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Repulsive guidance molecule b inhibits neurite growth and is increased after spinal cord injury

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ABSTRACT

Neuronal axons are guided by attractive and repulsive cues in their local environment. Since the identification of the repulsive guidance molecule (RGM) a (RGMa) as an axon repellent in the visual system, diverse functions, as part of the developing and adult central nervous system (CNS), have been ascribed to it. The binding of RGMa to its receptor neogenin has been shown to induce RhoA activation, leading to inhibitory/repulsive behavior and the collapse of the neuronal growth cone. In this paper, we provide evidence to suggest the involvement of RGMb, another member of the RGM family, in the rat CNS. RGMb inhibits neurite outgrowth in postnatal cerebellar granule neurons (CGNs) *in vitro*. RGMb is expressed by oligodendrocytes and neurons in the adult rat CNS, and the expression of this molecule is upregulated around the site of spinal cord injury. RGMb is present in myelin isolated from an adult rat brain. RGMb and neogenin are coexpressed in CGNs and entorhinal cortex neurons. These findings suggest that RGMb is a myelin-derived inhibitor of axon growth in the CNS. Inhibition of RGMb may provide an alternative approach for the treatment of spinal injuries.

Introduction

Repulsive guidance molecule (RGM) was originally identified as a membrane-associated glycoprotein with a molecular weight of 33/35 kDa; it exhibits repulsive and growth cone collapse-inducing activities in the chick retinotectal system [1]. At least three homologues of RGM have been found in vertebrates, namely, RGMa, RGMb, and RGMc. Mouse RGMa is highly homologous (sharing 80% amino acid identity) to chick RGM [2]. We have previously reported that RGMa inhibits neurite outgrowth in postnatal cerebellar neurons by a mechanism dependent on the activation of the RhoA/Rho-kinase pathway [3]. RGMa protein is expressed in purified myelin obtained from the central nervous system (CNS) and in the adult rat spinal cord, and the expression of RGMa is upregulated after spinal cord injury (SCI). Interestingly, local administration of the neutralizing antibody to RGMa resulted in the enhancement of locomotor recovery and growth of axons after SCI, suggesting that RGMa plays a role in suppressing the regeneration of the injured axons in the CNS [3].

Like RGMa, RGMb is expressed in the developing and adult nervous system [4,5]. Notably, RGMb is expressed at high levels in developing retinal ganglion cells (RGCs), and is also present in the embryonic superior colliculus. In mouse, RGMb shows a distribution complementary and non-overlapping with RGMa. RGMb expression has been detected in neuronal cell bodies and proximal axonal segments. An analysis of the organization of the respective genomic loci of RGMs in mouse suggests that RGMa and RGMb may have evolved by gene duplication, and that RGMb may be more closely related to RGMa than RGMc. These findings prompted us to hypothesize that RGMb may play other roles in the CNS. In this study, we examined the expression of RGMb after SCI, and examined whether RGMb inhibits axon growth *in vitro*.

Materials and methods

Cell culture. Samples of cerebellar granule neurons (CGNs) from Wistar rats at postnatal days 7–9 were prepared as previously described [6]. Briefly, small pieces of rat cerebella were trypsinized [0.25% trypsin in phosphate-buffered saline (PBS) for 10 min at 37 °C] and dissociated into single cells by pipetting. These cells were then suspended in serum-free Dulbecco's modified Eagle's

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medium (DMEM) supplemented with penicillin/streptomycin and B27 (Invitrogen) and seeded onto poly-L-lysine-coated (PLL-coated) plates or chamber slides. For neurite outgrowth assay, CGNs were treated with recombinant RGMa [2 μg/mL RGMa in 0.1% bovine serum albumin (BSA)/PBS, R&D Systems] or recombinant RGMb [2 or 10 μg/mL RGMb in 0.1% BSA/PBS, R&D Systems] 1 h after plating. Control CGNs were treated with the same volume of vehicle. All CGNs were incubated overnight (24 h).

Entorhinal cortex neurons obtained from Wistar rats at postnatal days 0–1 were trypsinized (0.25% trypsin in PBS for 15 min at 37 °C) and dissociated into single cells by pipetting. These cells were then plated in serum-free Neurobasal medium (Invitrogen) supplemented with penicillin/streptomycin, B27 (Invitrogen), and L-glutamine (Nacalai Tesque) on PLL-coated plates and incubated overnight for Western blot analysis.

Production of polyclonal rabbit anti-RGMb antibody. Japanese white rabbits (Japan Laboratory Animals, Inc Jla:JW) weighing 2–3 kg were immunized subcutaneously with 10 μg of recombinant human RGMb emulsified with complete Freund's adjuvant (Sigma–Aldrich, No. F5881). The animals were boosted three times with the same immunogen in incomplete Freund's adjuvant (Sigma–Aldrich, No. F5506) every 2 weeks. Seven days after the final immunization, the sera were obtained, and the specific antibody was purified with an affinity column. To this end, RGMb was coupled to an NHS-activated Sepharose column (GE Healthcare, No. 17-0716-01), and the antisera was applied to the column. The column was washed with phosphate-buffered saline (PBS), and the antibody was eluted with 0.1 M glycine–HCl (pH 2.7). The antibody was dialyzed against PBS. The cross-reactivity to RGMa was tested by ELISA. ELISA plates were coated with either RGMa or RGMb at 2 μg/mL for 1 h at room temperature, and blocked with PBS containing 2% bovine serum albumin for 1 h. The serially diluted antibody was added to the wells for 1 h, and the bound antibody was detected with horse radish peroxidase-conjugated goat anti-rabbit antibody (DAKO, No. P0448). The anti-RGMb antibody did not bind to RGMa.

