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Sustained intraspinal delivery of neurotrophic factor encapsulated in biodegradable nanoparticles following contusive spinal cord injury

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ABSTRACT

Glial cell line derived neurotrophic factor (GDNF) induces neuronal survival and tissue repair after spinal cord injury (SCI). A continuous GDNF supply is believed to gain greater efficacy in the neural restoration of the injured spinal cord. Accordingly, nanovehicle formulation for their efficient delivery and sustained release in injured spinal cord was examined. We first used fluorescence-labeled bovine serum albumin (FBSA) loaded in biodegradable poly(lactic acid-*co*-glycolic acid) (PLGA) for intraspinal administration after SCI and for in vitro study. Our results showed that the preservation of PLGA–FBSA was observed in the injured spinal cord at 24 h, and PLGA–FBSA nanoparticles were well absorbed by neurons and glia, indicating that PLGA as a considerable nanovehicle for the delivery of neuroprotective polypeptide into injured spinal cord. Furthermore, intraspinal injection of GDNF encapsulated in PLGA (PLGA-GDNF) nanoparticles into the injured spinal cord proximal to the lesion center had no effect on gliosis when compared to that observed in SCI rats receiving PLGA injection. However, local administration of PLGA-GDNF effectively preserved neuronal fibers and led to the hindlimb locomotor recovery in rats with SCI, providing a potential strategy for the use of PLGA-GDNF in the treatment of SCI.

1. Introduction

Spinal cord injury (SCI) causes the destruction of long ascending and descending axonal fiber tracts and triggers inflammation to induce the neural cell death, which makes spinal cord repair difficult [1,2]. Accordingly, neuroprotection by administration of exogenous neurotrophic growth factors to improve axonal outgrowth has been examined as a potential therapeutic treatment for SCI [1–3]. GDNF is a transforming growth factor (TGF)-β super family member which was originally isolated from a glial cell line, and the molecule functions as a trophic factor for dopaminergic neurons [4], as well as nondopaminergic neurons, including spinal cord motoneurons and peripheral ganglia. Previous studies have shown that GDNF given by factor infusion, cell-based transplantation or gene therapy can enhance neuronal survival, fiber regeneration, and recovery of hindlimb movement in rats with SCI [5–9], indicating that this molecule is considered as a potent agent for CNS treatment.

However, the up-regulated proteolytic enzyme activities after SCI may degrade the administered growth factors and repeated penetrating injections for replenishment may aggravate tissue damage and hinder functional recovery. Accordingly, an effective controllable release factor delivery system is needed to effectively protect therapeutic molecules from degradation and successfully target therapeutic agents to the injured site of the spinal cord. A tremendous number of nanodevices have been exploited for therapeutic purpose to carry a drug in a controlled release manner from the site of administration into the therapeutic target and several drug-targeting systems have been approved for the treatment of certain cancer and serious infectious diseases [10]. Biodegradable polymers such as poly(lactic acid-*co*-glycolic acid) (PLGA) have long been used for controlled release drug delivery systems [11–13]. Neuroactive compounds including neurotrophic factors can be encapsulated into PLGA for their controllable and sustained release [11–14]. For example, nerve growth factor (NGF) formulated in PLGA microspheres holds a promising approach for the treatment of the central nervous system (CNS) disorders [15], because NGF microspheres can effectively protect neurons against unilateral

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transection of the septohippocampal pathway or from intrastriatal insult by the excitatory amino acid quinolinic acid [15]. Striatal implantation of GDNF loaded in micro-scaled microspheres has recently been found to be capable of sustained release for at least up to two months in vivo, and subsequently induce the survival of the remaining dopaminergic fibers and functional improvement in Parkinson disease animal model [16]. Although sub-micron or nano-scaled PLGA nanoparticles encapsulating neuroactive molecules have also been developed for in vitro biomedical applications [10,17–19], they have not yet been applied for in vivo investigations in the injured spinal cord.

