Biomedicine & Pharmacotherapy 101 (2018) 682–690

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

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

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ARTICLE INFO

Keywords:
HGF
Gene therapy
Nerve regeneration

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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https://doi.org/10.1016/j.biopha.2018.02.138
Received 21 November 2017; Received in revised form 26 February 2018; Accepted 26 February 2018

[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

programming environment. The following parameters of averaged potentials were determined: 1) latency period, 2) amplitude of the first peak, 3) time to reach the maximum value for the first peak, 4) peak-to-peak amplitude.

Axons number was evaluated in formalin-fixed frozen sections of common peroneal nerve using immunofluorescent staining and morphometric analysis as previously described [6]. Samples of nerve tissues were immersed in a freezing medium and frozen in vapor of liquid N_2. After preparation sections (7 μm thick) on glass slides were fixed in 4% paraformaldehyde supplemented by 0.2% Triton-X100; prior to GFAP immunostaining antigen unmasking solution was used. Slides were blocked by 10% normal donkey serum and incubated overnight with primary antibodies (anti-NF-H antibody, #8135, Abcam, UK; anti-GFAP antibody #556329, BD Pharmingen, USA; anti-CD11b, #101208, Biolegend, USA).After incubation with the primary antibody, the sections were stained with AlexaFluor®488-conjugated secondary antibody (#A21206, Thermo Scientific, USA) or with AlexaFluor594-conjugated secondary antibody (#A11032, Thermo Scientific, USA); all slides were counterstained with DAPI (Sigma-Aldrich). Staining was visualized on a fluorescent microscope Zeiss AXIO Observer A1 (Zeiss, Germany) fluorescent microscope. Obtained images were analyzed using ImageJ freeware. Microphotographs were taken under 200× magnification. Morphological evaluations were performed on Day 14 after surgery and following electrophysiological study.

Hind limb functional recovery was analyzed 1, 2, 6 and 13 days after surgery using peroneal function index (PFI) as described previously [6,15,31]. Among numerous ways to assess peripheral nerve recovery after an injury gait analysis is one of the most important, because it directly reflects the regeneration of the nerve. Common peroneal nerve is a branch of sciatic nerve after its separation in popliteal region. Its terminal branches innervate the anterior tibial muscle and extensors of fingers and thumb. Therefore, functional state of the sciatic nerve is directly related to integrity of the peroneal nerve. After injury foot print changes dramatically and it becomes narrow and long. To assess function recovery, we selected the SFI proposed by the Inserra et al. in 1998 for mice [34], taking into account the change in both – trace width and length. This formula, in our opinion, is more objective than PFI, based only on a change in the width of the track, thus, in this study we used SFI as parameter of functional state of peroneal nerve. Tracks were obtained and analyzed for each animal before surgery (Normal) and 13 days after it according to Medinaceli et al. [35,36]. The left hind limb of tested animal was moistened with ink and mouse was placed to a corridor with paper at the bottom. From obtained tracks the most accurate footprints were selected for analysis (4–6 pieces) and width of toe spread (TS) and print length (PL) were measured. To process the data obtained, the digitized tracks were analyzed and the corresponding ratios were calculated for each animal: NTS and ETS – normal toe spread and experimental toe spread, respectively; NPL и EPL – normal print length and experimental print length, respectively. The SFI was calculated using the formula proposed by Inserra et al.: SFI = 118.9 [(ETS-NTS)/NTS] − 51.2 [(EPL-NPL)/NPL] − 7.5.

2.7. Statistical analysis

Data is expressed as mean ± standard error of the mean (SEM) for each group. Statsoft “Statistica 8.0” was used for analysis of obtained data. Statistically significant difference between two groups was determined Mann–Whitney rank sum *U*-test depending on sample distribution profile. Multiple groups were compared using ANOVA with Bonferroni correction for level of significance where required. *P*-values less than 0.05 were considered indicative of significance.

3. Results

3.1. Human HGF is expressedin vivo after plasmid-based gene transfer

Prior to assessment of hHGF influence on nerve repair we evaluated expression of hHGF in explant culture of *m. tibialis anterior* as described in our previous work [36–38]. After gene delivery of human HGF we detected its production in explant culture of skeletal muscles injected with 100 and 200 μg of pC4W-hHGF on day 3 after plasmid administration. Maximal level of hHGF (1.1 ± 0.163 ng/ml) was detected in conditioned medium from explants of muscles injected with 200 μg of pC4W-hHGF. Explants of muscles injected with 100 μg of pC4W-hHGF showed lower content of hHGF (0.9 ± 0.3 ng/ml), but this difference was statistically insignificant ($p > 0.05$). This suggested that we have reached the relative “plateau” of hHGF production in skeletal muscle explants as far as no significant difference between 100 and 200 μg was found. In vehicle-injected muscle specimen and after empty pC4W transfection we failed to detect hHGF above lower limit of ELISA kit indicated as 125 pg/ml by manufacturer’s protocol. Obtained data on plasmid-based hHGF expression in muscles explants coincide with our earlier studies [27].

