Neuroscience 223 (2012) 114–123

GELDANAMYCIN ACCELERATED PERIPHERAL NERVE REGENERATION IN COMPARISON TO FK-506 *IN VIVO*

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Abstract—FK-506 accelerates nerve regeneration and improves functional recovery *in vivo*; its immunosuppressive properties, however, limit its clinical utility. Geldanamycin (GA), a non-immunosuppressive agent, shares a common binding target (heat shock protein 90) with FK-506 and may accelerate nerve regeneration through a similar mechanism. GA has been shown to augment neurite outgrowth *in vitro* but has not been tested *in vivo*. The current study investigated the effect of GA on the rate of axonal regeneration and functional recovery following peripheral nerve injury. In the first experiment, Thy1-GFP transgenic rats underwent serial transmuscular imaging to quantify the rate of axonal regeneration following saphenous nerve crush injury. In subsequent experiments, Lewis rats underwent tibial nerve crush or transection-and-repair injuries and were assessed for functional recovery by walking track analysis. All animals were randomized to receive daily administration of FK-506 (2 mg/kg), GA (0.2 mg/kg), or a control vehicle (dimethyl sulfoxide, 1 mL/kg) starting 3 days prior to injury. Both GA and FK-506 significantly increased the rate of axonal regeneration following crush injury in Thy1-GFP rats. In Lewis rats undergoing tibial nerve crush injury, earlier functional recovery occurred at day 5 and day 6 in animals treated with FK-506 and GA respectively, vs. day 13 for controls. Over a truncated 21-day timeframe, Lewis rats undergoing tibial nerve transection-and-repair injury and treated with FK-506 regained function at day 16, whereas those treated with GA or the control vehicle did not regain normal function. GA-treated animals, however, did exhibit significant functional improvement vs. controls. The current study demonstrated that GA accelerates axonal regeneration and enhances functional recovery *in vivo*. Its ability to increase the rate at which peripheral axons regenerate is comparable to that of FK-506. GA, however, did not match the performance of FK-506 in injury models where Wallerian degeneration (WD) is ongoing in the distal stump. This provides evidence that FK-506 accelerates axonal regeneration through two parallel mechanisms: the first being its well-established effect on neurons; the second is likely a newly described, as-yet poorly defined mechanism that affects WD. Finally, given the decrease in observed toxicity with GA administration, it might be a suitable non-immunosuppressive alternative to FK-506 for accelerating peripheral nerve regeneration in cases of clinical nerve injury.

Key words: geldanamycin, FK-506, tacrolimus, nerve regeneration, live imaging, Thy1-GFP rat.

INTRODUCTION

Restoration of normal motor function following peripheral nerve injury is limited by the time required for regenerating axons to reach the appropriate denervated target muscle. Physiologic changes due to chronic end-organ denervation, including a loss of regenerative support in the distal stump (Fu and Gordon, 1995a,b; Gordon and Fu, 1997; Hoke et al., 2002), and irreversible degeneration of the target muscle (Gutmann and Young, 1944; Roytta and Salonen, 1988; Schmalbruch and Lewis, 1994; Rodrigues Ade and Schmalbruch, 1995; Kobayashi et al., 1997; Viguie et al., 1997; Jejurikar et al., 2002; Borisov et al., 2005a,b; Chen et al., 2010), contribute to limited functional outcomes seen clinically despite advancements in surgical techniques.

