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Rapamycin increases neuronal survival, and reduces inflammation and astrocyte proliferation after spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) frequently leads to a permanent functional impairment as a result of the initial injury followed by secondary injury mechanism, which is characterised by increased inflammation, glial scarring and neuronal cell death. Finding drugs that may reduce inflammatory cell invasion and activation to reduce glial scarring and increase neuronal survival is of major importance for improving the outcome after SCI.

In the present study, we examined the effect of rapamycin, an mTORC1 inhibitor and an inducer of autophagy, on recovery from spinal cord injury. Autophagy, a process that facilitates the degradation of cytoplasmic proteins, is also important for maintenance of neuronal homeostasis and plays a major role in neurodegeneration after neurotrauma. We examined rapamycin effects on the inflammatory response, glial scar formation, neuronal survival and regeneration *in vivo* using spinal cord hemisection model in mice, and *in vitro* using primary cortical neurons and human astrocytes. We show that a single injection of rapamycin, inhibited p62/SQSTM1, a marker of autophagy, inhibited mTORC1 downstream effector p70S6K, reduced macrophage/neutrophil infiltration into the lesion site, microglia activation and secretion of TNFα. Rapamycin inhibited astrocyte proliferation and reduced the number of GFAP expressing cells at the lesion site. Finally, it increased neuronal survival and axonogenesis towards the lesion site. Our study shows that rapamycin treatment increased significantly p-Akt levels at the lesion site following SCI. Similarly, rapamycin treatment of neurons and astrocytes induced p-Akt elevation under stress conditions. Together, these findings indicate that rapamycin is a promising candidate for treatment of acute SCI conditions and may be a useful therapeutic agent.

1. Introduction

The primary mechanical damage to the spinal cord initiates a secondary damage that includes inflammatory cell infiltration (such as neuotrophils and macrophages) and microglia activation. These cells together with reactive astrocytes that respond to the injury at the lesion site, release a large number of pro-inflammatory cytokines and neurotoxins leading to further neuronal cell death and tissue degeneration (Beattie, 2004). These secondary effects mediate further glial scarring, which mainly consists of reactive astrocytes and proteoglycans and create a physical and chemical barrier for any axonal regeneration (Silver and Miller, 2004).

Autophagy is the process of self-digestion of cellular components (Klionsky and Emr, 2000; Wang and Klionsky, 2003). It is important for normal growth control and may be defective in diseases (Larsen and Sulzer, 2002; Ogier-Denis and Codogno, 2003). Autophagy plays an important role in maintaining the balance between the synthesis and the degradation of proteins. It also serves as a cell-survival mechanism under stress conditions such as absence of nutrients. Thus, autophagy is important for normal cell growth, differentiation, and survival (Reggiori and Klionsky, 2002). Recent studies demonstrated that in some neurodegenerative diseases autophagy may rescue neuronal cells (Angliker and Ruegg, 2013; Berger et al., 2006; Ravikumar et al., 2004) Therefore, understanding the role of autophagy in neurodegenerative processes will help in developing new therapeutic strategies. Several studies have demonstrated that autophagy is activated in many neurotrauma models, including cerebral ischemia (Carloni et al., 2008; Puyal et al., 2009; Rami et al., 2008), traumatic head injury (Diskin et al., 2005; Erlich et al., 2006, 2007; Smith et al., 2011), and spinal cord injury (Codeluppi et al., 2009; Kanno et al., 2011; Tang et al., 2014).

Rapamycin is a macrolide antibiotic specifically inhibits mTORC1 and its downstream effector p70S6K, which regulates transcription, translation, cell cycle and autophagy (Kamada et al., 2003; Vignot

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et al., 2005). Rapamycin mediates anti-inflammatory responses, which can prevent neuronal cell death. Several studies demonstrate beneficial effect of rapamycin treatment at the cellular and functional levels, including decreased inflammation and improvement of locomotor recovery (Erlich et al., 2007; Song et al., 2014; Tang et al., 2014; Xie et al., 2014). However, several other studies report no such beneficial effect of rapamycin treatment on recovery following neurotrauma (Hu et al., 2010; Tachibana et al., 2005). Thus, the neuroprotective effect of rapamycin after spinal cord injury has to be further studied.

In the present study we examined the effect of rapamycin treatment on the inflammatory response, glial scar formation and neuronal survival following spinal cord hemisection in mice. We also explored the direct effect of rapamycin on astrocyte and neuronal cells under stress conditions *in vitro*.

2. Materials and methods

2.1. Mice

Adult (2–4 months) male C57BL/6 mice were used. All procedures were approved by Monash University Animal Ethics Committee in accordance with the requirements of the National Health and Medical Research Council of Australia (MARP/2011/075), and the Animal Care Committee of Tel-Aviv University (L-13-071).

2.2. Spinal cord hemisections

As described (Goldshmit et al., 2004), mice (20–30 g) were anaesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg) in phosphate buffered saline (PBS) injected intraperitoneally. The spinal cord was exposed at the low thoracic to high lumbar area. After laminectomy a complete left hemisection was made at T12 and the overlying muscle and skin were sutured. Mice were randomly assigned to the control-DMSO or rapamycin injection groups and allowed to survive for 2 days to 2 months post injury.

