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Research report

Treatment of a relapse-remitting model of multiple sclerosis with opioid growth factor

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ABSTRACT

Relapse-remitting multiple sclerosis (MS) is an immune-mediated disease of the central nervous system that affects more than 2.5 million individuals worldwide. While the etiology of MS is unclear, disease manifestation involves proliferation and activation of lymphocytes and astrocytes, leading to demyelination and neuronal damage. Current therapies are not completely effective, and few target the underlying pathophysiology of MS. The purpose of this study was to examine the therapeutic efficacy of a novel biological pathway, the opioid growth factor (OGF)–OGF receptor (OGFr) axis. OGF inhibits DNA synthesis and has been shown to repress proliferation of T lymphocytes, microglia, and astrocytes in other autoimmune disorders. An animal model for relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) was established by immunization of SJL/J mice with proteolipid protein. Treatment with OGF or saline was initiated simultaneously with immunization, and within 9 days, behavioral signs of RR-EAE were observed. OGF-treated RR-EAE animals had less severe clinical disease than mice receiving saline and exhibited 66% reductions in median cumulative disease scores, as well as prolonged periods of remission and diminished number and length of disease relapses. Neuropathological examination of lumbar spinal cord revealed reductions in the number of T lymphocytes, microglia/macrophages, and activated astrocytes, with cell proliferation being the mechanism targeted by OGF. Areas of demyelination and neuronal damage were markedly reduced during the 55-day observation period. These data are the first to demonstrate that OGF prevented relapses in RR-EAE and diminished underlying neuropathology, corroborating the potential of the OGF–OGF receptor pathway for treatment of MS.

1. Introduction

Multiple sclerosis (MS) is a chronic, debilitating immune-mediated disease of the central nervous system (CNS) that affects more than 2.5 million individuals worldwide, with nearly 85% of the patients afflicted with the relapse-remitting form (RR-MS) (NMSS, 2013). Disease manifestation involves proliferation and activation of T-lymphocytes, microglia, and astrocytes, leading to inflammation, demyelination, and axonal damage. Over a period of time, neurodegeneration in the spinal cord and brain are associated with disease progression. Current therapies are designed to target one or more of the symptoms of the disease, but few are disease-modifying in nature (Stoll et al., 2012). Despite some differences in the cause of relapse-remitting disease, the mouse model of relapse-remitting experimental autoimmune encephalomyelitis (RR-EAE) represents an animal model that responds to proteolipid protein immunizations by proliferation of T-cells and microglia, and activation of astrocytes (Pollinger et al., 2009; Summers De Luca et al., 2010). The behavioral course of disease can be charted and utilized as an endpoint for therapeutic interventions.

Opioid growth factor (OGF), chemically termed [Met^5]-enkephalin, and its receptor, OGFr, form a physiological pathway that maintains homeostasis and can be modulated to shift the course of disease (Zagon et al., 2002; McLaughlin and Zagon, 2012). Modulation of the OGF–OGFr axis either by chronic treatment with the endogenous peptide OGF, or by upregulation of OGF and OGFr following low dosages of naltrexone (LDN), in mice immunized with myelin oligodendrocytic glycoprotein (MOG) to establish progressive EAE was neuroprotective against encephalitogenic processes (Zagon et al., 2009b, 2010; Rahn et al., 2011). Signs of behavioral deficits are delayed in appearance, reduced in severity, or reversed in EAE mice receiving 10 mg/kg OGF beginning at the time of disease induction in comparison to mice receiving daily injections of saline (Zagon et al., 2010; Rahn et al., 2011; Campbell et al., 2012). Evaluation of lumbar spinal cord sections revealed significant reductions in the number of activated astrocytes and regions of demyelination (Zagon et al., 2010; Rahn et al., 2011). Treatment of mice with exogenous OGF initiated at the time of established EAE reversed the progression of clinical disease within 6 days (Campbell et al., 2012). Mice with MOG-induced

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EAE and receiving OGF treatment initiated with established disease exhibited a reduced number of activated astrocytes and damaged neurons, decreased areas of demyelination, and repressed T cell proliferation (Campbell et al., 2012). Within 3 weeks of MOG immunization, EAE mice treated with saline had 3.5-fold elevated numbers of Iba1+ cells in the lumbar spinal cord in comparison to normal mice. OGF-treated EAE mice had 30% reductions in the number of microglia/macrophages relative to EAE mice receiving saline (Campbell et al., 2012). OGF therapy reduced the number of T lymphocytes in the spinal cord (detected by CD3 staining) by 56% relative to EAE mice receiving saline. The mechanism targeted by OGF was cell proliferation, with Ki67 staining markedly reduced in spinal cord sections from OGF-treated EAE mice. Sections stained with both Ki67 and GFAP revealed only 3% of cells in OGF-treated EAE mice being double labeled in comparison to ~14% of cells in spinal cord sections from saline-injected EAE mice. OGF has been shown in a number of models to up-regulate cyclin-dependent inhibitory kinases and protract cell passage from G_0G_1 to S (Cheng et al., 2009). This mechanism to reduce cell proliferation has been documented for T and B lymphocytes stimulated *in vitro* to replicate (Zagon et al., 2011a,b).

