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Hyaluronan oligosaccharides promote functional recovery after spinal cord injury in rats

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ABSTRACT

Hyaluronan is a component of the extracellular matrix of the central nervous system, and forms perineuronal nets around neurons. It has been recently reported that the hyaluronan-degrading enzyme hyaluronidase promotes lateral mobility of AMPA-type glutamate receptors and enhances synaptic plasticity. However, the biological significance of hyaluronan-degrading products (oligosaccharides) has not been studied in depth. Here we investigated the effects of hyaluronan oligosaccharides on motor function recovery after spinal cord injury in rats. The disaccharide HA2 and especially the tetrasaccharide HA4, significantly improved motor function, unlike the case with oligosaccharides composed of 6–12 saccharides. Consistent with this finding, HA4 treatment enhanced axonal regeneration/sprouting, as assessed by corticospinal tract tracer fiber counts. HA4 treatment also significantly suppressed accumulation of Iba-1-positive cells in a lesion two weeks after injury. *In vitro* experiments demonstrated that NMDA-induced neuronal cell death was partly blocked by HA4, but not by other oligosaccharides, whereas proteoglycan-mediated inhibition of neurite outgrowth was not affected by treatment with any oligosaccharide examined. Taken together, the present results revealed that due in part to its neuroprotective activity, HA4 promotes motor function recovery after spinal cord injury.

The extracellular matrix (ECM) surrounding neurons in the central nervous system (CNS) forms a lace-like structure, the so-called perineuronal net (PNN) [20]. PNN is composed of hyaluronan (hyaluronic acid; HA), proteoglycans and tenacin R [17]. The globular domains located in the N-terminal of unique proteoglycans (referred to as "lecticans") bind to HA, and the C-terminal domains of proteoglycans are linked to tenacin R, so that a complex, lace-like structure is formed. Although PNN surrounding GABAergic interneurons is prominent, it can be detected on virtually all neurons [2,3,9]. A recent report suggested that the HA-degrading enzyme hyaluronidase and the chondroitin sulfate-degrading enzyme chondroitinase-ABC enhance the lateral mobility of the AMPA receptor, and consequently promote short-term synaptic plasticity [4]. Considering that chondroitin sulfate proteoglycans are the major type of proteoglycan in the PNN, both hyaluronidase and chondroitinase ABC may degrade various PNN components, and consequently destroy the PNN structure. PNN may thus inhibit structural rearrangements at synapses, and consequently contribute to the maintenance of neuronal networks. This idea is further supported by recent studies showing that chondroitinase-ABC restores the plasticity of not only sensory networks but also of networks associated with emotion [5,14]. However, the hyaluronidase used is a testicular enzyme, and chondroitinase ABC is a bacterial enzyme. Thus, these enzymes are absent in mammalian CNS. Therefore, the proof and characterization of neuronal enzymes, which degrade glycosaminoglycans, are still missing.

In contrast to extensive investigations of the effects of PNN-degrading enzymes on the structural rearrangement of neuronal networks, little attention has been paid to degradation products. In the present study, we investigated biological effects of HA oligosaccharides, preparing HA oligosaccharides composed of 2–12 saccharides (HA2–HA12), and applied them to spinal cord injury

Abbreviations: AMPA, DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; HA, hyaluronan acid; NMDA, N-methyl-D-aspartic acid; Iba-1, ionized calcium binding adaptor protein; SCI, spinal cord injury; BBB scores, Basso, Beattie and Bresnahan scores; CMF-PBS, calcium–magnesium free PBS; PG, proteoglycans; BSA, bovine serum albumin; CST, corticospinal tract; BDA, biotin-dextran amine.

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sites in a rat model. We observed a prominent functional recovery in animals treated with HA4, and detected HA4 neuroprotective activity.

HA oligosaccharides were prepared according to the method reported previously [21] with minimal modification. Briefly, HA (supplied from Sikagaku Corporation. Tokyo) was depolymerized by partial digestion with testicular hyaluronidase and separated into size-uniform HA oligosaccharides by anion exchange chromatography after removal of hyaluronidase. After desalting by size-exclusion chromatography, the purity and size of each HA oligosaccharide were confirmed by high-performance liquid chromatography (HPLC). Endotoxins, proteins, and DNA were absent in these oligosaccharide preparations. High-molecular-weight hyaluronan (HMW-HA; 600–1200 kd) was purchased from the Seikagaku Biobusiness Corporation (Tokyo, Japan).

Adult female Sprague-Dawley rats weighing 200–230 g were used. The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After Th9 laminectomy, we exposed the dura mater and induced an injury force, 200 kdyn, with a commercially available spinal cord injury device (Infinite Horizon impactor; Precision Systems & Instrumentation). Immediately after including the spinal cord contusion, we performed a Th12 partial laminectomy, and inserted a thin silicone tube with an osmotic mini-pump into the subarachnoid cavity under a surgical microscope. Osmotic mini-pumps (200 μl solution, 0.5 μl/h, 14-d delivery; Alzet pump model 2002 [Durect Co.]) were filled with each of the oligosaccharides and either HMW-HA (10 μg/200 μl), or saline (as a vehicle control). The tube was sutured to the spinous process to anchor it in place, and the mini-pump was placed under the skin on the animal's back. Afterward, muscles and skin were closed in layers. The bladder was compressed by manual abdominal pressure twice a day until bladder function was restored. All animals were given antibiotics in drinking water (1.0 ml Bactramin [Roche] in 500 ml acidified water) for 2 weeks after SCI. We used animals which showed complete paralysis (BBB score = 0), the day after the operation. Other animals which could move their hind paw or died immediately were excluded.

