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ErbB1 epidermal growth factor receptor is a valid target for reducing the effects of multiple inhibitors of axonal regeneration

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ABSTRACT

Pharmacological inhibitors of epidermal growth factor receptor (ErbB1) attenuate the ability of CNS myelin to inhibit axonal regeneration. However, it has been claimed that such effects are mediated by off-target interactions. We have tested the role of ErbB1 in axonal regeneration by culturing neurons from ErbB1 knockout mice in the presence of various inhibitors of axonal regeneration: CNS myelin, chondroitin sulfate proteoglycans (CSPG), fibrinogen or polyinosinic:polycytidylic acid (poly I:C). We confirmed that ErbB1 was activated in cultures of cerebellar granule cells exposed to inhibitors of axonal regeneration and that ErbB1 kinase inhibitors promoted neurite outgrowth under these conditions. In the presence of myelin, fibrinogen, CSPG and poly I:C ErbB1 −/− neurons grew longer neurites than neurons expressing ErbB1. Furthermore, inhibitors of ErbB1 kinase did not improve neurite outgrowth from ErbB1 −/− neurons, ruling out an off-target mechanism of action. ErbB1 kinase activity is therefore a valid target for promoting axonal elongation in the presence of many of the molecules believed to contribute to the failure of axonal regeneration in the injured CNS.

Introduction

Axons do not normally regenerate in the adult mammalian CNS. Conditions such as complete spinal cord injuries therefore result in permanent and very serious functional deficits. There are many potent inhibitors of axonal regeneration in the injured CNS including myelin-associated proteins, CSPG, fibrinogen and axonal guidance molecules. These are believed to combine to make the damaged spinal cord very unfavourable for axon regrowth (Anderson et al., 2007; Bolsover et al., 2008; Busch and Silver, 2007; Sandvig et al., 2004; Tang, 2003; Verma and Fawcett, 2005; Wehrle et al., 2005). The EGF receptor ErbB1 may play an important role in regulating axonal regeneration. In some studies, stimuli that increase ErbB1 phosphorylation have been shown to promote axon outgrowth (Evangelopoulos et al., 2009; Goldshmit et al., 2004; Tsai et al., 2010). However, other studies indicate that ErbB1 may be a critical mediator of outgrowth-inhibitory cues in the adult CNS. Exposure to Nogo-66 or OMgp (inhibitory components of CNS myelin) or CSPG, causes phosphorylation of ErbB1 in an intracellular calcium-dependent manner (Koprivica et al., 2005). Inhibitors of ErbB1 kinase activity allow axons to grow over an inhibitory substrate of myelin or CSPG *in vitro* and through a crush injury of the optic nerve *in vivo* (Koprivica et al., 2005). Fibrinogen, which enters CNS lesions after trauma, acts through β3 integrin to cause ErbB1 phosphorylation; axon outgrowth is inhibited but can be rescued by application of an ErbB1 kinase inhibitor (Schachtrup et al., 2007). Axon outgrowth over fibroblasts is enhanced by treatment with ErbB1 inhibitors (Povlsen et al., 2008). Inhibiting ErbB1 kinase activity greatly enhanced axonal regeneration through a crush injury of the mouse optic nerve *in vivo* (Koprivica et al., 2005) and it has been reported that treatment with an ErbB1 kinase inhibitor enhanced functional recovery following spinal injury in rats (Erschbamer et al., 2007). However, an attempt at replication of the latter finding on spinal injury was not successful (Sharp et al., 2012). These results therefore suggest a model in which a large number of clinically important inhibitors of CNS axonal regeneration activate ErbB1, and the activated ErbB1 in some way acts to reduce or even eliminate axon outgrowth or regeneration. Since the inhibitors of ErbB1 that have been shown to enhance axonal regeneration include the licensed drug Erlotinib, these observations have potentially important clinical applications.

