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Synergistic effects of self-assembling peptide and neural stem/progenitor cells to promote tissue repair and forelimb functional recovery in cervical spinal cord injury

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ABSTRACT

While neural stem/progenitor cells (NPCs) show promise for traumatic spinal cord injury (SCI), their efficacy in cervical SCI remains to be established. Moreover, their application to SCI is limited by the challenges posed by the lesion including the glial scar and the post-traumatic cavitation. Given this background, we sought to examine the synergistic effect of self-assembling peptide (SAP) molecules, designed to optimize the post-traumatic CNS microenvironment, and NSCs in a clinically-relevant model of contusive/compressive cervical SCI. We injected K2(QL)6K2 (QL6) SAPs into the lesion epicenter 14 days after bilateral clip compression-induced cervical SCI in rats, combined with simultaneous transplantation of neural stem/progenitor cells (NPCs) intraspinally adjacent to the lesion epicenter. The QL6 SAPs reduced the volume of cystic cavitation in the spinal cord lesion. Simultaneously engrafted NPCs preserved motor neurons and attenuated perilesional inflammation. The combination of QL6 and NPCs promoted forelimb neurobehavioral recovery and was associated with significant improvement in forelimb print area and stride length. In summary, we report for the first time histologic and functional benefits in a clinically-relevant model of cervical SCI through the synergistic effects of combined SAP and NPCs.

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1. Introduction

Various approaches have been used in attempts to treat animal spinal cord injury (SCI) with very limited success to date. In the clinic, more than 50% of SCI are at the cervical level [1–3]. Cervical lesions cause severe disability of both lower and upper extremities, which results in poorer quality of life and significantly higher cost of treatments. In addition, the cervical cord has a different anatomy and pathology from the thoracic cord in terms of the distribution of blood, the size of gray/white matter, and neural segmental function. Accordingly, cervical SCI models are more desirable for translation to clinical trials.

It is well known that SCI results in a loss of neural tissues and formation of cystic cavities that inhibit regenerating axons from crossing the lesion area. The hostile microenvironment negatively influences the preservation and regeneration of neural tissues [4–6]. In order to enhance therapeutic efficacy, many studies have reported benefits from remodeling the extracellular matrix to provide a suitable scaffold for regeneration of cells and axons [7,8]. Specifically, self-assembling peptides (SAP) that can form 3D structures of nanofibers *in situ* under physiological conditions without apparent immune response have been developed. Numerous approaches have been used to produce synthetic extracellular matrix, which are classified into natural polymers including fibrin, collagen, agarose and alginates, and synthetic polymers such as poly a-hydroxil acids and polyvinylchloride [9]. SAPs are self-assembling nanofibers that have refined features that construct a robust scaffold, are amenable to injection due to their water solubility and exhibit dramatic morphology changes immediately after injection due to the addition of salts from the cerebrospinal fluid [10]. Recently, injection of K2(QL)6K2 (QL6) at 24 h after injury showed significant histologic tissue preservation by attenuating inflammation and astrogliosis, reducing post-traumatic apoptosis of neural cells, and promoting axonal regeneration in

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thoracic injured rats in our lab [11]. We have since developed this strategy as a combined therapy with transplantation of neural stem/progenitor cells (NPCs) to exploit these benefits and apply them to a challenging delayed SAP-based therapy into a rat model of cervical compression SCI. In the current research, we use adult NPCs isolated from transgenic mice combined with SAPs implanted into rats with a clip-compression cervical injury. We assessed the animals' behavioral outcomes in addition to examining parameters including tissue sparing and inflammation. We hypothesized that rats receiving the combinatorial strategy of NPCs with QL6 would have improved outcomes in all parameters, particularly with regard to reduced inflammation and astrogliosis.

2. Materials and methods

All neurobehavioral and neuroanatomical protocols in this study were performed using appropriate randomization and blinding. Unbiased stereological approaches were used to undertake all neuroanatomical assessments.

2.1. Animal care

A total of 77 adult female Wistar rats (250 g; Charles River Laboratories) were used for this study. All experimental protocols were approved by the animal care committee of the University Health Network (Toronto, Canada) in accordance with the policies established in the guide to the care and use of experimental animals prepared by the Canadian Council of Animal Care.

