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Plasmid-based gene therapy with hepatocyte growth factor stimulates peripheral nerve regeneration after traumatic injury

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ABSTRACT

Peripheral nerve injury remains a common clinical problem with no satisfactory treatment options. Numerous studies have shown that hepatocyte growth factor (HGF) exerts neurotrophic effect in motor, sensory, and parasympathetic neurons in addition to mitogenic, morphogenic, angiogenic, antiapoptotic, antifibrotic, and anti-inflammatory effect on various tissues and cells. In our study we examined efficacy of gene therapy with HGF-bearing plasmid (pC4W-hHGF) to improve consequences of traumatic nerve injury in mice.

Treatment by pC4W-hHGF led to restoration of nerve structure and functional recovery compared to similar parameters in control animals. Compound action potentials (CAP) in experimental groups treated with 100 or 200 μg of pC4W-hHGF demonstrated increased amplitude and latency decrease compared to spontaneous recovery control group. In HGF-treated mice histological analysis showed a three-fold increase in axon number in nerve portion located distal to the lesion site compared to control. Moreover, significant functional recovery of *n. peroneus communis* triggered by pC4W-hHGF gene therapy was observed using the footprints analysis. Obtained results provide evidence for plasmid-based HGF gene therapy as a potential treatment for traumatic injury of peripheral nerve.

1. Introduction

Peripheral nerve injury (PNI) has a prevalence of 3%–5% in patients with polytrauma [1,2] and may lead to long-term functional deficiencies significantly affecting quality of life and causing significant financial burden due to patient's disability [3]. Peripheral nervous system has certain potential for full regeneration after trauma in contrast to central nervous system known to have a very limited regenerative capacity [4,5]. However, in case of severe peripheral nerve injury functional recovery is often unsatisfactory and clinical intervention is necessary.

Hepatocyte growth factor (HGF) exhibits unique features that make it a promising agent for PNI treatment. Several studies showed that HGF and its receptor c-Met are expressed in peripheral nervous system cells [6] and in adult brain [7,8]. HGF/c-Met axis is involved in mitogenic, morphogenic, angiogenic and antiapoptotic effects in various kinds of cells and tissues [9,10]. Early studies have demonstrated that HGF functions as a guidance and survival factor in the developing nervous system being an essential component of muscle-derived support for motoneurons in development [11,12]. HGF receptor c-Met is expressed by Schwann cells as well as by peripheral sensory and motor neurons [13,14]. Both in vitro and in vivo there is substantial evidence that HGF is essential for peripheral sensory, sympathetic and motor neurons and enhances neuronal survival and axonal outgrowth [6,15–17].

Neuroprotective effects of HGF in diseases affecting central and peripheral nervous systems were demonstrated in several studies. Treatment by HGF in acute phase induces long-term neuroprotection and recovery from stroke via induction of proliferation and differentiation of neural precursor cell [18]. After optic nerve injury HGF promotes long-term survival and axonal regeneration of retinal ganglion cells [19]. Adenoviral transfer of HGF gene prevents death of injured adult motoneurons in a rat model of peripheral nerve avulsion

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[20]. Furthermore, non-viral HGF gene therapy by intramuscular injections in patients with painful diabetic neuropathy provided symptomatic relief with improvement in quality of life [21]. In addition, plasmid-based HGF gene therapy by intrathecal injection significantly attenuated pain induced by nerve injury in mice through direct inhibition of spinal cord microglia and astrocyte activation due to anti-inflammatory action of HGF [22].

Besides neuroprotective activity HGF is considered as the most promising factor for angiogenic gene therapy because it can stimulate angiogenesis without induction of inflammation and vascular permeability [23,24]. Recently two double blind placebo-controlled phase II clinical trials demonstrated that HGF plasmid-based gene therapy significantly improved primary end-points and tissue oxygenation in critical limb ischemia compared to placebo [25,26].

Basing on these findings we hypothesized that plasmid-based gene therapy by HGF may be a promising approach to treat traumatic PNI due to neuroprotective, angiogenic, anti-inflammatory and antifibrotic activity of HGF. In present study we report efficacy of gene therapy by non-viral gene delivery of human HGF to alleviate consequences of traumatic PNI in a mouse model of this lesion.

2. Material and methods

2.1. Animal strain and ethical approval

We used 9–10 week-old C57/Bl6 male mice (purchased from "Andreevka" animal husbandry facility, Russia) for nerve traumatic injury model. After acclimation animals received standard food and water ratios according to in-house rules of husbandry. All animals were narcotized by intraperitoneal injection of avertin (300 µl of 2.5% solution) before surgery. Euthanasia was conducted under isoflurane narcosis by secondary cervical dislocation. Surgical manipulations and euthanasia procedures were developed in compliance with national and European Union directives and were approved by the Institutional Ethics Board for Animal Care (*National Medical Research Center for Cardiology*; permit #385.06.2009).