A therapeutic that accelerates the rate at which axons regenerate would shorten the time needed for axons to reinnervate the appropriate end organ and expand the time window for effective surgical intervention. FK-506 (tacrolimus) is the only therapeutic intervention that has consistently demonstrated the ability to accelerate the rate at which axons regenerate. In addition to being a widely-used immunosuppressant in transplant patients (Goto et al., 1987; Kino et al., 1987a,b), it has been shown to accelerate axonal regeneration *in vivo* and improve motor outcomes after injury (Gold et al., 1995; Gold, 1997; Doolabh and Mackinnon, 1999; Wang and Gold, 1999; Becker et al., 2000; Jost et al., 2000; Lee et al., 2000; Feng et al., 2001; Navarro et al., 2001; Grand et al., 2002; Myckatyn et al., 2002; Sobol et al., 2003; Udina et al., 2003, 2004; Yang et al., 2003; Brenner et al., 2004; Jensen et al., 2005; Lopez-Vales et al., 2005; Snyder et al., 2006; Yan et al., 2011, 2012). It is hypothesized that FK-506 mediates its neuroregenerative effects through binding to heat shock protein 90 (Hsp90) in neurons via the immunophilin FK-506-binding protein 52 (FKBP-52). Binding results in activation of a calcineurin-independent (non-immunosuppressive)

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Abbreviations: DMSO, dimethyl sulfoxide; EPL, experimental print length; FKBP-52, FK506-binding protein 52; GA, geldanamycin; GFP, Green fluorescent protein; Hsp90, heat shock protein 90; NPL, Normal print length; PLF, print length factor; WD, Wallerian degeneration.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.07.026

pathway resulting in accelerated axonal regeneration (Gold et al., 1999, 2005; Gold and Zhong, 2004). FK-506 may also accelerate regeneration through a parallel pathway that increases the efficiency with which the axonal debris is cleared from the distal nerve stump during Wallerian degeneration (WD) by inhibiting calcineurin (Kang et al., 2007) (immunosuppressive pathway), and altering the innate immune response (Boivin et al., 2007). However, the contribution of this second pathway has yet to be clearly defined. Although FK-506 has been used clinically in nerve allotransplantation (Mackinnon et al., 2001) its use in cases of non-life-threatening peripheral nerve injuries is difficult to justify because of the significant risks associated with systemic immunosuppression (Brenner et al., 2002).

The ansamycin antibiotic geldanamycin (GA), like FK-506, has strong binding affinity for Hsp90 (Whitesell and Cook, 1996; Stebbins et al., 1997; Gold, 1999; Gold et al., 1999; Lele et al., 1999; Bucci et al., 2000; Qin and Panek, 2008); unlike FK-506, however, it does not induce immunosuppression. GA was initially developed as a potential anti-cancer agent (Supko et al., 1995; Zagzag et al., 2003; Miyata, 2005; Fukuyo et al., 2010) and has been shown to possess neuro-protective properties against cerebral ischemic insults, (Giffard et al., 2004; Kwon et al., 2008; Ge et al., 2009; Manaenko et al., 2010), as well as neuronal plaque formation in animal models (Auluck and Bonini, 2002; Chun et al., 2002; McLean et al., 2004; Flower et al., 2005; Fortun et al., 2007; Herbst and Wanker, 2007). In addition, GA has been shown to augment neurite outgrowth *in vitro* (Gold et al., 1999; Lopez-Maderuelo et al., 2001; Jin and Sano, 2008), though evaluation of its effect on *in vivo* nerve regeneration has never been performed.

The current study was designed to evaluate the ability of GA to accelerate axonal regeneration in comparison to FK-506. Serial imaging of the crushed saphenous nerve in transgenic Thy1-GFP rats, whose axons express green fluorescent protein (GFP), was used to demonstrate that treatment with GA significantly increases the rate at which axons regenerate after a nerve injury. Subsequent functional evaluations demonstrated that, while GA accelerates functional recovery, it does not match the performance of FK-506. It was, however, considerably less toxic and better tolerated by animals than FK-506.

EXPERIMENTAL PROCEDURES

Animals

Thy1-GFP transgenic rats (genOway, Lyon, France) (Moore et al., 2011a) expressing GFP in axons under the control of the neuron-specific Thy1 promoter were used for serial transmuscular *in vivo* imaging of the crushed saphenous nerve. Male Lewis rats (250 mg; Charles River Laboratories, Wilmington, MA, USA) were used in subsequent behavioral evaluation involving tibial nerve crush and transection.

