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Decorin promotes robust axon growth on inhibitory CSPGs and myelin via a direct effect on neurons

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ABSTRACT

Inhibitory chondroitin sulfate proteoglycans (CSPGs) and myelin-associated molecules are major impediments to axon regeneration within the adult central nervous system (CNS). Decorin infusion can however suppress the levels of multiple inhibitory CSPGs and promote axon growth across spinal cord injuries [Davies, J.E., Tang, X., Denning, J.W., Archibald, S.J., and Davies, S.J., 2004. Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. Eur. J. Neurosci. 19, 1226–1242]. A question remained as to whether decorin can also increase axon growth on inhibitory CSPGs and myelin via a direct effect on neurons. We have therefore conducted an *in vitro* analysis of neurite extension by decorin-treated adult dorsal root ganglion (DRG) neurons cultured on substrates of inhibitory CSPGs or myelin membranes mixed with laminin. Decorin treatment promoted 14.5 and 5-fold increases in average neurite length/neuron over untreated controls on CSPGs or myelin membranes respectively. In addition to suppressing inhibitory scar formation, our present data shows that decorin can directly boost the ability of neurons to extend axons within CSPG or myelin rich environments.

Introduction

The failure of axon regeneration in the adult mammalian brain and spinal cord has been attributed to a combination of intrinsic neuronal limitations and an environment within the injured central nervous system that is poorly supportive of axon growth. In addition to the immediate impediment to axon regeneration presented by the rapid upregulation of scar-associated axon growth inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) at sites of injury (Morgenstern et al., 2002; Tang et al., 2003), it is thought that myelin-associated proteins such as NOGO, MAG and OMgp also present an inhibitory environment for axon regeneration immediately adjacent and beyond sites of injury (reviewed in Yiu and He, 2006).

Two potential means of overcoming the axon growth inhibitory effects of scar and myelin-associated inhibitors are to lower their levels within the injured nervous system or to block neuronal sensitivity to them. Decorin is a small leucine proteoglycan that is expressed by neurons, astrocytes and Schwann cells in neural tissues (Hanemann et al., 1993; Stichel et al., 1995). Importantly, decorin is known to have the ability to inhibit the activity of transforming growth factors betas (TGFβs) (Yamaguchi et al., 1990) and the epidermal growth factor receptor (EGFR) (Csordas et al., 2000), both of which have been shown to regulate the synthesis of axon growth inhibitory CSPGs (Asher et al., 2000; Dobbertin et al., 2003). We have previously demonstrated that infusion of human recombinant decorin core protein can suppress the core protein levels of the CSPGs neurocan, phosphacan, brevican and NG2 by as much as 90% at sites of acute rat spinal cord injury (Davies et al., 2004). This marked reduction in the levels of multiple axon growth inhibitory CSPGs, resulting from suppression of CSPG synthesis and/or a potential increase in CSPG degradation (Davies et al., 2004; Davies et al., 2006), correlated with the ability of adult sensory axons to cross decorin-treated injuries (Davies et al., 2004). Thus decorin clearly has the ability to effectively "lower the hurdle" presented to growing axons attempting to traverse sites of CNS injury by reducing the levels of multiple scar-associated CSPGs.

In light of previous *in vitro* experiments demonstrating that decorin had no effect, either growth promoting or inhibitory, on neurite extension by adult DRG neurons grown on laminin (Davies et al., 2004), we hypothesized that the decorin mediated reduction of scar-associated inhibitors, and not a direct effect of decorin on neurons,

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was therefore the prime mechanism supporting axon growth across decorin-treated spinal cord injuries (Davies et al., 2004). However these experiments did not rule out the possibility that the axon growth across decorin-treated spinal cord injuries and beyond may in part have been supported by a direct effect of decorin on neurons that increased their ability to grow axons in the presence of remaining CSPG and myelin-associated axon growth inhibitors.

To test this hypothesis, we have conducted *in vitro* assays of the ability of human recombinant decorin core protein to promote neurite outgrowth by adult DRG neurons seeded onto substrates rich in axon growth inhibitory CSPGs and myelin membranes. Our data demonstrates for the first time that decorin can promote robust increases in both neurite extension and branching by adult DRG neurons plated on to substrates containing relatively high concentrations of multiple axon growth inhibitory molecules. Our study shows that decorin can promote axon growth by acting directly on injured neurons to effectively suppress their sensitivity to a variety of both scar and myelin-associated axon growth inhibitors.

