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The human natural killer-1 (HNK-1) glycan mimetic ursolic acid promotes functional recovery after spinal cord injury in mouse☆

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Abstract

Human natural killer-1 (HNK-1) cell antigen is a glycan epitope involved in several neural events, such as neuritogenesis, myelination, synaptic plasticity and regeneration of the nervous system after injury. We have recently identified the small organic compound ursolic acid (UA) as a HNK-1 mimetic with the aim to test its therapeutic potential in the central nervous system. UA, a plant-derived pentacyclic triterpenoid, is well known for its multiple biological functions, including neuroprotective, antioxidant and anti-inflammatory activities. In the present study, we evaluated its functions in a mouse model of spinal cord injury (SCI) and explored the molecular mechanisms underlying its positive effects. Oral administration of UA to mice 1 h after SCI and thereafter once daily for 6 weeks enhanced the regaining of motor functions and axonal regrowth, and decreased astrogliosis. UA administration decreased levels of proinflammatory markers, including interleukin-6 and tumor necrosis factor-α, in the injured spinal cord at the acute phase of inflammation and activated the mitogen-activated protein kinase and phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin pathways in the injured spinal cord. Taken together, these results suggest that UA may be a candidate for treatment of nervous system injuries.

Keywords: HNK-1; Ursolic acid; Spinal cord injury; Mouse; Axonal regrowth

1. Introduction

Human natural killer-1 (HNK-1) cell antigen is a glycan epitope that is predominantly expressed in central and peripheral nervous system tissues. HNK-1 is involved in several neural functions, such as cell interactions during development, synaptic plasticity and regeneration of nerve connections after damage in the adult [1,2]. The HNK-1 carbohydrate is carried by many recognition molecules, including NCAM, L1, P0, TAG-1/axonin-1, F3/F11/contactin, several types of proteoglycans and molecules of the tenascin family [1–3]. In particular, the interaction of HNK-1 with one of its binding proteins, the high mobility group box 1 (HMGB1), also called HMG1, amphoterin or sulfoglucuronyl carbohydrate binding protein 1, has been suggested to regulate cell–cell recognition, neurite outgrowth and neuronal migration [4–6]. HNK-1 is associated with myelin profiles of motor axons but not of sensory axons in the mouse femoral nerve [7–9]. Interactions of the HNK-1 epitope with chondroitin sulfate proteoglycans enhance neuronal cell adhesion and neurite outgrowth [10], and an HNK-1 glycomimetic peptide promotes functional recovery after femoral nerve injury in adult mice and monkeys [11–13]. In our previous study, although an HNK-1 mimetic peptide positively influenced remyelination and reestablishment of glutamatergic synapses, it failed to improve functional recovery after spinal cord injury (SCI) in mice [14]. The inability to achieve functional recovery was related to its inability to reinnervate the cholinergic and monoaminergic axons caudal to the lesion site. However, a more detailed study of the causes of this failure was not carried out. Recently, we observed that HNK-1 contributes to functional recovery after SCI in adult zebrafish [15]. Since under physiological conditions a potential instability of the HNK-1 mimicking peptide could pose difficulties for therapies in humans, we have screened a library of small organic molecules for compounds structurally and functionally mimicking HNK-1 and identified ursolic acid (UA) as a mimetic. We found that UA promoted functional recovery, axonal regrowth/sprouting and remyelination in a mouse model of severe spinal cord compression injury.

UA (3-beta-hydroxyurs-12-en-28-oic acid), a natural pentacyclic triterpenoid acid, is one of the major components of several medicinal plants. UA exhibits a broad range of biological functions, such as neuroprotective [16–22], antioxidant [23] and anti-inflammatory [24] activities. Behavioral tests suggested that UA not only displays antidepressant- and anxiolytic-like effects but also promotes

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neurogenesis and improves cognitive impairments in different animal models of nervous system disorders [17,25–27]. Neuroprotective effects of UA are thought to involve inhibition of oxidative stress and inflammation in different neurotoxic models and activation of the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway in cerebral ischemia [17–19]. In the MPTP-intoxicated mouse, UA improves behavioral deficits, restores altered dopamine levels and protects dopaminergic neuronal viability [27], calling for characterizing the molecular targets and mechanisms underlying UA functions which remain to be understood. Also, the effects of UA in SCI have not been investigated.