Fluorescent nanoparticles have been used to trace pathways in the brain, because neurons are able to uptake and transport beads in the size range of 20–200 nm with the fast axonal transport to the soma [20,21]. To understand whether nano-scaled PLGA nanoparticles can serve as a vector to carry GDNF for SCI treatment, we first used fluorescent-labeled latex nanoparticles to investigate the effects of the particle size on the transport of nanoparticles following the injection into normal or injured spinal cord. Considering that systemic injection could either result in the lack of efficacy due to the existence of the blood brain barrier or induce adverse side effects [1,22], intraspinal injection was performed in the study. PLGA-based nanoparticles encapsulating fluorescent-labeled bovine serum albumin (FBSA) was further used for in vitro and in vivo studies to examine the distribution of PLGA–FBSA and sustained release of FBSA in the injured spinal cord and cultured neural cells. The therapeutic effect of PLGA-GDNF on the injured spinal cord was evaluated by examining the preservation of neurofilament (NF)-stained neuronal fibers and functional recovery using the Basso Beattie Bresnahan (BBB) locomotor rating scale. In addition, we also investigated whether local administration of PLGA-GDNF affected glial activity in the injured spinal cord by immunofluorescence against glial fibrillary acidic protein (GFAP) for reactivated astrocytes, and ionized calcium binding adapter molecule (Iba1), a marker for activated microglia.

2. Materials and methods

2.1. Preparation of PLGA nanoparticles loaded with FBSA and GDNF

FITC-labeled BSA or GDNF (R&D) encapsulated PLGA nanoparticles (PLGA–FBS) were synthesized by the double emulsion solvent evaporation method. Briefly, 600 μL of FBSA (Molecular Probes) solution in phosphate buffer (5 mg/mL) was dropped into PLGA (PLA: PGA = 50:50, mw. 40,000-75,000, Sigma) chloroform solution (15 mg/mL) with a magnetic bar stirred at 500 rpm and then sonicated with a microtip sonicator (MICROSON XL2000, MISONIX) for 2 min on ice to create the first emulsion. The first emulsion was transferred into 36 mL of 2.5% polyvinyl alcohol (PVA) (mw. 30,000–70,000; Sigma) solution with a magnetic bar stirred at 500 rpm. The mixture was then sonicated for 5 min with the microtip sonicator on ice. With the magnetic bar stirred at 500 rpm, the organic solvent gradually evaporated in the hume hood. The nanoparticle suspension was centrifuged at 10,000 rpm for 30 min and the large nanoparticles were removed. The supernatant was washed with Labscale ™ TFF system (Millipore) with Pin = 25 psi and Pout = 10 psi. The dialysate was stored at −80 °C until use.

The preparation of GDNF (R&D) loaded PLGA nanoparticles (PLGA-GDNF) was similar to the PLGA–FBSA. Briefly, 25 μL of GDNF solution in citrate buffer (2 mg/mL, pH = 8) was dropped into 250 μL of PLGA solution in methyl chloride (2 mg/mL) and then sonicated with a microtip sonicator for 15 s on ice to create the first emulsion. The first emulsion was transferred into 1.5 mL of 10% sucrose solution and then sonicated for 20 s with the microtip sonicator on ice to form the second emulsion. The organic solvent within the second emulsion was rapidly removed by using rotory evaporator (EYELA). Finally, the PLGA-GDNF solution was concentrated by ultrafiltration centritube (Amicon Ultra, 10 kDa, Millipore) and stored at −80 °C until use.

2.2. In vitro release profile

The in vitro release of FBSA or GDNF from PLGA nanoparticles was evaluated in a franz cell mounted with a 0.1 μm VCTP (Millipore) membrane. In the upper chamber of the franz cell, 8 mg of PLGA–FBSA nanoparticles was put in 5 mL of 0.01 M phosphate-buffered saline (pH 7.4) and in the lower chamber 20 mL PBS was added. At predetermined times, 100 μL was removed from the lower chamber and the FBSA concentration was determined by a protein assay kit (Bio-Rad).

The in vitro release of GDNF from PLGA nanoparticles was evaluated in Neurobasal medium (Life Technologies) at 37 °C. In 96-well titer plate, 100 μg of PLGA-GDNF nanoparticles were suspended in 20 mL medium and the solution was dispensed into each well. At predetermined times, 80 μL was collected from the well and the concentration was determined by a GDNF ELISA kit (R&D system).