2.2. Plasmid design and preparation

Mammalian expression plasmid vector pC4W as well as codon-optimized human HGF gene sequence have been described earlier [27]. All plasmids were amplified in *E. coli* (DH-5α strain), grown in LB medium and purified using EndoFree Plasmid Giga Kit (Qiagen, USA). Standard LAL-test was performed to assay pyrogenicity of isolated DNA; all tested samples did not exceed 10 EU/mg of plasmid DNA, which complies to manufacturer's range and Institute regulations for in vitro and animal tests.

2.3. Nerve injury model

Efficacy of gene therapy was evaluated in vivo in a model of unilateral traumatic crush injury of common peroneal nerve (*n. peroneus communis*). Common peroneal nerve innervates the toe extensor muscles, therefore its damage is accompanied by visible functional changes. Loss of ability to straighten hind limb fingers allows to assess degree of damage and the rate of subsequent nerve recovery.

Common peroneal nerve damage was induced according to a previously published protocol with minor modifications [28–31]. Animals were narcotized by intraperitoneal injection of 2.5% avertin as described above and surgery was performed under a binocular microscope. The left common peroneal nerve was isolated from surrounding tissue under sterile conditions and crushed for 20 s using 1 mm wide needle holder with silicone coated forceps approximately 2–3 mm distal from neural trifurcation [28,29,31,32]. Plasmid was administered by intramuscular injections as described below. The wound was sutured immediately after injury (catgut, 5–0) and skin was sterilized with antiseptic. After surgery the animals remained on a warm mat to avoid hypothermia until recovery.

2.4. Intramuscular plasmid delivery

Plasmid delivery method was developed in our previous studies using injections of pc4W-β-gal plasmid with different electroporation regimen [31,33]. In accordance to these results we have chosen 100 V/cm electroporation for plasmid delivery which provided with up to 10% of transfected muscle fibers and < 8% of necrotized fibers [31]. Amount of injected DNA was 100 or 200 µg per animal respectively with solution total volume of 100 µl. Dose was equally divided between two injection regions: 50 µl was administered by three intramuscular injections around the damaged nerve area, and the other 50 µl was injected into *m. tibialis anterior* containing the area of neuromuscular synapses of the damaged nerve. Needle of an insulin syringe was injected parallel to the longitudinal axis of the muscle and solution was slowly introduced into the muscle to avoid rupture of perimysium. Total of three experimental groups were formed:

1 Control group (empty pC4W plasmid, n = 9);
2 Experimental group HGF100 (plasmid pC4W-hHGF; 100 µg per animal, n = 15),
3 Experimental group HGF200 (plasmid pC4W-hHGF; 200 µg per animal, n = 6).

2.5. Detection of human HGF expression in explant cultures

After intramuscular delivery of plasmid we detected production of hHGF in mouse skeletal muscles. Samples of *m. tibialis anterior* from experimental mice were isolated at day 3 after injection of plasmid. Explant culture was prepared on Matrigel as previously described [34]. Briefly, *m. tibialis anterior* was excised, cut in half, plated on Matrigel and cultured in M199 medium supplemented by 2% FBS. After 3 days of culture conditioned medium was collected and HGF concentration was measured by ELISA (Quantikine hHGF ELISA Kit, R&D systems, Cat#SHG00).

2.6. Nerve recovery assessment

Nerve conduction velocity was analyzed at day 14 after surgery on isolated nerve as previously described with minor modifications [6,31]. Compound action potentials (CAP) were recorded in isolated common peroneal nerve of experimental and control mice. Before the CAP registration nerves were placed in Hanks' solution (HBSS) for 30 min. Pilot studies on intact nerves showed that immediately after extraction the amplitude and the latency of the potential varies greatly whereas after 30 min stabilization of all recorded parameters occurs and CAP profile remains unchanged up to 1.5–2 h. Recordings were performed in the bath filled with Hanks solution to ensure the optimal functioning of the nerve fibers in vitro. Aspirating recording electrode with a silver wire (diameter of 200 µm; chlorinated) was used with 1 mm distance between wires in the nichrome (Ni-Chr) stimulating electrodes. For stimulation of isolated nerves we used FHC Pulsar 6b. The nerve was affected by sequential monophasic pulses of 5 µs (frequency of 1 Hz). Action potentials were averaged from 10 potentials obtained in response to stimulation by 10 pulses with amplitude of 10 V. This value of potentials allows simultaneous activation of all fibers of the common peroneal nerve. Signal was recorded using an in-house made biopotential amplifier with a gain of 7500 fold and an integrated broadband filter with a range 300–7000 Hz. Additional filtration and amplification of the electrophysiological signal was carried out using an LPF-202 amplifier (Warner Instrument Company, USA). Analogue signal was digitalized on a USB 1208fs device (Measurement Computing Corporation, USA) that provided a sampling frequency of 50 kHz. Digitalized signal was processed using in-house software in the Mathlab