However, experiments using siRNA to knock down ErbB1 expression *in vitro* have yielded results inconsistent with this growing consensus. Cultures in which ErbB1 expression had been dramatically reduced by treatment with siRNA showed undiminished inhibition of axon outgrowth by myelin, and the ErbB1 kinase inhibitor AG1478 retained its

Abbreviations: CNS, Central nervous system; CSPG, Chondroitin sulfate proteoglycans; DRG, Dorsal root ganglia; EGF, Epidermal growth factor; TLR3, Toll-like receptor 3.

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ability to rescue axon outgrowth. On the basis of this and other evidence it was suggested that AG1478 exerted its axon-promoting effect through an action on a protein other than ErbB1 (Ahmed et al., 2009; Douglas et al., 2009). However, siRNA rarely eliminates the target protein completely. We therefore re-examined this question by using neurons from ErbB1 knockout mice in which the protein is completely absent. If PD168393 and AG1478 attenuate the effects of inhibitors of CNS axonal regeneration in these neurons, then they would be certainly acting off-target. However, we saw no such protection. Rather, our results confirm the central role of ErbB1 in mediating the inhibition.

In addition we sought to examine whether the nucleic acids can also inhibit axonal growth through ErbB1. Double stranded RNA and its analogue poly I:C, acting upon Toll-like receptor 3 (TLR3), have been reported to inhibit axon outgrowth from sensory neurons (Cameron et al., 2007). TLR3 may be activated by RNA released from damaged mammalian cells (Kariko et al., 2005), or by viral RNA. We asked whether this dramatically different cue also operated through ErbB1 and whether this effect, like that of CNS myelin, involved changes of intracellular calcium.

Materials and methods

ErbB1 +/− mice were obtained from the Jackson Labs (Strain *Egfr*^{tm1Mag}/J) and maintained in a CD1/MF1 mixed outbred colony. Genotyping used primers and PCR conditions suggested by Jackson Labs, specifically the common primer 5′GCCCTGCCTTTCCCACCAT A3 ′, the mutant-specific primer 5′TTGCAGCACATCCCCCTTTC3 ′ generating a 450 bp amplicon and the wild type specific primer 5′ATCAACTTTGGGAGCCACAC3′ generating a 350 bp amplicon. Genotyping was performed in the UCL Sequencing and Genotyping Facility and gave unequivocal results (Supplementary Fig. 1A). ErbB1 −/− pups were recognised at birth by their open eyes and at later days by lack of hair. All pups used for dissection were genotyped to confirm identification. Western blots of brains dissected from E18.5 embryos identified as ErbB1 −/− confirmed the absence of the protein (Supplementary Fig. 1B).

To culture cerebellar granule neurons (CGNs), cerebella were dissected from humanely killed P5–P7 pups and minced manually followed by digestion with 0.05% trypsin for 12 min at 37 °C in phosphate buffered saline. Digestion was terminated by adding excess foetal bovine serum. The suspension was centrifuged and the pellet resuspended in 2000 units/ml DNAase (Invitrogen) in Neurobasal medium (Invitrogen). After trituration large pieces were allowed to settle and removed; the suspension was then centrifuged and the pellet resuspended in growth medium comprising Neurobasal medium plus 2% B27 supplement, 0.4 mM additional glucose, 20 mM additional KCl, 20 μM glutamine, 100 units/ml penicillin and 100 μg/ml steptomycin followed by plating in 24 well plates at 65,000 cells per well.

Sensory neuron cultures used a growth medium of F12 with Glutamax (Invitrogen) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and either 10% foetal calf serum or, where noted, 1% N2 supplement (Invitrogen). Dorsal root ganglia from P7–P9 pups were dissected and incubated in a mixture of collagenase (5 mg/ml), dispase (2 mg/ml) and DNAase (1000 U/ml) for 40 min at 37 °C. The enzyme reaction was stopped by addition of excess growth medium. The cells were then centrifuged for 10 min at 1000 rpm. After centrifugation, the supernatant was discarded and 1 ml of growth medium was added, triturated and centrifuged for 5 min at 400 rpm. The resultant supernatant was discarded and 1 ml of growth media was added and triturated to form the cell suspension. In order to reduce the number of non-neuronal cells, a BSA cushion (15%) was prepared in growth medium. The cell suspension was slowly added through the sides of the tube such that the suspension rested on the BSA solution. It was then centrifuged at 1000 rpm for 10 min. The neuronal cells formed the pellet at the bottom of the tube and the non-neuronal cells were seen as a white layer at the interface. The pellet was then resuspended in growth medium and plated in eight chambered glass slides (VWR).