In the present study, we demonstrate the potential of UA for the treatment of SCI and provide evidence that the HNK-1-mimetic UA contributes to functional recovery after SCI in mouse model. UA treatment promoted remyelination and axonal regrowth in the injured spinal cord of mice. UA treatment also up-regulated the HNK-1 glycan expression and enhanced the intracellular signaling cascades known to favor spinal cord regeneration after injury. We here also demonstrated that UA treatment after spinal cord compression injury in mice reduces inflammation and glial scar formation. Since UA was originally purified from edible plants of the Oleaceae family, we believe that it could have a favorable safety profile, allowing for clinical application.

2. Materials and methods

2.1. Screening for the HNK-1 mimetic compound

The HNK-1 carbohydrate was synthesized as described [28]. The screening for small organic molecules was carried out by competitive enzyme-linked immunosorbent assay (ELISA) using the HNK-1 antibody from the Developmental Studies Hybridoma Bank. The carbohydrate was substrate-coated [28], and the antibody was used at a concentration of 1:500 dilution in a competitive ELISA essentially as described for the polysialic acid carbohydrate [29] by screening a library of small organic compounds from the NIH National Library of Medicine. UA was the most potent inhibitor of HNK-1 antibody binding as assayed by titration of the concentrations of the small organic compounds.

2.2. Animals

Female C57BL/6J mice (4 to 5 months old and with an average weight of 22 g) were purchased from the Guangdong Medical Laboratory Animal Center (Guangdong, China), maintained at 27°C under a reverse 12-h dark/light cycle and provided food and water *ad libitum*.

Experiments were approved by the Committee on Animal Experimentation of Shantou University Medical College, and all attention was paid to minimize pain and the number of animals used. All experiments were carried out by an experimenter that was blinded to the treatment of animals.

2.3. SCI in C57BL/6J mice

SCI was performed as described [14] using 4- to 5-month-old female C57BL/6J mice. For surgery, mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg, Ketanest, Parke-Davis/Pfizer, Karlsruhe, Germany) and xylazine (5 mg/kg, Rompun, Bayer, Leverkusen, Germany). Laminectomy was performed [30] at the T7–T9 level with mouse laminectomy forceps (Fine Science Tools, Heidelberg, Germany) following compression injury elicited by a mouse spinal cord compression device (Fine Science Tools). Muscles and skin were then closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany). After the surgery, mice were kept on a heated pad (37°C) for 8 h to prevent hypothermia and thereafter singly housed in a temperature-controlled (26°C) room with water and soft food. During the postoperative period, the bladders of the animals were manually voided twice daily.

2.4. Drug treatment

UA was dissolved in 1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). Based on a literature review of potency of UA concentrations [19,31,32], 100 mg/kg and 200 mg/kg of UA were chosen as doses for this study. UA was administered orally 1 h after injury followed by one daily administration for 6 weeks. DMSO (1%) in PBS was administered orally for vehicle control. For the sham-operated controls, animals underwent a T7–T9 laminectomy without compression injury and no treatment with UA.

2.5. Locomotion of mice

The recovery of ground locomotion was evaluated using the Basso Mouse Scale (BMS) [33]. In addition, we used a numerical assessment of locomotion by using a single-frame motion analysis to determine the foot-stepping angle (FSA) and rump-height index (RHI) in the beam walking test [34,35]. The limb extension–flexion ratio (EFR) was evaluated from video recordings of voluntary movements with the "pencil" test [34]. Assessment was performed before and at 1, 2, 3, 4, 5 and 6 weeks after injury. Values for the left and right extremities were averaged.