Recovery of hind-limb motor function was assessed by measuring the Basso, Beattie and Bresnahan (BBB) scores [1]. Quantification was performed in a blinded manner by two observers.

CGNs were prepared from Sprague-Dawley rats on postnatal days 7–8 by essentially using a described method [7,16]. Briefly, the cerebella were cleared of meninges and blood vessels, roughly homogenized by chopping, and trypsinized. Neurons were washed in the presence of DNAse 1 and soybean trypsin inhibitor (Sigma–Aldrich), and cellular debris was pelleted by centrifugation. The starting cell suspension was applied immediately to a two-step gradient of Percoll (35/60% in calcium and magnesium-free PBS containing 2 mM EDTA [Pharmacia Fine Chemicals]). Gradients were centrifuged at 2000 × *g* for 10 min in a clinical centrifuge. The two resulting bands, one at the interface of the CMF-PBS/35% Percoll, and the other at the interface between the layer of 35% Percoll and that of 60% Percoll, were removed with a fire-polished Pasteur pipette, diluted to 10 ml with CMF-PBS, pelleted at 600 rpm for 5 min, washed twice in Neurobasal medium supplemented with B27 (Gibco), and counted.

CGN cultures were plated on 24-well plates (TPP) coated with 0.01% poly-L-lysine (Sigma–Aldrich) at a density of 1.0×10^6 cells/ml. Cells were maintained at 37 °C with 5% CO_2 for 24 h. After cells were kept for 24 h in culture in Neurobasal medium supplemented with B27, cytosine D-arabinoside (Sigma–Aldrich) was added at a final concentration of 10 μg/ml in order to arrest any additional growth of non-neuronal cells. Cultures were supplemented twice per week. At 14 days *in vitro*, we added each of the HA oligo preparations to the neuronal cell cultures 24 h prior to administration of the glutamate analogue, NMDA (Tocris). Concentrations of the HA preparations and NMDA were 10 μg/ml and from 10 to 500 μM. In some experiments, HA oligosaccharides were washed off twice with fresh medium from those cultures pretreated with HA (10 μg/ml) for 24 h, after which NMDA was added to the cultures. After an additional 24 h culture, cells were incubated (37 °C with 5% CO_2, 15 min) with 10 mM propidium iodide (PI; Dojindo), 1 mM calcein-AM (Dojindo), and DAPI (Vector Laboratories), which stained the nuclei of dead cells, viable cells, and all cells, respectively. Then, cells were mounted on coverslipped glass slides. Fluorescent images of each well were inspected and photographed using an inverted phase-contrast microscope (Olympus, Model BX60). The ratio of dead-to-viable cells was calculated using the following formula: (PI positive cells/PI positive cells + calcein-AM positive cells) × 100. In addition, neuronal cell death was estimated by measuring the activity of lactate dehydrogenase (LDH) released from damaged or destroyed cells into the culture media. All LDH activity was measured using an LDH-cytotoxic test kit according to the protocol recommended by the manufacturer (Wako).

All substrates were prepared prior to the assay and stored at −80 °C until needed. CGN cultures were plated on two-chamber poly-L-lysine-(0.01%) coated Lab-Tek slides (Nunc) coated with PG (300 ng/ml) obtained from chicken brain (CC117, Chemicon) with each of the HA oligo at a density of 5.0×10^5 cells/ml. After cells were maintained at 37 °C with 5% CO_2 for 24 h, CGN cultures were fixed in 4% (w/v) paraformaldehyde, followed by blocking in 1% (w/v) BSA and permeabilization with 0.3% Triton X-100 in PBS for 15 min at room temperature. Then, cultures incubated with anti-beta-tubulin class III mouse monoclonal antibody at 1:500 (Tuj-1; Convance) for 1 h. After rinsing, plates were incubated with secondary FITC goat anti-mouse antibodies at 1:100 (Invitrogen). The neurite length per cell was evaluated using an inverted phase-contrast microscope (Olympus, Model BX60). Processes with a length equivalent to one or more diameters of the cell body were considered to reflect neuritis and were measured by Image J software. Neurite lengths were measured with at least 100 neurons per condition from duplicate wells, and quantified as described previously [18].

After terminal anesthesia by ether hyperaspiration, rats were perfused transcardially with buffered 4% paraformaldehyde. Spinal cords were removed, postfixed overnight, and cryoprotected in buffered 30% sucrose overnight. Tissues were cut into 20-μm sections with a cryostat and mounted on glass slides. Sections were blocked in PBS containing 1% BSA and 10% normal goat serum for immunohistochemical analysis. Sections were then incubated overnight at 4 °C with polyclonal rabbit anti-Iba-1 antibody (Wako Pure Chemical Industries, 1:200) for microglia staining, and monoclonal mouse anti-CD68 antibody (ED1; Chemicon International, 1:100) for activated microglia staining. After rinsing, sections were incubated with the secondary antibody, Alexa Fluor 488, 594–conjugated streptavidin (Invitrogen, 1:400) in PBS with 0.05% Tween20 for 60 min at room temperature.

The midpoint of a lesion was determined by hematoxylin-and-eosin staining of several serial sagittal 20 μm sections. Light intensity and thresholding values were maintained at constant levels for all analyses by a computer-driven microscope stage. All the image analyses shown hereafter were performed using spinal cord samples prepared from five sagittal sections at 20 μm intervals (a midsagittal section and two sections on either side of the midline, which was identified by the appearance of the central tube). In all sagittal sections shown here, the left side is rostral. The measured area was set between 10 mm caudal and 10 mm rostral to the lesion center.

Eight weeks after injury, descending CST fibers were labeled with biotin-dextran amine (BDA; 10% in saline, 3.5 μl per cor-