Substrates were primed by treatment with poly-L-lysine (Sigma, 100 μg/ml overnight). An inhibitory myelin substrate for sensory neuron culture was prepared by incubating primed slides for 4 h with myelin (100 μg/ml) followed by incubation for 2 h with 2 μg/ml laminin. In all other preparations, primed substrates were incubated for 4 h with laminin (10 μg/ml for CGNs, 2 μg/ml for sensory neurons) mixed where appropriate with myelin or CSPG (100 μg/ml and 5 μg/ml respectively). In contrast the inhibitory agents fibrinogen and poly I:C were added to the growth medium.

Cerebellar granule cell cultures were fixed with 4% paraformaldehyde after 16 h, whilst sensory neurons were fixed after 24 h (48 h for cultures on myelin). Cultures were stained for neuron-specific β3 tubulin (rabbit polyclonal, Sigma) and with Hoechst 33342 (Sigma). Images were acquired using a Zeiss Axiophot microscope, Hamamatsu C4742 camera and Improvision software. The total neurite length for each imaged neuron was measured using the Neurite Tracer plug-in for the ImageJ software package. For cerebellar granule cells, neurite lengths on one individual cover slip (10 images at 20× magnification) were averaged to yield a single value. In most experiments statistical assessment was performed on measurements obtained from 2 coverslips from each of 3 animals (N = 6). For sensory neurons, neurite lengths from all four wells with the same culture conditions, comprising measurements of more than 30 sensory neurons, were averaged to yield a single value from each animal.

Data from each experiment were analyzed by ANOVA followed by a set of post tests using Bonferroni correction. These comprised three types of comparison. First, for each culture environment, neurite outgrowth from ErbB1 knockout neurons was compared with outgrowth from heterozygote neurons under the same culture conditions. Second, neurite outgrowth in experimental conditions was compared with outgrowth from neurons of the same genotype under control conditions. Lastly to assess whether a drug treatment gave significant rescue of neurite outgrowth, neurite length in the presence of a neurite outgrowth inhibitor and additional drug was compared with neurite length of the same genetic type with the same outgrowth inhibitor but without the drug.

Phosphorylation of ErbB1 in cerebellar granule cells was assessed by scraping cells off the substrate, washing twice with phosphate buffered saline, and lysing for 45 min at 4 °C in CelLytic lysis buffer (Sigma Aldrich) supplemented with 1% protease inhibitor (Sigma) and 1%phosphatase inhibitor (Active Motif). Total protein extracts (10 μg/lane) were separated electrophoretically in 7% Tris-Acetate gel and transferred to an Immobilon membrane (Millipore). Immunodetection used rabbit primary antibodies against total and Y1068 phosphorylated EGFR (1:1000, Cell Signaling, Beverly, Massachussetts) together with monoclonal anti-tubulin (1:3000, Sigma). Finally, the primary antibodies were probed with horseradish peroxidase conjugated polyclonal goat antibodies (Cell Signaling) for chemiluminescence detection with the ECL System (GE Healthcare).

ErbB1 inhibitors PD168393 and AG1478 were obtained from Merck. BAPTA-AM and BCECF-AM were from Invitrogen.

Results

ErbB1 phosphorylation in the presence of the inhibitors of axonal regeneration, CNS myelin or fibrinogen

First we confirmed that CNS myelin and fibrinogen, potent inhibitors of axonal regeneration, elevated levels of ErbB1 phosphorylation. Phosphorylation of ErbB1 was detectable in cerebellar granule neurons on a permissive polylysine/laminin substrate, but was markedly increased in cells cultured in the presence of fibrinogen or cultured on CNS myelin. As expected, under all conditions, the ErbB1 kinase inhibitor PD168393 at 10 nM or 100 nM reduced ErbB1 activation to trace levels