Experimental Neurology 280 (2016) 106–114

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

RAD001 (everolimus) attenuates experimental autoimmune neuritis by inhibiting the mTOR pathway, elevating Akt activity and polarizing M2 macrophages

Ranran Han [1], Juan Gao [1], Hui Zhai, Jinting Xiao, Ya'nan Ding, Junwei Hao *

Department of Neurology, Tianjin Neurological Institute, Tianjin Medical University General Hospital, Anshan Road, Heping District, Tianjin, 300052, China

ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 28 March 2016
Accepted 5 April 2016
Available online 8 April 2016

Keywords:
Experimental autoimmune neuritis
Guillain–Barre' syndrome
Macrophages
mTOR
RAD001

ABSTRACT

Guillain–Barre' syndrome (GBS) is an acute, postinfectious, immune-mediated, demyelinating disease of peripheral nerves and nerve roots. As a classical animal model of GBS, experimental autoimmune neuritis (EAN) has become well-accepted. Additionally, the potent immune modulation exerted by mammalian target of rapamycin (mTOR) inhibitors has been used to treat cancers and showed beneficial effects. Here we demonstrate that the mTOR inhibitor RAD001 (everolimus) protected rats from the symptoms of EAN, as shown by decreased paralysis, diminished inflammatory cell infiltration, reductions in demyelination of peripheral nerves and improved nerve conduction. Furthermore, RAD001 shifted macrophage polarization toward the protective M2 phenotype and modified the inflammatory milieu by downregulating the production of pro-inflammatory cytokines including IFN-γ and IL-17as well as upregulating the release of anti-inflammatory cytokines such as IL-4 and TGF-β. Amounts of the mTOR downstream targets p-P70S6K and p-4E-BP1 in sciatic nerves decreased, whereas the level of its upstream protein p-Akt was elevated. This demonstrated that RAD001 inhibited the mTOR pathway and encouraged the expression of p-Akt, which led to M2 macrophage polarization, thus improved the outcome of EAN in rats. Consequently, RAD001 exhibits strong potential as a therapeutic strategy for ameliorating peripheral poly-neuropathy.

1. Introduction

Guillain–Barre' syndrome (GBS) is an autoimmune disease and an acute inflammatory disorder that afflicts the peripheral nervous system (PNS). GBS is presently the most frequent cause of acute flaccid paralysis worldwide, thereby becoming a leading source of serious neurologic emergencies (Yuki and Hartung, 2012). Proven effective immunotherapies for GBS are plasma exchange and intravenous immunoglobulin G. Nevertheless, despite receiving immunotherapy, approximately 5% of these patients die, and up to 20% suffer from severe disabilities (Hughes et al., 2007). Clearly this affliction is in urgent need of resolution.

Experimental autoimmune neuritis (EAN) has been developed as an animal model of GBS, because it mimics its clinical, histopathological and electrophysiological features. EAN is pathologically characterized by breakdown of the blood-nerve barrier, robust accumulation of reactive T cells and macrophages into the PNS and demyelination of peripheral nerves (Hughes and Cornblath, 2005). During the acute phase, pro-inflammatory cytokines including IL-6, IFN-γ, IL-17 and TNF-α predominate in sciatic nerves and lymphoid organs and mediate inflammatory damage to the peripheral nerves, whereas during the recovery period, anti-inflammatory cytokines such as IL-4 and IL-10 play an essential role in ending the disease course (Zhang et al., 2013; Zhu et al., 1998).

In addition, phenotypically polarized macrophages generally appear either in a pro-inflammatory M1 mode, *i.e.*, classically activated, or in an anti-inflammatory M2, alternatively activated form (Biswas and Mantovani, 2010; Sica and Mantovani, 2012). Such changes of macrophage polarization in the local environment can have a decisive role in the pathogenesis of autoimmune and inflammatory diseases (Jiang et al., 2016; Wynn et al., 2013). IFN-γ has given rise to classically activated macrophages, which secrete IL-1, IL-6 and IL-23. These M1 phenotype macrophages are involved in the induction stage of EAN and are the main contributors to its pathogenic effects and destructive course. As antibody-dependent cellular cytotoxicity and phagocytosis evolve, damage to the myelin sheath may ensue due to the production of reactive oxygen intermediates (Hartung et al., 1988). Meanwhile, alternatively activated M2 macrophages arise mainly in response to IL-4