2.6. Western blot analysis

To determine HNK-1 glycan expression and intracellular signal transduction products in mice, an approximately 1-cm-long segment of spinal cord proximal to the lesion site and an approximately 1.5-cm-long segment of spinal cord distal to the lesion site, including approximately 1.2 cm of both the sensory and motor branches, were taken at 6 weeks after SCI. Tissue homogenization and Western blot analysis were performed as described [36]. The following primary antibodies were used: HNK-1 monoclonal antibody (1:500, Thermo Fisher); mouse monoclonal extracellular signal-regulated kinases 1 and 2 (Erk1/2) antibody (1:1000, Santa Cruz); mouse monoclonal phosphorylated Erk (p-Erk) antibody (1:1000, Santa Cruz); mouse monoclonal protein kinase B (Akt1) antibody (1:1000, Santa Cruz); mouse monoclonal phosphorylated Akt (p-Akt1) antibody (1:500, Santa Cruz); mouse monoclonal B cell lymphoma 2 (Bcl-2) antibody (1:500, Santa Cruz); rabbit polyclonal Bcl-2 associated X protein (Bax) antibody (1:1000, Santa Cruz); rabbit mechanistic target of rapamycin (mTOR) antibody (1:1000, Santa Cruz); rabbit phosphorylated mTOR (p-mTOR) antibody (1:1000, Santa Cruz); rabbit polyclonal myelin basic protein (MBP) antibody (1:500, Boster Biological Technology, Wuhan, Hubei, China) and mouse monoclonal β-actin antibody (1:1000, Santa Cruz). Goat anti-rabbit IgG and goat anti-mouse IgG (1:1000, Jackson ImmunoResearch, West Grove, PA, USA) conjugated to horseradish peroxidase were used as secondary antibodies.

2.7. Immunohistology

Serial coronal sections (25 μm thick) were collected proximal and distal to the lesion site from tissues collected at 6 weeks after SCI and processed for immunofluorescence as described [37]. Briefly, the sections were incubated overnight at 4°C with the following primary antibodies: rabbit glial fibrillary acidic protein (GFAP; 1:400, Boster) and rabbit MBP (1:500, Boster), rabbit ionized calcium-binding adaptor molecule (Iba-1; 1:500, Boster), rabbit 5-HT (1:500, Boster) and mouse neurofilament 200 (NF200; 1:400, Boster). The appropriate secondary antibodies were as follows: donkey anti-mouse antibody conjugated to Dylight 488 (1:1000, Jackson ImmunoResearch) and donkey anti-rabbit antibody conjugated to Dylight 568 (1:400, Invitrogen, Eugene, OR, USA) were incubated at room temperature for 2 h. Immunoreactivities were evaluated in coronal sections 4 mm proximal and 4 mm distal (in 20 corresponding areas, 200 μm apart in consecutive sections) to the lesion site with a 40× objective. Groups consisted of 3 animals (n=60 sections per group). Relative immunofluorescence intensities in spinal cords were measured using Image-J Pro Plus 6.0 software (Wayne Rasband, NIH).

2.8. ELISA measurement of cytokines

For the measurement of proinflammatory cytokine levels in the spinal cord, an approximately 2-cm-long segment of the spinal cord, including lesion site in the center, was taken on days 1, 7 and 14 after SCI. Estimations of proinflammatory cytokines were performed with commercially available ELISA kits: mouse interleukin (IL)-1β (GenStar), mouse IL-6 (GenStar) and mouse tumor necrosis factor (TNF)-α (GenStar). All assays were carried out in triplicate using the recommended buffers, diluents and substrates. Absorbance was determined using a microplate reader at 450 nm (Tecan Infinite M200 Pro, Tecan, Switzerland). The intraassay coefficients of variation for these experiments were less than 10%.

3. Results

3.1. Application of UA improves locomotor recovery after SCI in C57BL/6J mice

We evaluated motor recovery of UA-treated C57BL/6J mice after severe spinal cord compression injury by weekly assessing BMS scores up to 6 weeks after SCI (Fig. 1A). Better recovery was observed at 5 and 6 weeks after SCI in mice treated with 200 mg/kg UA compared with control mice that had received only vehicle solution. Similarly, the BMS recovery index showed a tendency for better recovery with 200-mg/kg UA treatment (Fig. 2A). FSA, an indicator for better recovery, was changed from approximately 30° before injury to 170° after injury in all groups. A decrease of FSA was found only at 6 weeks with 200-mg/kg UA-treated mice compared to vehicle-treated control mice (Fig. 1B).