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Research Report

Hyaluronan tetrasaccharide promotes regeneration of peripheral nerve: *In vivo* analysis by film model method

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ABSTRACT

Hyaluronan (HA) is known to inhibit neurons from regenerating in the central nervous system. However, hyaluronan tetrasaccharide (HA4) was found in *in vitro* experiments to promote outgrowth of neurons. To investigate the promotion by HA4 of nerve regeneration *in vivo*, we analyzed outgrowth of regenerating axons treated with HA4, using a film model method. After the common peroneal nerve in mice was transected, the proximal end of cut nerve was placed on a sheet of thin plastic film, immersed in several drops of HA4 solution, covered with another sheet of film, and then kept *in vivo*. Six hours after the procedure, terminal sprouts had grown out from ending bulbs formed at the cut end of parent nerve administered with HA4 solution 100 or 1000 μg/mL, while no sprouts were observed in groups treated with 10 μg/mL of HA4 or in controls. On the 2nd day after axotomy (day 2), many regenerating axons in the group treated with 100 μg/mL of HA4 extended onto the flat film for a longer distance than those treated with 1000 μg/mL of HA4 and controls. With the optimal dose of HA4 (100 μg/mL), axonal outgrowth was significantly ($p<0.01$) greater than that in controls at each time point. Schwann cells appeared migrating from parent nerve onto the film from day 3 as well as in controls. Thus, enhanced outgrowth of regenerating axons and normal behavior of migratory Schwann cells suggested that HA4 promoted regeneration of neurons without the mediation of Schwann cells.

1. Introduction

1.1. *Hyaluronan and hyaluronan tetrasaccharide*

Hyaluronan (HA) is a linear macromolecular glycosaminoglycan formed from repeating disaccharide units of D-glucuronate and N-acetyl-D-glucosamine. Intrinsic HA is included in the extracellular matrix of various organs, e.g., dermis, epidermis, hyaline cartilage, ovary, mesenteric tissue (e.g., umbilical cord), and also in extracellular fluid (e.g., synovial fluid and vitreous humor), but is degraded by liver and the lymphatic system so rapidly that it does not remain in the blood and

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lymph. Intrinsic HA possesses viscoelastic properties and easily aggregates or binds the other components of tissue and cells (Volpi et al., 2009). In inflammatory tissues and tumors, low-molecular-weight HA (10–500 KDa) binds CD 44 receptors on the surface of cells to induce genes for chemokines and cytokines, angiogenesis, and activation of tumor necrosis factors, while these responses were suppressed by high-molecular-weight HA (800 KDa–1.2 MDa) (Volpi et al., 2009). Following injury in the peripheral nervous system, no hyaluronan was found. However, after lesions in the central nervous system, HA was abundantly produced by astrogliosis to enclose progenitor cells and suppress their differentiation to neurons and glia (Fraser et al., 1997). HA is therefore considered one of the inhibitors of regeneration in the central nervous system (Fraser et al., 1997; Marret et al., 1994; Struve et al., 2005). Hyaluronan is natively depolymerized by hyaluronidase or radicals *in vivo* to HA oligosaccharides, including di-, tetra-, hexa-, octa-, deca-, and dodecasaccharides (Xu et al., 2002). These HA oligosaccharides acquired novel functions different from intact HA. In particular, hyaluronan tetrasaccharide (HA4) suppressed the death of K562 cells by up-regulating heat shock protein 72 under conditions of stress, as demonstrated by Xu et al. (2002), and promoted outgrowth of PC12 cells, as reported by Yamanokuchi et al. (2010, unpublished). It has been hypothesized that HA4 can keep neurons *in vivo* from dying after injury and promote regeneration of them. In the present study, to test this hypothesis, we examined promotion by HA4 of nerve regeneration.