Materials and methods

Preparation of inhibitory substrates

Glass coverslips were pre-treated with 100 μg/ml poly-L-lysine and then rinsed and dried. For CSPG experiments, a solution of 10 μg/ml of laminin (Sigma, St. Louis, MO) and 10 μg/ml CSPG mixture (Chemicon, Temecula, CA) was applied to the coverslips for 3 h. For experiments using CSPGs and myelin membranes, laminin was added as a supportive substrate for neuronal attachment and neurite outgrowth. Previous studies (Holmberg et al., 2006; Snow et al., 2003) have also investigated the inhibitory effects of CSPGs and myelin membranes in the presence of laminin *in vitro* as laminin is an important growth promoting molecule that is upregulated at sites of CNS injury (Sosale et al., 1988) and is known to be a binding partner for inhibitory CSPGs (Snow et al., 1996). The addition of laminin therefore more closely models the environment presented to growing axons *in vivo* and also provides a growth supportive substrate for neurites that have been rendered insensitive to the inhibitory effects of CSPGs and myelin membranes by decorin. Coverslips were washed with PBS immediately prior to plating neurons. For preparation of myelin enriched substrates, myelin membranes were isolated from adult rat brain and spinal cord using a method originally developed by Norton and Poduslo (1973). Standard Western blot analysis (non-reduced) techniques were used for detection of MAG (mouse anti-MAG, Chemicon, Temecula, CA) and OMgp (mouse anti-human OMgp, AbD Serotec, Raleigh, NC) to confirm the isolation of axon growth inhibitory myelin membranes. Myelin membranes isolated with this method have been shown to be highly inhibitory to neurite growth *in vitro* (Benson et al., 2005; Cai et al., 1999; Holmberg et al., 2006; Karnezis et al., 2004; Ng et al., 1996b; Ng et al., 1996a; Shen et al., 1998; Wang et al., 2002b). Coverslips were incubated with a solution of 15 ng/mm^2 myelin membranes and 10 μg/ml of laminin overnight. Coverslips were then washed immediately prior to plating neurons. At no point in all experiments were the CSPG or myelin enriched substrates allowed to dry.

Preparation of adult DRG neuron cultures

Purified suspensions of adult rat sensory neurons were isolated from 3 month old Sprague Dawley rat dorsal root ganglia (DRG) as previously described (Davies et al., 1997). Briefly, DRG were rapidly dissected, incubated in a mixture of 2.5 U/ml dispase (Roche, Switzerland) and 200 U/ml collagenase II (Sigma, St. Louis, MO) in DMEM at 37 °C for 1.5 h. Enzyme treated DRG were then carefully triturated and the resulting suspension fractionated by centrifugation for 5 min at 1500 rpm in a Beckman-Coulter Microfuge 18. Supernatants containing non-neuronal cells were discarded and cell pellets enriched for neurons were re-suspended in DMEM without growth factors or serum. Neurons were then cultured for 48 h on the CSPG substrate and for 18 h on the myelin membranes. Human recombinant decorin core protein was added to some wells at a concentration of 20 μg/ml 2 h after the neurons were plated. A concentration of 20 μg/ml of decorin core protein was chosen in the present study so as to match the optimal concentration of decorin previously shown to be effective at promoting neurite outgrowth by identically prepared adult DRG neurons grown on monolayers of normally inhibitory meningeal fibroblasts (Davies et al., 2004).

β-III-tubulin immunohistochemistry

Standard immuno-fluorescence staining for β-III-tubulin was conducted to visualize neurons and neurites. Briefly, cultures were fixed with 2% paraformaldehyde for 5 min, rinsed with PBS (phosphate buffered saline) and incubated with a monoclonal anti-type III β tubulin antibody (Sigma, St. Louis, MO) in 0.1% triton/PBS for 30 min at room temperature. Coverslips were then washed for 30 min in PBS followed by a 30 min incubation at room temperature with an AlexaFluor 488 conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR) in triton/PBS. Coverslips were then washed and mounted on glass slides.

Quantification of neurite growth

Each experimental comparison of neurite outgrowth for decorin versus untreated control neurons was conducted in triplicate and a minimum of three coverslips per experimental condition was used for analysis of neurite outgrowth. Images of all neurons on individual coverslips were captured using an Olympus DP70 camera and an Olympus BX epifluorescent microscope under constant magnification and resolution.

Analysis of neurite initiation

The number of neurons that extended at least one neurite was divided by the total number of neurons in each experimental condition. This data was used to calculate the percentages of neurons per experimental group that either failed or were able to initiate neurite outgrowth.

Analysis of neurite length

The total number of β-III-tubulin+neurons per coverslip and the lengths of their neurites were quantified using a custom script for Zeiss AxioVision Image Analysis Software. Neurons that did not extend any neurites were included in this analysis and assigned a neurite length of zero μm. The average neurite length (μm) per neuron for each coverslip was then calculated. The average neurite lengths per neuron for each coverslip within an experimental treatment or control group were then averaged. Finally, data was analyzed by *T*-test ($p<0.05$) and standard deviations calculated.

Analysis of neurite branching

A random number generator was used to select archived images from coverslips within each experimental group, and the numbers of branch points for 90 neurons from each group were counted. A branch point was defined as the point at which a neurite bifurcated. The origin of a primary neurite from the soma was not scored as a branch point.

Western blot analysis of plasminogen/plasmin expression

Control untreated and decorin-treated neuron cultures on CSPGs substrates were analyzed for decorin induction of plasmin and its zymogen plasminogen. Tissue culture dishes were pre-coated with a solution of 10 μg/ml CSPG mixture and 10 μg/ml laminin for 3 h and rinsed with PBS immediately prior to plating adult DRG neurons.