2.2. Isolation and culturing of adult neural precursor cells

Adult NPCs were isolated from 10 YFP-expressing transgenic mice [129-Tg (ACTB-EYFP) 2Nagy/J, The Jackson Laboratory]. Briefly, mice were killed by cervical dislocation, and the brains were excised under sterile conditions and transferred to artificial CSF (aCSF) solution containing 2 M NaCl, 1 M KCl, 1 M $MgCl_2$, 155 mM $NaHCO_3$, 108 mM $CaCl_2$, 1 M glucose, and 1% penicillin/streptomycin (Pen/Strep) (Sigma). The subventricular zone of the forebrain was dissected and transferred to a low-calcium aCSF solution (10 ml) containing 40 mg of trypsin, 20 mg of hyaluronidase, and 4 mg of kynurenic acid for 30 min at 37 °C. Then, trypsin was inactivated, and the tissue was mechanically dissociated into a cell suspension with a fire-polished glass pipette and cultured in serum free medium (200 ml) containing of 20 ml of DMEM/F12, 4 ml of 30% glucose, 3 ml of 7.5% $NaHCO_3$, 1 ml of 1 M HEPES, 200 mg of transferrin, 50 mg of insulin, 19.25 mg of putrescine, 20 μl of selenium, 20 μl of progesterone, 1 μg of FGF2, 2 μg of EGF, and 1% Pen/Strep for 7d. The neurospheres generated were passaged weekly by mechanical dissociation in the same medium.

2.3. Experimental groups and surgical procedures

2.3.1. Cervical spinal cord injury

The aneurysmal clip contusion-compression model of thoracic SCI has been extensively characterized and reported previously (Rivlin and Tator, 1978; Fehlings and Tator, 1995; Nashmi and Fehlings, 2001). This injury model was then adapted for use in the cervical enlargement. Under isoflurane anesthesia (2–2.5%) and a 1:1 mixture of O_2/N_2O, the surgical area was shaved and disinfected with 70% ethanol and povidone iodine. A midline skin incision was made at the cervical area (C4–T2), and paraspinal muscles were retracted. The rats underwent a C6 and C7 laminectomy and then received an 18 g clip (Walsh) compression injury applied by a quick-release applicator for 1 min at the C6 level of the spinal cord [12]. The wound was sutured in layers, and the animals were given postoperative analgesia (0.05 mg/kg buprenorphine) and saline (5 ml) to prevent dehydration and received Clavamox (amoxicillin plus clavulanic acid) for 7d. Animals were allowed to recover and housed in standard rat cages with absorbent bedding at a temperature of 26 °C. Their bladders were manually expressed three times daily until return of reflexive bladder control.

2.3.2. Experimental groups and treatments

At 2 weeks after injury, all injured rats were block randomized into four experimental groups based on grip strength meter (GSM) test (see below) to ensure equivalent deficits across the experimental groups before starting the treatments (Table 1).

2.3.3. Injection of QL6 and transplantation of NPCs

At 2 weeks after injury, the animals underwent intraspinal injections of NPCs or vehicle using well-established methods developed by our group [13,14]. Briefly, the rats were anesthetized as before and then the injured spinal cord was carefully re-exposed extradurally under surgical microscope. Assessment of cell viability by Trypan Blue staining indicated the presence of ~90% live cells in the cell suspension. The cells were diluted in the growth medium (50×10^3 live cells/μl) and then used for cell transplantation. Using a Hamilton syringe connected to a stereotactic frame with a microglass pipette (100 μm OD), a total volume of 8 μl of cell suspension,

Table 1
Description of the experimental design.