1.2. *Film model method and nerve regeneration*

To examine the early phase of regeneration in the peripheral nervous system, we developed a film model method (Torigoe et al., 1996). After the common peroneal nerve in mice was transected, the proximal end of cut nerve was placed on a sheet of thin plastic film, moistened with several drops (approximately 20–50 μl) of Ringer's solution, then covered with another sheet of film. The two films were sewn together and maintained *in vivo*. The neural specimen on the flat film is so thin (about 20 μm in thickness) that no semi-thin sectioning is required for histological procedures. Therefore, under light microscopy, we can observe regenerating axons growing out entirely from the cut end of parent nerves to the tips of them, i.e., growth cones, as if observation were performed *in vitro*. According to studies with the film model method, regenerating axons, called nodal sprouts, started to sprout from the first or second nodes of Ranvier adjacent to the proximal end of cut nerve from 3 h after axotomy (Torigoe et al., 1996), extended along the parent nerve stump, reaching the cut end of parent nerve 18 h after axotomy (Torigoe and Lundborg, 1998). Besides nodal sprouts, another type of sprout, called the terminal sprout, also grew out from the ending bulb, which resulted from swelling of the transected nerve itself at the cut end of parent nerve (Torigoe and Lundborg, 1998). Examination of the time course of outgrowth of terminal sprouts (Fig. 1a–d) revealed that ending bulbs were formed 6 h after axotomy, and thereafter, terminal sprouts grew out from the ending bulbs 12 h after axotomy. Since terminal sprouts already had appeared at the ending bulbs before nodal ones reached there, we could easily examine the outgrowth of terminal sprouts from 6 to 12 h after axotomy. Regenerating axons, either terminal or nodal sprouts, were kept naked for at least 2 days after axotomy (Torigoe et al., 1996). From the 3rd day after axotomy (day 3), Schwann cells appeared from parent nerve stump, sheathed regenerating axons, and accelerated outgrowth of regenerating axons (Torigoe et al., 1996, 1999).

In this study, the film model method was used to examine the regenerative effects of HA4. Six hours after axotomy, terminal sprouts were examined at the ending bulb; on day 2, outgrowth of naked axons; and from day 3 to day 4, acceleration by migratory Schwann cells. We found that HA4 at an optimal dose promotes neural regeneration.

2. Results

We moistened the surface of the film with several drops of HA4 solution at doses of 10, 100, or 1000 μg/mL instead of Ringer's solution (HA4 groups 10, 100, or 1000, and controls, respectively). Six hours after axotomy, ending bulbs had formed at the proximal tip of transected nerve in each group. Terminal sprouts were found extending at many ending bulbs in the HA4 groups 100 and 1000 (Fig. 1e and f), but few sprouts were visible in the HA4 group 10 (Fig. 1g) or the controls.

Groups administered with HA4 at 100 or 1000 μg/mL continued the experiment. On day 2, regenerating axons extended from the cut end of parent nerve onto the film. The length of axons in HA4 group 100 was markedly longer than that in HA4 group 1000 and the controls (Fig. 2). The optimal dose of HA4 solution was thus 100 μg/mL.

The mean length of regenerating axons in HA4 group 100 at each interval was significantly ($p<0.01$) longer than that in the controls (Figs. 3a and b, 4). In the initial phase of naked axons until day 2, the growth rate of regenerating axons in HA4 group 100 was 268 μm/day and approximately 3 times faster than that in the controls (77 μm/day). In the accelerated phase with migratory Schwann cells from day 3, the growth rate in HA4 group 100 was 281 μm/day and almost the same as that in the controls (283 μm/day). Migratory Schwann cells in HA4 group 100 were visualized with antibody to S-100 (Fig. 3c). They were distributed along regenerating axons, and each of them had a spindle-shaped cell body with several long branches. The distribution of migratory Schwann cells and their configuration were almost the same as those in the controls (Torigoe et al., 1999).

When HA4 was given to mice via intraperitoneal administration at a dose of 100 or 1000 μg/mL every day, instead of direct immersion of HA4, no promotion of axonal outgrowth was observed.

3. Discussion

Enhanced regeneration after administration with HA4 is characterized by reduction of the lag time between axotomy and the onset of terminal sprouting and by accelerated outgrowth of regenerating axons. These characteristics are induced by increasing the intrinsic growth state, i.e., metabolism in neurons (Hoffman, 2010).