Abbreviations: GBS, Guillain-Barre' syndrome; EAN, experimental autoimmune neuritis; mTOR, mammalian target of rapamycin; PNS, peripheral nervous system; PBS, phosphate-buffered saline; dpi, days post-immunization; EMG, electromyography; CMAP, compound muscle action potential; MNCV, motor nerve conduction velocity; MNC, mononuclear cell; LFB, luxol fast blue; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

* Corresponding author.
E-mail address: hjw@tmu.edu.cn (J. Hao).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.expneurol.2016.04.005

(Mosser and Edwards, 2008). These M2 macrophages contribute to recovery by promoting T-cell apoptosis, secreting anti-inflammatory cytokines like IL-10 and TGF-β (Mantovani et al., 2013) and generating myelin repair and axonal regeneration (Kiefer et al., 2001). A switch of macrophage phenotype from classical activation (M1) to alternative activation (M2) could change the functions of macrophages from inflammatory to anti-inflammatory accompanied by tissue repair (Mosser and Edwards, 2008). Therefore, therapies that facilitate the polarization of macrophages toward the beneficial M2 phenotype may offer protective effects against EAN.

RAD001 (everolimus), an analog of rapamycin, belongs to a group of inhibitors for mammalian target of rapamycin (mTOR), which plays a crucial role in cell proliferation, growth and survival, and mediates its effects mainly by inhibiting mTORC (mTOR complex) 1, with limited or no effect on mTORC2 activity (Saran et al., 2015). Currently, RAD001 is the only mTOR inhibitor approved by the U.S. FDA (Food and Drug Administration) for the treatment of papillary renal carcinoma, pancreatic neuroendocrine tumor, some types of breast cancer and subependymal giant-cell astrocytoma associated with tuberous sclerosis (Agarwala and Case, 2010; Baselga et al., 2012; Curran, 2012; Dorris and Jones, 2014; Yim, 2012). RAD001 has proven effective in reducing those tumors mainly by attenuating the growth of cancer cells under *in vitro* and *in vivo* conditions (Saran et al., 2015). To date, RAD001 has also exerted a protective effect *via* the beneficial regulation of such autoimmune diseases as experimental autoimmune uveoretinitis (Hennig et al., 2012) and autoimmune hepatitis (Ytting and Larsen, 2015). Furthermore, as recently shown, activating the mTOR pathway can downregulate the level of p-Akt, and thus reduce M2 macrophage polarization (Byles et al., 2013). These results prompted our detailed examination of the mTOR pathway and macrophages polarization in treating EAN.

In this study of EAN, we applied RAD001 in preventative and therapeutic patterns and evaluated clinical scores, pathological changes, the distribution of M1/M2 macrophages and inflammatory milieu. As a result, we found that inhibition of the mTOR pathway and activation of Akt attenuated the destructive symptoms of EAN.

2. Materials and methods

2.1. Experimental animals and group assignments

Conditions used here for experiments with rats were approved by the Animal Ethics Committee of the Tianjin Medical University. Lewis rats (male, 160–190 g, 6–8 weeks old) were purchased from the Vital River Corporation (Beijing, China). All rats had access to food and water *ad libitum* and were acclimated to the vivarium environment, which was maintained under temperature-control (23 °C ± 2 °C, 12-h light/dark periods) for one week. Animals were then randomly assigned to one of three groups (preventative, therapeutic or control, $n = 5$ per group). Animal suffering and numbers killed were minimized to the greatest extent possible.

2.2. Induction of EAN and evaluation of clinical signs

To induce EAN, 300 μl of an inoculant containing 300 μg of dissolved P0 peptide 180–199 (10 mg/ml; Bio-Synthesis Corporation) was injected into both hind footpads of each rat. The peptide was dissolved in phosphate-buffered saline (PBS) (2 mg/ml) and then emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco) containing *Mycobacterium tuberculosis* (strain H37RA) to a final concentration of 1 mg/ml. Following immunization, clinical signs of EAN were scored daily in a blinded protocol by two different examiners as follows: 0 = normal; 1 = reduced tonus of the tail; 2 = limp tail; 3 = absent righting reflex; 4 = gait ataxia; 5 = mild paresis of the hind limbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia of the hind limbs; 8 = tetraparesis; 9 = moribund; and 10 = death.