Neurite outgrowth assay. CGNs, which were cultured overnight, were fixed with 4% (wt/vol) paraformaldehyde (PFA). The fixed neurons were treated with PBS containing 5% BSA (Sigma) and 0.1% Triton X-100. Subsequently, the neurons were labeled with a monoclonal antibody (Tuj1) recognizing the neuron-specific β-tubulin III protein (1:1000; Covance) and a secondary anti-mouse IgG antibody conjugated with Alexa Fluor 568 (1:1000; Invitrogen). The length of the longest neurite of a β-tubulin III-positive neuron was measured. More than 400 CGNs in each group were examined. Two-way ANOVA followed by Scheffe's multiple comparison test were performed to compare the average values of axon length. P values <0.01 were considered to be statistically significant.

Isolation of myelin from rat brain and preparation of spinal cord/brain sample for Western blot analysis. The brains from 4-week-old Wistar rats were homogenized in 0.32 M sucrose. The homogenate was layered over 0.85 M sucrose. After centrifugation at 25,000 rpm for 30 min, crude myelin was obtained. Next, we obtained purified myelin by sucrose washout, osmotic shock repeated twice, and discontinuous gradient centrifugation. The purified myelin was suspended in 20% octylglucoside (Dojindo). All steps were carried out at 0–4 °C [7].

Spinal cords or brains obtained from Wistar rats, with or without SCI, were finely minced and homogenized in a lysis buffer using an electric homogenizer. The homogenate was centrifuged for 20 min at 15,000 rpm at 4 °C. The supernatant was then aspirated for Western blot analysis.

Western blot analysis. CGNs, entorhinal cortex neurons, adult rat spinal cords or brains were lysed in 50 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, 10% glycerol, and 0.5% Brij-58 (Sigma–Aldrich),

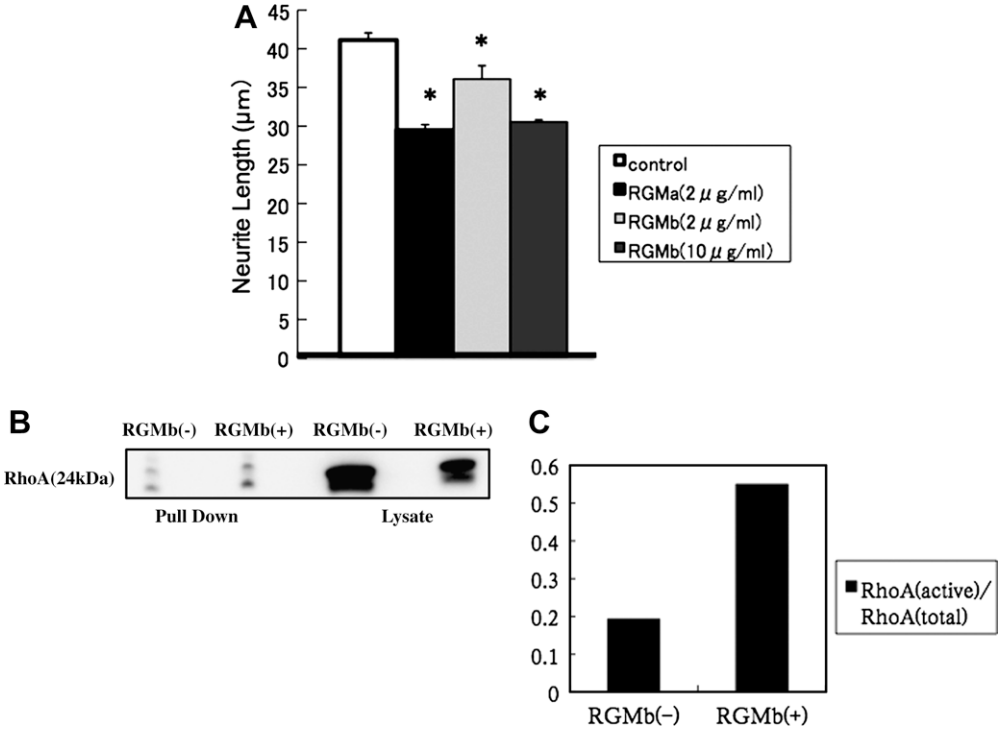

Fig. 1. (A) RGMb inhibits neurite outgrowth in cultured cerebellar granule neurons (CGNs). The CGNs obtained from Wistar rats at postnatal days 7–9 were incubated overnight (24 h) in the absence (control) or presence of soluble RGMa (2 μg/mL) or RGMb (2 or 10 μg/mL) on PLL-coated chamber slides. The length of the longest neurite per neuron was measured. Results are indicated as means ± SEM (n = 4). Asterisks indicate statistical significance. $^{*}P < 0.01$ compared with the control. Two-way ANOVA was performed, followed by Scheffe's multiple comparison test. (B) Activation of RhoA in the CGNs by RGMb. CGNs from Wistar rats at postnatal days 7–9 were serum-starved overnight and treated with RGMb (1.5 μg/mL) at 37 °C for 30 min. Control CGNs were treated with the same volume of vehicle. The activity of RhoA was examined by RhoA pull-down assay. (C) The ratio of active RhoA to total RhoA increased by RGMb treatment.