	Injury	Vehicle	NPC	QL6	Mino	CsA	*n*	Mortality
i) Laminectomy	−	−	−	−	−	−	−	−
ii) Plain injured	+	−	−	−	−	−	−	−
iii) Vehicle	+	+/+	−	−	+	+	10	4
iv) NPC	+	+/−	+	−	+	+	9	3
v) QL6	+	−/+	−	+	+	+	9	3
vi) NPC + QL6	+	−	+	+	+	+	9	2

Abbreviations: CsA, cyclosporine A; Mino, minocycline; *n*, number randomized; NPC, neural stem/progenitor cell; QL6, self-assembling peptide.

containing 4×10^5 live cells, was intraspinally injected into the dorsolateral spinal cord, next to the midline. Four intraspinal injections were made bilaterally 2 mm rostral and 2 mm caudal to the injury site. Immediately following NPC transplantation, a total of 10 μl QL6 was injected into the injury site. QL6 was prepared as a 1% (w/v) concentration for gel formation that can mimic an extracellular matrix [15,16]. Four intraspinal injections were made bilaterally surrounding the lesion epicenter, 1.5 mm below the dorsal surface, and the volume of injection was 2.5 μl at each point using the same method as for the injection of NPCs. An injection rate of 0.5 μl/min was used, and at the end of injection the microglass pipette was left in the cord for at least 2 min to allow diffusion from the injection site. The animals received a daily subcutaneous injection of cyclosporine A (10 mg/kg, Sandimmune, Novartis) starting 1 d before transplantation and continuing until the end of the experiments. The rats also received a daily injection of the anti-inflammatory drug minocycline (50 mg/kg, Sigma) intraperitoneally for 10 d starting 1 d before transplantation. Note that animals in all other groups, except the "plain injured" control, received the same number of injections to the spinal cord using only culture medium, and received cyclosporine A and minocycline in the same manner as the NPC-transplanted rats.

2.4. Tissue processing

2.4.1. Animal perfusion

Animals were deeply anesthetized with isoflurane and then perfused transcardially with cold PBS (0.1 M) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. For cryotomy, the spinal cord was post-fixed in PFA overnight at 4 °C, and then cryoprotected in 30% sucrose in PBS for 48 h at 4 °C. Then, a 2 cm length of the spinal cord centered at the injury site was dissected and embedded in mounting media (HistoPrep, Fisher Scientific) on dry ice. Cryostat sections were cut 30 μm thick and stored at −80 °C.

2.5. Assessment of tissue sparing

Serial spinal cord sections, ranging approximately 5 mm centered at the epicenter, were stained with myelin-selective luxol fast blue (LFB) and the cellular stain hematoxylin-eosin (HE) to identify the lesion epicenter and assess the residual cross-sectional and volume tissue ($n = 5$/group). The area of cavity, lesion (including cavity), gray matter, white matter, preserved tissue (white matter + gray matter) and total sectional area was measured with StereoInvestigator after tracing the region at each section. For normalizing with respect to total cross-sectional area, parameters were divided by "total area" at each section, and presented as "% area" of each parameter. Volumes were calculated automatically with StereoInvestigator ranging from 960 μm rostral to 960 μm caudal to the epicenter by 240 μm interval and presented as total and percent volume.

2.6. Immunohistochemical procedures and image analysis

For all immunohistochemical staining, the blocking solution contained 5% nonfat milk, 1% BSA, and 0.3% Triton X-100 in 0.1 M PBS unless otherwise has been mentioned. Frozen slides were blocked and incubated with DAPI and the following primary antibodies: Chicken anti-ChAT (1:200, abcam) for motor neurons, mouse anti-NeuN (1:500, Millipore) for neurons, rabbit anti-Iba-1 (1:500, Wako) for microglia/macrophages, mouse anti-CD68 (1:200, Serotec) for activated microglia, rabbit anti-GFAP (1:1000, Millipore) for astrocytes, mouse anti-CSPGs (CS56, 1:200, Sigma). The slides were washed in PBS three times and incubated with fluorescent Alexa 647, 568, 488, and 350 (1:400, Invitrogen) secondary antibody for 1 h. The slides were washed and coverslipped with Mowiol mounting medium. The images were taken using a Zeiss 510 laser confocal microscope or Leica epifluorescence microscope.

2.7. Image processing and analysis

In all neuroanatomical procedures, quantification was executed in an unbiased manner by examiners blinded to the treatment groups based on the previously described methods by our group and others [14,17–19].

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