, due to both extrinsic inhibitory factors, *e.g.*, the chondroitin sulfate proteoglycans (CSPGs) and myelin-associated growth inhibitors (MAIs), and a developmental loss of intrinsic growth capacity. The latter is suggested by findings in lamprey that the 18 pairs of individually identified reticulospinal neurons vary greatly in their ability to regenerate their axons through the same spinal cord environment. Moreover, those neurons that are poor regenerators undergo very delayed apoptosis, and express common molecular markers after SCI. Thus the signaling pathways for retrograde cell death might converge with those inhibiting axon regeneration. Many extrinsic growth-inhibitory molecules activate RhoA, whereas inhibiting RhoA enhances axon growth. Whether RhoA also is involved in retrograde neuronal death after axotomy is less clear. Therefore, we cloned lamprey RhoA and correlated its mRNA expression and activation state with apoptosis signaling in identified reticulospinal neurons. RhoA mRNA was expressed widely in normal lamprey brain, and only slightly more in poorly-regenerating neurons than in good regenerators. However, within a day after spinal cord transection, RhoA mRNA was found in severed axon tips. Beginning at 5 days post-SCI RhoA mRNA was upregulated selectively in pre-apoptotic neuronal perikarya, as indicated by labelling with fluorescently labeled inhibitors of caspase activation (FLICA). After 2 weeks post-transection, RhoA expression decreased in the perikarya, and was translocated anterogradely into the axons. More striking than changes in RhoA mRNA levels, RhoA was continuously active selectively in FLICA-positive neurons through 9 weeks post-SCI. At that time, almost no neurons whose axons had regenerated were FLICA-positive. These findings are consistent with a role for RhoA activation in triggering retrograde neuronal death after SCI, and suggest that RhoA may be a point of convergence for inhibition of both axon regeneration and neuronal survival after axotomy.

1. Introduction

RhoA is a member of the Rho family of small GTPases, which cycle between inactive GDP-bound and active GTP-bound states and function as molecular switches in signal transduction cascades. By regulating actin cytoskeletal dynamics, RhoA plays a role in determining cell shape, attachment, motility and proliferation. In recent years, RhoA has been implicated in two important responses of neurons to spinal cord injury (SCI): 1) apoptotic neuronal death near the injury; and 2) failure of severed axons to regenerate. In mammalian models of SCI *in vivo*, technical limitations make interpretation of data regarding the precise nature of these two effects ambiguous. We have used the sea lamprey as a model to address these ambiguities because its large, individually identified reticulospinal neurons show great heterogeneity in the ability of their axons to regenerate through the same spinal cord environment (Davis Jr and McClellan, 1994b; Jacobs et al., 1997) and to survive long term after axotomy (Shifman et al., 2008). These and other advantages of the lamprey have allowed us to investigate the neuron-intrinsic factors underlying axotomy-induced neuronal death as well as failure of axonal regeneration. Those neurons that are poor regenerators also are poor long-term survivors after axotomy (Shifman et al., 2008), suggesting a possible convergence of pathways for inhibition of axon regeneration and neuronal survival, possibly through RhoA.

1.1. Post-axotomy neuronal apoptosis

Rho induced apoptosis in hippocampal (Donovan et al., 1997) and cortical neurons (Zhang et al., 2007) *in vitro*, but *in vivo* studies have delivered conflicting evidence. Application of a Rho antagonist (C3–05) reduced the number of TUNEL-labeled cells in spinal cord injured mouse and rat, suggesting a role for Rho activation in cell death near a SCI (Dubreuil et al., 2003). A dominant negative form of RhoA reduced

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apoptosis in the developing cortex, while overexpression of RhoA in cortical neurons increased apoptosis (Sanno et al., 2010). Similarly, knockdown of RhoA increased retinal ganglion cell (RGC) survival after optic nerve injury (Koch et al., 2014). On the other hand, earlier investigators had found a decrease in developmental motor neuron survival in spinal cords of embryonic mice conditionally expressing a dominant negative form of either RhoA or Rho kinase (ROCK) that blocks activities of all Rho or ROCK isoforms, respectively (Kobayashi et al., 2004). Thus, RhoA seems to modulate neuronal death, but precisely how is not clear. In particular, we wish to know whether RhoA participates in retrograde neuronal death after axotomy. The occurrence of retrograde neuronal death in supraspinal neurons after SCI has been questioned (Kwon et al., 2002; Nielson et al., 2010; Nielson et al., 2011) (but see (Novikova et al., 2000)), while the neuronal apoptosis near a SCI and attributed to RhoA activation (Dubreuil et al., 2003) could be due to factors other than axotomy, *e.g.*, inflammatory influences (Beattie et al., 2002), particularly because the apoptosis is not restricted to neurons. Indeed, after SCI, many of the apoptotic cells are glia (Liu et al., 1997), particularly oligodendrocytes, both near the lesion and remotely in association with axons undergoing Wallerian degeneration (Crowe et al., 1997). These other mechanisms can be ruled out in the very delayed retrograde apoptosis seen selectively among the reticulospinal neurons known to be poor regenerators (Shifman et al., 2008), since their cell bodies are located far from the site of spinal cord transection.