2.3. Spinal cord contusion

Female adult SD rats (250 g ± 30) were anesthetized, and their spinal cord was exposed by laminectomy at the level of T9/T10. A 10-guage rod was dropped onto the laminectomized cord from a height of 25 mm (moderate SCI) and 50 mm (severe SCI) using a device developed at the New York University [6,7]. During surgery the rectal temperature was maintained at 37 °C using a thermostatically regulated heating pad, and bladder evacuation was then daily applied. Antibiotics (sodium ampicillin 80 mg/kg) were injected post-surgery. Animal care was provided in accordance with the *Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals proved by* Institutional Animal Care and Use Committee of National Cheng Kung University.

2.4. Injection of nanoparticles

The injection of 20 and 100 nm fluorescent labeled polystyrene latex nanospheres (Millipore) was performed right after SCI. A 5-μL Exmire micro-syringe with a 31-gauge needle was positioned at the midline of the L1 level and stereotaxically inserted 0.7–0.8 mm below the dura (Fig. 1B). The fluid containing 1 μL of fluorescent labeled nanospheres in the sizes of 20 nm (2×10^7 particles/μl/rat; $n = 3$) and 100 nm (4×10^8 particles/μL/rat; $n = 3$) in diameter were administered by intraspinal injection at the amount of 2×10^7 particles/rat. The injection of FBSA (400 ng/5 μL/injection; 800 ng/rat; $n = 3$), PLGA–FBSA (400 ng/5 μL/injection; 800 ng/rat; $n = 3$), PLGA-GDNF (2 μg/5 μL/injection; 4 μg/rats; $n = 6$), PLGA (5 μL/injection; 10 μL/rats; $n = 6$) was followed by the procedure as previously described [6,7]. Briefly, FBSA, PLGA–FBSA, PLGA-GDNF or PLGA nanoparticles were injected into approximately 1 mm rostral and caudal to the lesion epicenter (Fig. 3K). After each injection, the 31-gauge needle was maintained in the spinal cord for an additional 2 min to reduce the possibility of the leakage of the injected fluid from the site. Animals were then housed in pairs, and manually bladder evacuation was performed at least twice a day. In addition, the injured animals received antibiotics (sodium ampicillin, 80 mg/kg/day) daily for a week after SCI.

2.5. Evaluation of hindlimb locomotor function

As previously described [6], animals received either PLGA or PLGA-GDNF were weekly assessed for locomotor function by two blinded observers, using BBB hindlimb locomotor rating scale [23]. Locomotor activities were evaluated by placing animals for 4 min in the open-field with a molded plastic surface. Hindlimb locomotor recovery in animals was scored on the scale of 0 (no hindlimb movement) to 21 (normal mobility).

2.6. Tissue preparation

Experimental animals were perfused intracardially with 0.9% cold NaCl (400 ml/rat on average), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (500 ml/rat). Spinal cord tissues were removed, post-fixed in 4% paraformaldehyde overnight, and then cryoprotected in 30% (w/v) sucrose in PBS for one day. The cord (approximately 2 cm in length with the epicenter) was excised, embedded in Tissue Tek OCT (Miles), and then longitudinally sectioned at 20 μm thickness. The tissues were rinsed in PBS three times, and then subjected to nucleus staining using DAPI solution. Images were captured using a Nikon E-800 microscope equipped with a cooling CCD system.

2.7. Immunofluroescence

The staining was followed by the procedure as described previously [6,7]. Tissue sections were rinsed with PBS three times, and then incubated for 30 min with 0.1% Triton X-100 in PBS containing 5% normal goat serum to increase the permeability and reduce nonspecific binding. Primary rabbit antibodies, anti-neurofilament–200 kDa (NF; Sigma), anti-Iba1 (Wako), and anti-GFAP (Chemicon), were applied to tissue sections at the dilution of 1:200 overnight at 4 °C in a humidified chamber. Sections were rinsed three times with PBS followed by biotinylated secondary antibodies for 1 h and fluorescein–avidin for 45 min at room temperature, and finally subjected to nucleus staining using DAPI solution. The results were analyzed under a fluorescence microscope with cooling digital camera.

2.8. Astrocyte cell culture

Primary rat astrocytes were prepared as previously described [21]. Cerebral cortices from neonatal Sprague–Dawley rat brains (P1-2) were removed and carefully dissected. The tissue was dissociated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 0.0025% trypsin/EDTA and passed through a 70 μm pore nylon mesh (BD Biosciences). After centrifugation, the cell pellet was