2.3. RAD001 treatment

RAD001 (everolimus) (5 mg/tablet, Novartis) was dissolved in 2 ml ethanol and further diluted in ddWater (endotoxin-free) to a final concentration of 0.2 mg/ml. For preventative treatment, RAD001 solution was administered by oral gavage (1 mg/kg body weight/day) for 16 consecutive days post-immunization (dpi). For therapeutic treatment, the same dose of RAD001 solution was administered by oral gavage daily from the day when the first clinical signs were observed, namely from dpi 7 to 16. Control animals received the same volume of the vehicle solution (*i.e.*, 0.08% ethanol/ddWater). The dosage of RAD001 was based on our previous investigation using different doses (3, 1 and 0.3 mg/kg, daily). The animals treated with 3 mg/kg dose showed higher mortality rate and severe adverse effects: diarrhea, weight loss those were not observed with 1 mg/kg and 0.3 mg/kg daily. And it seemed that 1 mg/kg can exert more significant effect on ameliorating EAN symptoms.

2.4. Electrophysiological studies

Electromyographic (EMG) recordings of the left sciatic nerve in EAN rats were executed on dpi 16 with a fully digital Keypoint Compact EMG/NCS/EP recording system (Dantec Co.). A single blind trial method was applied to record evoked compound muscle action potential (CMAP) amplitudes and latencies of sciatic nerves, as previously described (Sarkey et al., 2007). Rats were anesthetized first with chloral hydrate (intraperitoneally, 3 mg/kg). Two pairs of monopolar needle electrodes were used to stimulate the sciatic nerve and record the signals, respectively. After exposing the left sciatic nerve from the hip (proximal) to the ankle (distal), one pair of needle electrodes was inserted at the sciatic notch (hip/proximal) or the ankle (ankle/distal). The nerve stimulation parameters used to elicit CMAPs were: 1 Hz pulses, with each pulse being 5 mA in amplitude and 0.3 ms in duration. The recording electrodes were positioned in the "belly" part of the gastrocnemius muscle to record evoked potentials from stimulating the sciatic nerve. The motor nerve conduction velocity (MNCV) was calculated by measuring the distance between stimulating cathode electrodes and then measuring the latency difference. The amplitude was calculated from the baseline to the maximal peak under the resulting CMAP curves. After electrophysiologic measurements were completed, the incision was sutured under an aseptic environment. Body temperatures of rats during electrophysiologic measurements were maintained above 34 °C by positioning a heating pad under the rat. For each animal, triplicate measurements were made.

2.5. Histopathological assessment

Following nerve conduction studies, rats were perfused pericardially with cold PBS followed by 4% paraformaldehyde (Solarbio). Then the sciatic nerves were rapidly removed and post-fixed in 4% paraformaldehyde overnight at 4 °C. After dehydration using graded ethanol and vitrification by dimethylbenzene, the nerves were embedded in paraffin (Aladdin). Cross sections 6 μm thick were cut with a microtome (Leica RM2255), mounted on poly-L-lysine-coated slides and stored at room temperature until stained. Hematoxylin-eosin (H&E) (Solarbio) and luxol fast blue (LFB) stains were applied separately to evaluate the extent of inflammatory cell infiltration and demyelination. Results were visualized with a Nikon Coolscope digital microscope. To quantify the inflammatory cell infiltration, 5 fields of each slide were acquired; pictures of each group were collected from 4 different rats. To evaluate the severity of demyelination, histological scores were calculated from fields acquired as described above according to the following semi-quantitative pathological/histological scale: 0, normal perivascular area; 1, mild cellular infiltrate adjacent to the vessel; 2, cellular infiltration plus demyelination in immediate proximity to the vessel; 3, cellular infiltration and demyelination throughout the section. Cell numbers