1.2. Inhibition of axon growth

In developing neurons, RhoA activation leads to collapse and retraction of the growth cone, thus affecting axonal projection, guidance and extension (Luo, 2000; Fujita and Yamashita, 2014). Both the myelin-associated growth-inhibitory proteins (MAIs) and the chondroitin sulfate proteoglycans (CSPGs) block axon regeneration *in vitro* and axon sprouting *in vivo*, at least in part by activating RhoA after neuronal injury (Kopp et al., 2012; Sharma et al., 2012). It is difficult to determine whether RhoA affects axon regeneration after SCI in mammals because axon regeneration does not occur spontaneously in mammalian CNS, and technical limitations require that most experiments on SCI employ partial injury models, in which true regeneration of severed axons is difficult to distinguish from collateral sprouting by spared ones. These limitations do not apply to the lamprey, in which axons of some reticulospinal neurons regenerate, even after complete transection (Rovainen, 1976; Selzer, 1978; Banerjee et al., 2016) and functional recovery is substantial (Cohen et al., 1986, 1988; Davis Jr and AD, 1993). Although early on, investigators focused on the inhibitory environment to explain failure of axon regeneration in CNS, manipulation of environmental growth inhibitors has produced limited and inconsistent results (Kim et al., 2003, Simonen et al., 2003, Zheng et al., 2003), perhaps because of the great variety of these factors, and their functional redundancy (Lee et al., 2010; Sharma et al., 2012). Thus attention is shifting toward neuron-intrinsic factors (Park et al., 2010; Sun and He, 2010; Kaplan et al., 2015; He and Jin, 2016), including the ability to respond to the environmental inhibitory cues. For example, our previous studies have found negative correlations between regenerative probability and mRNA expression of the CSPG receptors, leukocyte common antigen-related phosphatase (LAR) and protein tyrosine phosphatase σ (PTPσ) (Zhang et al., 2014), members of the receptor protein tyrosine phosphatase (RPTP) family. Recently, we reported in the lamprey that knockdown of RhoA by a translation-blocking morpholino antisense oligonucleotide both reduced activation of caspases in reticulospinal neurons and increased regeneration of their axons (Hu et al., 2017). In the present study, we report the cloning of RhoA, its mRNA expression in reticulospinal neurons, and its activation after axotomy in individually identified reticulospinal neurons whose caspase activities have been determined (Hu et al., 2013). The results show that caspases and RhoA both are activated selectively in neurons with poor regenerative/poor surviving abilities, and that activation of RhoA, rather than its mRNA expression, is the better correlate with both poor regeneration and apoptotic signaling after SCI. The findings are consistent with convergence of signaling pathways for failure of neuronal survival and axonal regeneration post-SCI.

2. Materials and methods

2.1. Identification and PCR cloning of the RhoA gene from a lamprey genomic database

The sea lamprey “ensemble database” and whole-genome sequencing (WGS) “trace database” maintained by the National Center for Biotechnology Information (NCBI, NIH) were used. These databases currently consist of assembled, partially assembled, and raw unassembled sequencing data from the sea lamprey genome (Smith et al., 2013). RhoA sequences from other species, including human, chicken and zebrafish, were used to query these databases, and the contigs found in the lamprey genomic library were confirmed using the basic local alignment search tool (BLAST) on NCBI servers. The contigs were further analyzed by aligning them with homologous genes of other species. Then oligonucleotide primers for polymerase chain reaction (PCR) were designed based on the lamprey RhoA gene sequence in regions highly identical with those of other species.

Total RNA from lamprey CNS was isolated using Trizol reagent (Invitrogen). The first- strand cDNA synthesis reaction from total RNA was catalyzed by Superscript III Reverse Transcriptase with oligo-dT or 8 bp random primers. The synthesized total cDNA served as templates for PCR cloning using the Expand™ Long Template PCR System (Roche Applied Science) as per the manufacturer's protocol. Following amplification, PCR fragments of expected size were purified on 1% agarose gels and ligated into the pGEM-T Easy Vector (Promega). The cloned fragments were sequenced (GENEWIZ, NJ), analyzed and compared by BLAST.

2.2. Riboprobe synthesis

The cloned lamprey RhoA cDNAs were used as templates for generating biotin-labeled antisense riboprobes. The lamprey RhoA protein contains 194 amino acids and shared > 90% amino acid identity with human, mouse, chicken and zebrafish (Fig. 1). A PCR strategy for rapid generation of template was used. The PCR primers were designed to amplify cDNA sequences (513 bp) of the underlined region in Fig. 1, which included almost the full length of the RhoA gene. A T7 promoter sequence was added to the 5′ end of the antisense primer. Biotin-labeled antisense RNA probes were constructed from the amplified cDNA template, which contained the T7 promoter sequence upstream of the antisense-strand, using T7 RNA polymerase (Promega) with Biotin RNA-labeling Mix (Roche Applied Science), as recommended by the manufacturer. PTPσ probes were described previously (Zhang et al., 2014).

2.3. Animals and spinal cord transection

Larval sea lampreys (*Petromyzon marinus*), 6–14 cm in length (3–5 years old), were obtained from streams of Lake Michigan and maintained in freshwater tanks at 16 °C until the day of use. The protocol was approved by the Temple University Institutional Animal Care and Use Committee. Lampreys were deeply anesthetized by immersion in saturated aqueous benzocaine until motionless to tail pinch, then pinned onto a Sylgard-coated dissecting dish filled with ice-cold lamprey Ringer (Lurie et al., 1994). The spinal cords were completely transected at the level of the 5th gill, and the animals allowed to recover up to 10 weeks. A total of 118 animals were investigated; 28 were used in RhoA *in situ* hybridization (ISH) studies on wholemounted lamprey brains, 13 in RhoA ISH on paraffin sections, and another 77