These observations were demonstrated using an animal model of MOG-induced EAE that most resembles chronic, progressive MS; however, most patients have RR-MS (NMSS, 2013). In the present study, we established a mouse model of RR-EAE using proteolipid protein (PLP) immunization of SJL/J mice (Summers De Luca et al., 2010), and determined the efficacy of daily injections of OGF initiated at the time of disease induction. Mice were observed daily over a 55 day period of time, and lumbar spinal cord tissue was collected on 10, 14, and 55 days after initiation of treatment in order to assess expression and proliferation of astrocytes, T lymphocytes, microglia/macrophages, as well as demyelination and neuronal damage.

2. Materials and methods

2.1. Mice

Female SJL/JOrlCRL mice (Charles River Labs, Wilmington, MA) were housed 5 per cage under standard conditions in a separate room from other rodents and acclimated for one week prior to disease induction; food and water were available *ad libitum*. As the course of EAE disease progressed, soft food and water packets were placed on the floor of the cages. All experiments were conducted in accordance with the National Institute of Health guidelines on animal care, and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

2.2. Induction of relapse-remitting EAE and treatment

RR-EAE was induced by immunization with proteolipid-protein following established protocols (Jackson et al., 2009; Mangano et al., 2009; Miller and Karpus, 2007). Mice were given multiple injections subcutaneously (total volume 300 μl) on the back with an emulsion of 100 μg $PLP_{139–151}$ (Peptides International, Louisville, KY) and 250 μg *Mycobacterium tuberculosis* (H37RA, Difco Laboratories, Detroit, MI) added to 0.15 ml incomplete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO); equal volumes of the PBS containing $PLP_{139–151}$ and Freunds adjuvant were emulsified by vortexing. Intraperitoneal (i.p.) injections of 200 ng pertussis toxin in phosphate buffered saline (List Biological Laboratories, Campbell, CA) were given on days 0 and 2. Mice were lightly anesthetized with 3% isoflurane (Vedco, Inc., St. Joseph, MO) for $PLP_{139–151}$ and *M. tuberculosis* injections; anesthesia was not required for daily OGF or saline treatments. Normal mice received equal volumes of sterile phosphate buffered saline in place of $PLP_{139–151}$ and pertussis toxin.

Mice immunized with $PLP_{139–151}$ were randomized to receive daily intraperitoneal injections (0.1 ml) of either 10 mg/kg OGF (Polypeptide Laboratories, Torrance, CA) (PLP + OGF) or an equal volume of sterile phosphate-buffered saline (PLP + Saline) administered to mice beginning on the day of immunization. All injections were given between 9.00 and 10.00 hr. Animals were weighed weekly in order to adjust drug dosages.

2.3. Behavioral observations

All mice were observed by 2 individuals, with one evaluator masked to the treatment group. Behavior was scored by placing each animal on a smooth surface and recording tail tonicity, gait and righting reflex. Limb strength was assessed by inverting the animal on a wire grid and observing the ability for each mouse to maintain grasp. To accommodate a broad series of behavioral observations, a modified scale of 0–10 (10 = death) was utilized (Campbell et al., 2012), with scoring based on summation of gradations for intermediate behavior observed for tail tonicity, gait, righting reflex, and individual limb tonicity. Paralysis of a limb was recorded when the limb was unable to support body weight.

Disease onset was considered the second consecutive day that a mouse had a behavioral score of 0.5 or greater. Cumulative disease scores for each treatment group were the summation of behavioral scores for all mice throughout the 55-day experimental period. Disease index was calculated as the cumulative score divided by the day of disease onset. Mean maximal severity scores were calculated as the average of the highest disease score for each mouse over the course of the experiment. A complete remission was considered when the animal returned to a score of 0.5 or less for two consecutive days. A relapse was defined as the period of time when behavioral scores for an individual mouse over 2 days were greater than or equal to two points higher than their average running behavioral score. Behavioral scores for mice that survived the entire 55 days were included in analyses; behavioral scores for mice that were euthanized for pathology were recorded, but not included in the long-term behavioral assessment.

2.4. Neuropathology

Spinal cord tissues were collected from mice in all groups at 10, 14, and 55 days of treatment which represented time prior to clinical signs of disease, the time of peak disease, and approximately 8 weeks of drug treatment, respectively. Prior to being euthanized by intracardial perfusion, mice were deeply anesthetized with a cocktail containing ketamine (30 mg/kg), xylazine (5 mg/kg) and acepromazine (2 mg/kg) diluted in sterile phosphate buffered saline. After perfusion with fresh 4% paraformaldehyde, intact vertebral columns were dissected and post fixed in 4% paraformaldehye for 18 h. Frozen sections (10 μm) of the lumbar region (L4-L5) were stained for microglia/macrophages (Venkatesan et al., 2010), T lymphocytes (Gourdain et al., 2012; McCandless et al., 2006), and activated astrocytes (Bannerman et al., 2007). Paraffin sections (10 μm) of the lumbar region (L5–L6) were stained as described below for demyelination (Jasmin et al., 2000) and markers of neuronal damage (Shafri et al., 2012; Jackson et al., 2009). Cell numbers, as well as areas of demyelination, were analyzed using published methodology (Zagon et al., 2009b, 2010; Rahn et al., 2011; Campbell et al., 2012). Overall cell proliferation, as well as specific astrocyte proliferation, was evaluated by Ki67 staining (Scholzen and Gerdes, 2000). Controls for immunostaining included sections stained with secondary antibody only. At least 2 sections/mouse from 3 to 8 animals/group were evaluated for each histopathological measure.

2.4.1. *Microglia/macrophages* were identified using Iba1 antibody (1:200, Wako, Osaka, Japan); the number of positively stained