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Short Communication

Intraspinal microinjection of chondroitinase ABC following injury promotes axonal regeneration out of a peripheral nerve graft bridge

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Abstract

Chondroitin sulfate proteoglycans (CSPG) within the glial scar formed after central nervous system (CNS) injury are thought to play a crucial role in regenerative failure. We previously showed that delivery of the CSPG-digesting enzyme chondroitinase ABC (ChABC) via an osmotic minipump allowed axonal regeneration and functional recovery in a peripheral nerve graft (PNG)-bridging model. In this study, we sought to overcome the technical limitations associated with minipumps by microinjecting ChABC directly into the distal lesion site in the PN bridging model. Microinjection of ChABC immediately rostral and caudal to an injury site resulted in extensive CSPG digestion. We also demonstrate that this delivery technique is relatively atraumatic and does not result in a noticeable inflammatory response. Importantly, microinjections of ChABC into the lesion site permitted more regenerating axons to exit a PNG and reenter spinal cord tissue than saline injections. These results are similar to our previous findings when ChABC was delivered via a minipump and suggest that microinjecting ChABC is an effective method of delivering the potentially therapeutic enzyme directly to an injury site.

Keywords: Spinal cord injury; Chondroitinase ABC; Peripheral nerve graft; Regeneration; Chondroitin sulfate proteoglycan; Glial scar

The glial scar formed after adult central nervous system (CNS) injury is both a chemical and physical barrier to axonal regeneration. The upregulation of various axonal growth inhibitors within the glial scar following injury is viewed as a major impediment for regeneration (Silver and Miller, 2004; Fawcett, 2006). One class of reactive astrocyte-associated inhibitors is the chondroitin sulfate proteoglycans (CSPG) (Morgenstern et al., 2002; Carulli et al., 2005). CSPGs function as potent inhibitory extracellular matrix (ECM) molecules and consist of a protein core to which many large, sulfated glycosaminoglycan (GAG) chains are covalently attached. These GAGs confer most of CSPG's inhibitory properties. The presence of CSPG within the lesion penumbra appears to be an important factor preventing the regeneration of axons (Davies et al., 1999; Tom et al., 2004). Use of the enzyme chondroitinase ABC (ChABC), which cleaves the inhibitory GAGs from the protein core of CSPG, has been shown to promote growth past the lesion and some functional recovery (Bradbury et al., 2002; Caggiano et al., 2005; Fouad et al., 2005; Cafferty et al., 2007). More recently, it was demonstrated that treating a lesion site with ChABC via an osmotic minipump permitted axons regenerating in a peripheral nerve graft (PNG) to exit into host spinal cord tissue, where functional synapses were formed (Houle et al., 2006).

While delivering ChABC with a minipump promoted regeneration, there are inherent limitations with the system. For example, the insertion of the pump into the lesion cavity is blind and air bubbles in the catheter will cause failure of ChABC delivery. We hypothesized that microinjecting ChABC directly into the lesion site would efficiently and more easily deliver ChABC to promote regenerating axons to exit a PNG and reenter the spinal cord.

Adult female Sprague–Dawley rats (225–250 g, Charles River) were anesthetized with isoflurane. A hemilaminectomy was performed on the fifth cervical vertebra (C5). The dura was cut to expose the right dorsal spinal cord and a dorsal quadrant lesion (DQ) of approximately 1 mm^3 was made via aspiration. The dura was closed using a 10-0 suture. One microliter of

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saline ($n=5$) or ChABC (Seikagaku; 20 U/mL, $n=3$) was injected over 10 min using a pulled glass pipette inserted directly into the ventral spinal cord tissue immediately rostral and caudal to the C5DQ. The exposed cord was covered with silastic membrane, the overlying musculature was sutured, and the skin was closed using wound clips. The animals were given ampicillin (200 mg/kg) and buprenorphine (0.1 mg/kg) and placed on a thermal barrier to recover. Two and four days later, the animals were reanesthetized, the C5DQ exposed, and the injections were repeated in the same general areas. Five days after injury, animals were given an overdose of Euthasol and were transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in PB (pH 7.4).

Twenty-five micrometer sections containing the C5DQ were cut and collected in series of six. Sections were rinsed in PBS, blocked for 1 h at room temperature with 5% normal goat serum, 1% bovine serum albumin, ±0.1% Triton X-100 in PBS (depending upon the primary antibody used), and then incubated in rabbit anti-glial acidic fibrillary protein (GFAP; Dako), mouse anti-ED1 (Serotec), and mouse anti-2B6 antibody (recognizes the 4-sugar "stub" following ChABC-digestion of CSPG; Seikagaku) overnight at 4 °C. The sections were rinsed and incubated in the appropriate pre-absorbed, fluorescently-tagged secondary antibody overnight at 4 °C. The sections were rinsed again, mounted on glass slides, coverslipped with VectaShield, and sealed with nail polish.