2.3. Rapamycin application

Rapamycin was dissolved in DMSO (25 mg/ml). For animal injection, the stock solution was diluted immediately before IP injections with 100 μl water containing 5% polyethylene glycol 400 and 5% Tween 80. Mice received a single injection 2 h post-injury of 1 mg/kg rapamycin and control animals received the drug vehicle.

2.4. Immunohistochemistry

Cryostat sections (20 μm) were stained using standard immunohistochemistry. Primary antibodies: rabbit anti-GFAP (1:1000, Dako); mouse anti-HuC/D (1:250; Invitrogen), rabbit anti-active caspase 3 (1:1000; Cell Signaling), mouse anti-NeuN (1:1000; Millipore), rat anti-CD11b (1:200; BioLegend); rabbit anti-Ki67 (1:400; Thermo); secondary antibodies: Alexa Fluor 488 and 568; 1:1000 (Invitrogen). Nuclei were visualised with DAPI (Sigma). Antigen retrieval was performed by incubation in 1 M Tris–HCl (pH:8.0) at 90 °C for 20 min (HuC/D).

2.5. Flow cytometry analysis of spinal cord tissue

After isolation of damaged spinal cords (1 mm from each side of the injury; at least n = 4 animals from each group), single cell suspensions were prepared using the 'rapid protocol' as described previously (Pinto et al., 2013). Flow cytometry analysis was conducted as previously described (Pinto et al., 2013), by immunostaining of the prepared single cell suspensions with rat anti-mouse CD45 (clone 30-F11; eBioscience), CD11b (clone M1/70; BioLegend) and CD14 (clone Sa2-8; eBioscience) antibodies. Flow cytometry data was analysed using FlowJo 7.6.4 software.

2.6. q-PCR of spinal cord tissue

Spinal cord tissue was dissected (at least n = 4 per group) 3 mm on either side of the lesion and homogenised by a polytron homogeniser (Kinematica, USA) in 1 ml TRI reagent according to manufacturer's instructions (TRI reagent, Sigma), extracted RNA from spinal cords were reverse transcribed (Superscript III RT kit, Invitrogen) and analysed by quantitative PCR (qPCR) performed using TaqMan Universal master mix (Applied Biosystems, Foster City, CA) and the 7900HT Fast Real-Time PCR system (Applied Biosystems). TaqMan gene expression assays were used for detecting *gfap* Mm01253033_m1 and *tnf-α* (Mm00443260_g1) (Applied Biosystems). Using the comparative CT method (ΔΔCT), mRNA levels were normalised against levels of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) mRNA (Mm99999915_g1) with the control (wildtype intact spinal cord) used as reference.

2.7. Cell counting

CD11b, GFAP density and Ki67 or HuC positive cells were quantified in a 200 μm^2 box at the lesion, in every third serial longitudinal 20 μm section. The density of staining or cell number was measured in 20× images. Measurements were analysed using Image J (Wayne Rasband, National Institutes of Health) and averaged from at least five boxes/section; five sections/spinal cord.

2.8. Lysate preparation and immunoblot

For protein analysis, spinal cords (3 mm from each side of the lesion) were homogenised by a polytron homogeniser (Kinematica, USA) in lysis buffer 7 days after SCI. For immunoblot analysis, equal amounts of protein from each sample were loaded and resolved by SDS-polyacrylamide gel electrophoresis through 7.5%–10% gels. The gels were electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked, blotted with the corresponding primary antibodies followed by secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected by chemiluminescence reaction. The protein levels were quantified by a densitometric analysis of protein bands using the ImageJ software. P70S6K, p62 and beclin were compared to β-actin levels of stripped membrane, and p-Akt was compared to total Akt of stripped membrane. Primary antibodies were rabbit anti-p70S6K (1:1000; Sigma), rabbit anti-Beclin 1 (1:5000; Santa Cruz), rabbit anti-p62 (1:2000; MBL), mouse anti-actin (1:10,000; MP Biomedical).

2.9. Anterograde axonal tracing

Axonal regeneration was examined using anterograde tracing (vehicle control n = 6, rapamycin n = 6). Seven weeks after SCI, TMRD ("Fluoro-Ruby", MW 10,000 kD; Molecular Probes, USA) was injected into the spinal cord at the level of the cervical enlargement, ipsilateral to the lesion as described (Goldshmit et al., 2004). After 7 days, mice were perfused with PBS followed by 4% paraformaldehyde (PFA). Spinal cords were removed and post-fixed for 1 h in cold 4% PFA followed by 20% sucrose in PBS overnight at 4 °C. Longitudinal (horizontal) serial cryostat sections were cut (50 μm) and slides were imaged using fluorescence and confocal microscopy. Labelled axons in the white matter were quantified 0–200 μm and 1 mm proximal to the lesion site at 400×. Photomontage of the regenerating axons was taken on a laser scanning confocal microscope, Zeiss LSM510 (Carl Zeiss).

2.10. Behavioural analyses

Two examiners tested mice weekly 24 h-5 weeks after SCI. The testing was done blindly. Horizontal grid walking (Goldshmit et al., 2004, 2011): After 2 min of free walking, mis-steps (normalised to total number of steps taken by the left hind limb) were quantified. Open field

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