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Biocompatibility of a coacervate-based controlled release system for protein delivery to the injured spinal cord

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ABSTRACT

The efficacy of protein-based therapies for treating injured nervous tissue is limited by the short half-life of free proteins in the body. Affinity-based biomaterial delivery systems provide sustained release of proteins, thereby extending the efficacy of such therapies. Here, we investigated the biocompatibility of a novel coacervate delivery system based on poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin in the damaged spinal cord. We found that the presence of the [PEAD:heparin] coacervate did not affect the macrophage response, glial scarring or nervous tissue loss, which are hallmarks of spinal cord injury. Moreover, the density of axons, including serotonergic axons, at the injury site and the recovery of motor and sensorimotor function were comparable in rats with and without the coacervate. These results revealed the biocompatibility of our delivery system and supported its potential to deliver therapeutic proteins to the injured nervous system.

1. Introduction

Endogenous repair of damaged nervous tissue and restoration of motor function is limited following spinal cord injury (SCI) [1]. Administration of therapeutic molecules such as growth factors is one promising strategy to promote repair; however, the short half-life of bioactive proteins in vivo limits the efficacy of direct injection [2–6]. Multiple injections or implantation of semipermanent cannulas may bypass this drawback but require invasive surgical techniques which limit their clinical relevance [7–9]. An injectable, biodegradable and biocompatible delivery system would provide sustained release of therapeutic proteins, thereby extending their efficacy and avoiding overly invasive delivery methods.

Here, we explored the utility of a unique biodegradable growth factor delivery system for use in the injured spinal cord. The delivery system is composed of a synthetic polycation, poly(ethylene argininylaspartate diglyceride) (PEAD), which is cationic and binds heparin electrostatically [10]. PEAD is biodegradable via hydrolysis of its ester bonds and undergoes ~40% degradation over 30 days in vitro [10]. When PEAD and heparin are combined, they interact to form submicron (~200 nm) sized liquid droplets that phase-separate from water. The droplet is called a "coacervate". The liquid coacervate suspension is thus injectable, and sustains the release of heparin-binding growth factors and morphogens with enhanced bioactivity in the skin and heart [11,12] and has been studied in the context of wound healing, cardiac repair, bone regeneration and therapeutic angiogenesis [13–16]. Protein release from the coacervate can be considered to be a dynamic process, with the ionic environment, the dissociation and reassociation of proteins from the coacervate, and hydrolytic degradation of PEAD all governing release kinetics. The stronger the interaction between protein and heparin, the slower the release will be.

The coacervate delivery system is composed of a recently designed polycation and heparin, which due to its anticoagulant properties may potentially exacerbate inflammation and bleeding in damaged nervous tissue. Therefore, we investigated the biocompatibility of the [PEAD:heparin] delivery vehicle in the contused spinal cord of adult rats. Inflammation, glial scarring, nervous tissue loss and functional impairments are well documented in this model of SCI, allowing for comprehensive biocompatibility analysis under conditions mimicking clinical neural trauma. In addition, we employed [PEAD:heparin] to administer sonic hedgehog (Shh) to

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the contused spinal cord as a potential therapeutic strategy [17]. Previously, we have successfully incorporated Shh at 95% loading efficiency into the coacervate and determined that its release is sustained over more than 21 days in vitro, therefore making it a viable candidate for in vivo tissue repair [14].

2. Materials and methods

2.1. Coacervate preparation

PEAD was synthesized as previously described [10]. PEAD and clinical-grade heparin (Scientific Protein Labs, Waunakee, WI) were each dissolved in 0.9% saline, and sterilized via filtration through 0.22 μm filters. To prepare the coacervate, heparin was complexed with Shh (Peprotech, Rocky Hill, NJ) before PEAD was added. Self-assembly of the PEAD and [heparin:Shh] complexes resulted in immediate phase separation to form the coacervate. Solutions were prepared at a final Shh concentration of 100 ng μl^{-1}. For vehicle controls, heparin was directly complexed with PEAD without the addition of Shh.

2.2. Spinal cord contusion model

All animal experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh, with strict adherence to guidelines of the National Institutes of Health and United States Department of Agriculture. Female Sprague–Dawley rats (225–250 g, n = 10 per group; Charles River Laboratory, Wilmington, MA, USA) were anesthetized via intraperitoneal injection of ketamine (60 mg kg^{-1}, Butlerschein, Dublin, OH) and dexdomitor (0.5 mg kg^{-1}, Pfizer, New York, NY). Following laminectomy, an Infinite Horizon impactor (Precision Systems and Instrumentation, LLC, Versailles, KY) was used to generate a 200 kDyne contusion injury at the 10th thoracic segment of the spinal cord. The injury was rinsed with sterile 0.9% saline containing 0.1% gentamicin (VWR, Radnor, PA), the muscles were sutured, and the skin closed with Michel wound clips (Fine Science Tools, Foster City, CA). Three days post-injury, animals were sedated, the injury site was exposed, and the contusion site was injected with 5 μl of either [PEAD:Heparin:Shh], [PEAD:Heparin], Shh or phosphate-buffered saline (PBS). For rats receiving Shh, the total administered dose was 500 ng.