3.2. Restoration of nerve conductivity after pC4W-hHGF gene therapy

To evaluate effects of HGF gene therapy on restoration of nerve conductivity we analyzed responses of *n. peroneus communis* in 6 intact nerves («Intact» group), in 9 nerves isolated at 14 days after surgery without gene therapy («Control» group) and in 13 nerves from animals subject to HGF gene therapy (7 nerves from pC4W-hHGF100 group and 6 nerves from pC4W-hHGF200 group). Fig. 1A illustrates changes of spike profiles between study groups at 14 days after crush injury. Potential jump during a 5 μs monophasic electric stimulation of the nerve is clearly detectable in each average wave. Relative to this the Latency of CAP (Fig. 1B) and Time to maximal response (Fig. 1C) were measured. Mean value of CAP latency was 880.0 ± 141.84 μs for the HGF200 group, and 1032.0 ± 115.53 μs for the HGF100 group; both values were significantly shorter than in control group with a value of 1511.43 ± 125.27 μs (Fig. 1B; Mann–Whitney *U*-test).

Maximum value of the CAP amplitude (0.65 ± 0.02 mV) was recorded for intact nerves, and the minimum values (0.05 ± 0.01 mV) were observed in nerves from control group (Fig. 1A). Significant difference between intact and spontaneously regenerating nerves was also observed for CAP latencies (240.0 ± 13.66 vs. 1511.43 ± 125.27; $^{**}p = 0.003$, Mann–Whitney *U*-test). Profiles of CAP in experimental groups HGF100 and HGF200 demonstrated increase in amplitude and decrease in latency compared to the negative control group, however CAP characteristics of intact nerves were not achieved. Mean value of amplitude in HGF100 and HGF200 groups were comparable and accounted for 0.10 ± 0.01 mV ($^{*}p < 0.05$ for HGF200, $^{**}p < 0.01$ for HGF100 vs. control group, Mann–Whitney *U*-test) (Fig. 1D). Mean value of Peak-To-Peak amplitude in HGF100 (0.22 ± 0.03, mV) and HGF200 (0.21 ± 0.03, mV) groups were comparable as well ($^{*}p < 0.05$ vs. control group, Mann–Whitney *U*-test) (Fig. 1E).

Although the highest level of HGF secretion was detected in muscles injected with 200 μg pC4W-hHGF (See Section 3.1) we did not find any significant differences in recovery of nerve conductivity between two doses of HGF plasmid.

3.3. pC4W-hHGF enhance nerve structure recovery after crush injury

At 14 days after nerve injury and plasmid injection we observed improved restoration of peroneal nerve structure in hHGF-treated groups compared to control animals which were injected with pC4W empty vector. Using immunofluorescent staining against axon marker protein NF-H (alternatively named NF200) we have shown that crush nerve injury causessignificant damage followed by axonal degeneration