To quantify ED1 expression, four sections from the center of the lesion per animal were selected. Images were taken and montaged together so that the entire half of the section unilateral to the lesion was visible (Corel Draw). Each montaged image was thresholded (MetaMorph). A template consisting of four regions was laid over each thresholded image. Each region was approximately 1 mm × 250 μm, and the regions were stacked so that the entire set of all four regions would span an area of 1 mm × 1 mm. The top-most side of the first region ran tangentially to the ventral edge of the C5DQ. The thresholded pixel area in each region was then calculated (MetaMorph). The pixel areas for each region were analyzed for statistical significance by performing a Student's *t*-tests (Microsoft Excel).

To determine the effect of microinjections of ChABC on regeneration out of a PNG, adult rats ($n=12$) were anesthetized, the caudal end of the second and the entire third cervical vertebrae (C3) was removed, and the dura was cut to expose the dorsal spinal cord. The entire right half of the C3 spinal cord was aspirated out, resulting in a cavity that was 2–3 mm in length. Predegenerated tibial nerves were isolated from donor rats and ~2–3 mm of the perineurium was reflected from the proximal nerve end. This end was placed into the C3 cavity site and secured in place by suturing the perineurium to the dura. The distal end of the PNG was left free outside of the spinal column along the C4–C8 vertebrae. The dura and overlying musculature were closed using sutures, and the skin was closed using wound clips. Two weeks later, the animals were anesthetized and a C5DQ was made as described above. One microliter of saline ($n=8$) or ChABC (20 U/mL, $n=4$) was injected rostral and caudal to the C5 injury as described above. The injections were repeated two and four days later. After the injection on day 4, the dura above the C5DQ was reopened. The free, distal end of the PNG was trimmed by 1 mm and inserted into the C5DQ cavity where it was secured in place by suturing the perineurium to the dura. Silastic membrane was placed over the graft. After the musculature was sutured, the skin was closed using wound clips. After at least three weeks, the animals were anesthetized and the cord and graft were exposed. The graft was cut and soaked with 10% biotinylated dextran amine (BDA; 3000 MW; Molecular Probes) to label regenerating axons by tracer diffusion though the distal end of the graft. Two days later, the animals were perfused with 4% PFA.

BDA-labeled axons were visualized by incubating sections in Avidin-HRP for 3 h at room temperature, rinsed in PBS, and then reacted with diaminobenzidine (DAB). Sections were rinsed again, mounted, counterstained with thionin, and coverslipped. To quantify the number of BDA+ axons emerging from the graft and back into spinal cord tissue, four representative DAB-reacted sections from each animal were analyzed. A virtual line 500 μm ventral to the PNG/spinal cord interface was made and the number of BDA+ labeled profiles that intersected with the line was counted. The numbers for each treatment group were analyzed for statistical significance by performing a Student's *t*-tests (Microsoft Excel).

We wanted to determine if the microinjection technique was traumatic to the tissue, which would limit its therapeutic potential. Therefore, we examined the inflammatory response at a subacute time point after injury and several injections. Five days after the lesion in the saline-treated spinal cords, ED1+ macrophages/microglia were found primarily in the lesion site (Figs. 1B, C, lesion site denoted by asterisk in Fig. 1A) and tissue just adjacent to them. The phagocytic cells did not appear to migrate much further than the lesion cavity wall that was surrounded by reactive, GFAP+ astrocytes (Figs. 1A, C). Microinjection of ChABC produced a similar response. As in saline-treated sections, ED1+ cells were located primarily in the lesion cavity (Figs. 1E, F, lesion site denoted by asterisk in Fig. 1D) and did not extend very far past the cavity wall that was surrounded by many GFAP+ astrocytes (Figs. 1D, F). Based upon densitometric measurements, there was no difference in ED1+ immunoreactivity between saline and ChABC-treated tissue (Fig. 1G).

To determine how effective microinjections were in delivering ChABC, we examined the resulting CSPG digestion pattern using the 2B6 antibody that recognizes the 4-sugar "stub" that remains following ChABC-digestion. Tissue that had been treated with ChABC had abundant 2B6 immunoreactivity within ipsilateral grey and white matter, including the lesion penumbra (Fig. 1I, arrowhead) and some of the contralateral tissue (Fig. 1I). Although there was some immunoreactivity for 2B6 in sections from saline-treated animals even though no ChABC was used (Fig. 1H), the staining pattern was identical to that of control sections incubated with secondary antibodies only (data not shown), suggesting that this pattern is non-specific binding due to the "sticky" nature of the scar region. Furthermore, there is strikingly less 2B6+ staining than in ChABC-treated sections.

We wanted to determine if digestion of scar-associated CSPG with microinjections of ChABC was sufficient to allow