2.3. Post-surgical care

Antisedan (1.5 mg kg^{-1}, Pfizer, New York, NY) was injected subcutaneously to wake the animals following procedures. Gentamicin (6 mg kg^{-1}, VWR, Radnor, PA) was administered intramuscularly for 7 days post-injury. Rimadyl (5 mg kg^{-1}, Pfizer, New York, NY) and Ringer's solution (5 ml, Butlerschein, Dublin, OH) were administered subcutaneously for 6 days post-injury. Bladders were manually expressed twice daily until the ability to urinate was regained (~2 weeks post-injury). All rats were checked at least once a day during the remaining survival time.

2.4. Motor function assessment

2.4.1. Overground walking

Overground walking ability was assessed using the Basso, Beattie and Bresnahan (BBB) open field test [18] at 1, 3, 7, 14, 21, 28, 35 and 42 days post-injury. Two experimenters blinded to the treatment groups observed the animals for 4 min and assigned a score between 0 and 21, with 0 representing complete paralysis and 21 representing normal motor function. BBB scores for each animal were normalized by their day 1 post-injury values and averaged per experimental group.

2.4.2. Sensorimotor function

Sensorimotor function was assessed using the horizontal ladder test. Rats were recorded while walking across a ladder with irregularly spaced rungs. Slips of the foot up to the ankle (small) and whole leg (large) were counted and expressed as a percentage of the total number of steps taken. Three runs were analyzed per animal and averaged per experimental group.

2.4.3. Gait and footprint

The DigiGait Analysis System (MouseSpecifics, Boston, MA) was used to analyze hindlimb gait and footprint during locomotion before and after contusion. Animals were acclimated to the treadmill for 4 days prior to taking baseline (pre-injury) and endpoint (6 weeks post-injury) measurements at a treadmill speed of 20 cm s^{-1}. The videos were then analyzed for paw area, paw angle and stride length. Endpoint data were expressed relative to baseline data and averaged per experimental group.

2.5. Histological procedures and immunocytochemistry

Six weeks post-injury, rats were transcardially perfused with 300 ml PBS, followed by 400 ml 4% paraformaldehyde (Sigma-Aldrich, Allentown, PA) in PBS. Spinal cords were dissected and post-fixed in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose in PBS for at least 48 h. A spinal cord segment measuring 12 mm centered on the lesion was cut into serial 14 μm thick cryostat sections. A series of sections was stained with cresyl violet and used to determine the spared tissue volume. Other series were immunostained with antibodies against glial fibrillary acidic protein (GFAP) (1:400, DAKO) to recognize reactive astrocytes, ED1 (1:250, Millipore) to recognize macrophages, 5-HT (1:2000, Immunostar) to detect serotonergic axons, and RT-97 (1:200, DSHB) to detect axons. Briefly, sections were blocked for 1 h at room temperature with 10% normal goat serum (NGS) containing 0.1% Triton, then incubated overnight at 4 °C in the primary antibody diluted in 2% NGS with 0.1% Triton. After washing, samples were incubated in the secondary antibody (goat anti-mouse 488 or goat anti-rabbit 594, 1:200, Invitrogen) for 2 h at room temperature, washed and counterstained with DAPI, then coverslipped.

2.6. Quantitative immunofluorescence analysis

Images were analyzed using Nikon NIS Elements software. For all immunohistochemical quantification, four or five sections centered on the dorsal–ventral axis of the lesion were analyzed and data were averaged per region over all sections. For macrophage quantification, the number of ED1-positive cells was quantified on large image composites of the entire lesion and expressed as a function of the tissue area. Images were taken at 20× and stitched together in order to generate entire lesion images. GFAP intensity was assessed on images taken rostral and caudal to the lesion, as well as at the epicenter. Composite images were obtained that spanned the entire width of the cord at each location. Total intensity was considered to be the average intensity of all regions. The intensities of the 5-HT and RT97 staining were quantified similarly as those of the GFAP staining. The total area of positive axon staining was expressed as a percentage of the analyzed area. The area of interest, intensity and cell counts were quantified in all analyses using NIS Elements.

2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses for endpoint outcomes such as DigiGait, tissue sparing and staining intensities were performed using one-way