All surgical procedures, experimental manipulations, and peri-operative care measures were approved by Washington University institutional Animal Studies Committee and were performed in compliance with National Institutes of Health guidelines. The animals were housed in a central facility, given food (PicoLab Rodent Diet; Purina Mills Nutrition International, St. Louis, MO, USA) and water *ad libitum*, and monitored post-operatively for weight loss, infection, and signs of distress.

Experimental design

A saphenous nerve crush model in Thy1-GFP transgenic rats was used to quantify the rate of axonal regeneration in response to GA. Thirty-six Thy1-GFP rats underwent two crush insults to the saphenous nerve. The first crush was created to eliminate GFP fluorescence via WD distal to the crush. The second crush was administered 7 days after the first crush at the same location, and marked the starting point for subsequent measurement regeneration lengths (Yan et al., 2011). Thy1-GFP rats were randomized into three subgroups ($n = 12$) receiving daily administration of FK-506 (2 mg/kg/day), GA (0.2 mg/kg/day), or the vehicle control (dimethyl sulfoxide (DMSO), volume matched) starting 3 days prior to the second crush injury. To minimize the total number of animals needed for the current study, a pilot group of animals consisting of one animal per treatment group (15 total) was evaluated at days 0, 3, 5, 7, and 10 days following the second crush. GA- and FK-506-treated animals demonstrated longer regeneration lengths when compared to controls at each evaluated time point with the 10-day post crush time point being the largest in magnitude. Seven additional animals per treatment group (21 total) underwent consecutive saphenous nerve crushes and were assessed at 10 days following the second crush. At the appropriate endpoint, the animals were sacrificed and the regenerating axons within the saphenous nerve were imaged transmuscularly and the length of regeneration was quantified as described in a previous paper (Yan et al., 2011).

The effect of GA on functional recovery following nerve injury was further evaluated using crush and transection injury models with subsequent behavioral analysis of the print length factor (PLF) (Hare et al., 1992; Yan et al., 2012). Twenty-four Lewis rats underwent tibial nerve crush and thirty Lewis rats underwent tibial nerve transection and immediate primary repair, both at 5 mm distal to the sciatic trifurcation. Animals were randomized into three subgroups ($n = 8$ per subgroup for tibial nerve crush and $n = 10$ per subgroup for tibial nerve transection) and received daily administration with FK-506, GA, or a vehicle (DMSO, 1 mL/kg; negative control) starting 3 days prior to nerve injury. All animal footprints were recorded on a walking track and analyzed to calculate the PLF at days 0, 7, and 14–21 (Hare et al., 1992; Yan et al., 2012). At the experimental endpoint (day 21), evoked force production in the gastrocnemius muscle was recorded and muscle mass was measured.

Agent preparation and administration

Fresh FK-506 (20 mg/mL) and GA (2 mg/mL) stock solutions were prepared every 3 days by dissolving FK-506 (LC Laboratories, Woburn, MA, USA) in 100% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA) and GA (LC Laboratories, Woburn, MA, USA) in DMSO (Sigma–Aldrich, St. Louis, MO, USA), respectively. The stock solutions were stored at −20 °C and protected from light. Each day the stock solutions were diluted 10-fold prior to administration to final concentrations of 2 mg/mL for FK-506 and 0.2 mg/mL for GA. Dosage selection for FK-506 (2 mg/kg/day) was based on a previous work demonstrating immunosuppression and nerve regeneration enhancement (Yang et al., 2003). Dosage selection for GA (0.2 mg/kg/day) was based on a preliminary dosing experiment (data not shown). The animals were weighed daily for accurate dosage. All injections were done via the subcutaneous route. The regimen for each agent included a 3-day preload (Snyder et al., 2006) and daily administration.