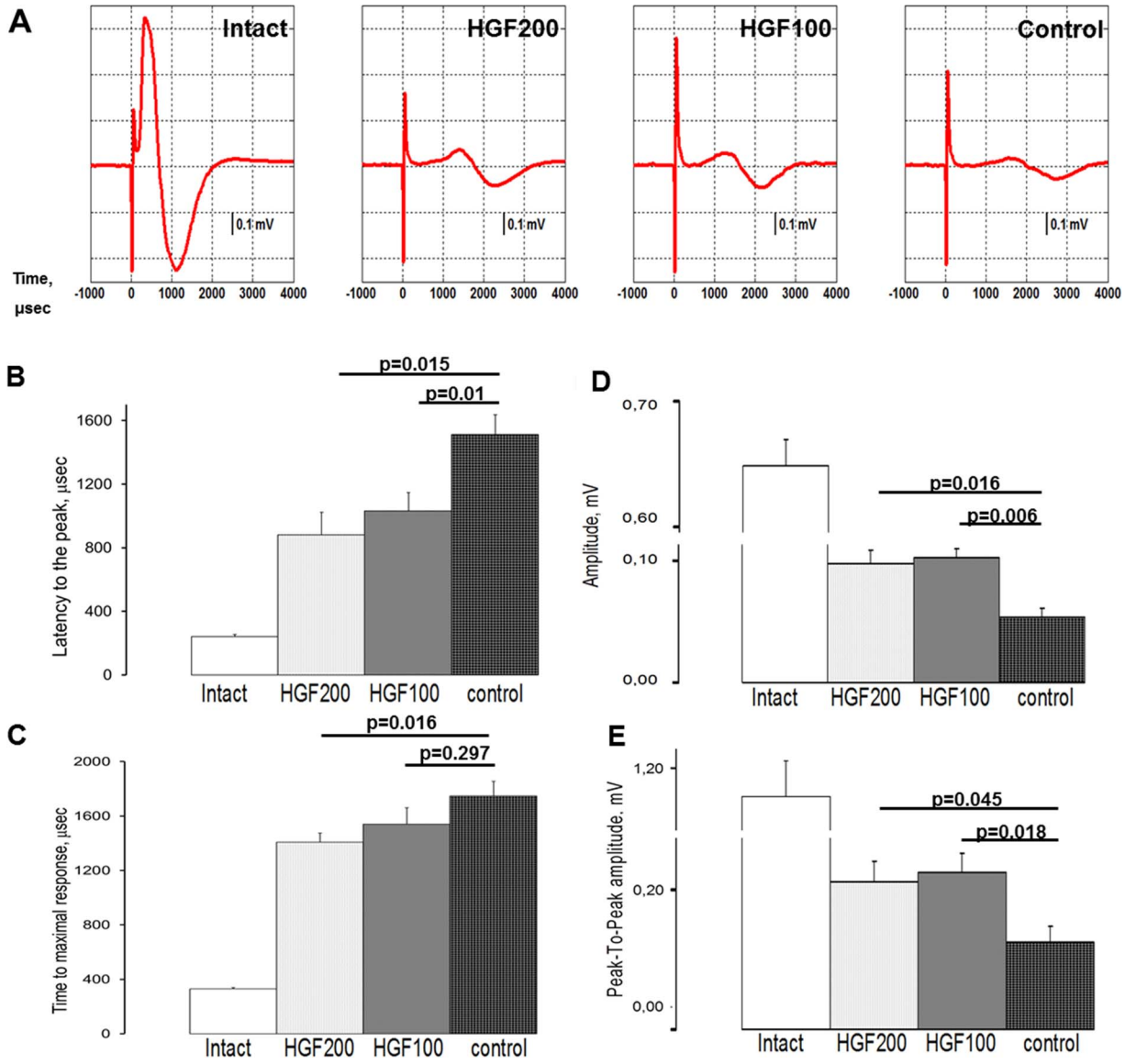

Fig. 1. Gene therapy by HGF restores CAP profiles after nerve crush injury.
A – Changes of CAP spike profiles 14 days after nerve crush injury in study groups compared to intact nerve CAP.
B – Latency to the peak of CAP (µs) was essentially lower in both experimental groups – pC4W-hHGF100 and 200 compared to control group.
C – Time to maximal response (µs) was significantly lower in HGF200 group vs. control group.
D, E – Amplitude of CAP and Peak-To-Peak amplitude (mV) were significantly higher in HGF100 and HGF200 groups compared to control group.
Data is presented as mean ± SEM, Mann–Whitney *U*-test.

distal to original lesion site.Injectionof 100 or 200 µg of pC4W-hHGF caused 3-fold increase of axon count in distal part of the nerve (29.16 ± 3.19 in HGF100 group (n = 9; $p < 0.001$) and 29.5 ± 3.76 in the HGF200 (n = 6; $p < 0.01$) vs. 9.17 ± 0.55 in control group, pcs/1000 µm^2) (Fig. 2 (A vs. B, C). Values in HGF100 and HGF200 groups were insignificantly higher compared to intact nerves, which had a density of 26.80 ± 1.34 pcs/1000 µm^2.Thus, no dose-dependent difference was observed; however, both doses used induced significant recovery of axonal density compared to empty pC4W-treated negative control animals.

3.4. Track profile analysis after HGF gene delivery

Hind limb function recovery was analyzed using sciatic function index (SFI) as described previously [6,16,30]. SFI was calculated using footprint analysis to compare values obtained before surgery vs. data obtained at days 1, 2, 6 and 13 of experiment. Intact animals usually land on the toes, which is clearly visible on the print (Fig. 3A). However, after nerve crush damage the track profile changes significantly indicating damage to the innervation of the toe extensor muscle (Fig. 3C).

As shown in Fig. 3B gene therapy by HGF has enhanced limb functional recovery in comparison with negative control group. SFI significantly improved in HGF-treated mice as early as on day 2 after injury (−32.56 ± 5.28 for pC4W-hHGF vs. −50.93 ± 5.52 for control; *p = 0.026); differences expanded both on the day 6 (−21.88 ± 3.53 pC4W-hHGF vs. −49.60 ± 9.28 for control; *p = 0.03) and day 13 after surgery (−18.38 ± 5.48 pC4W-hHGF vs. −42.16 ± 3.84 for control, **p = 0.01).

Obtained results indicate that plasmid-based HGF gene therapy efficiently restored traumatically injured nerve function. These results are of particular importance, since they directly reflect improvement of function in a limb with impaired nerve while electrophysiological and histological data have limited power to describe therapy outcome.

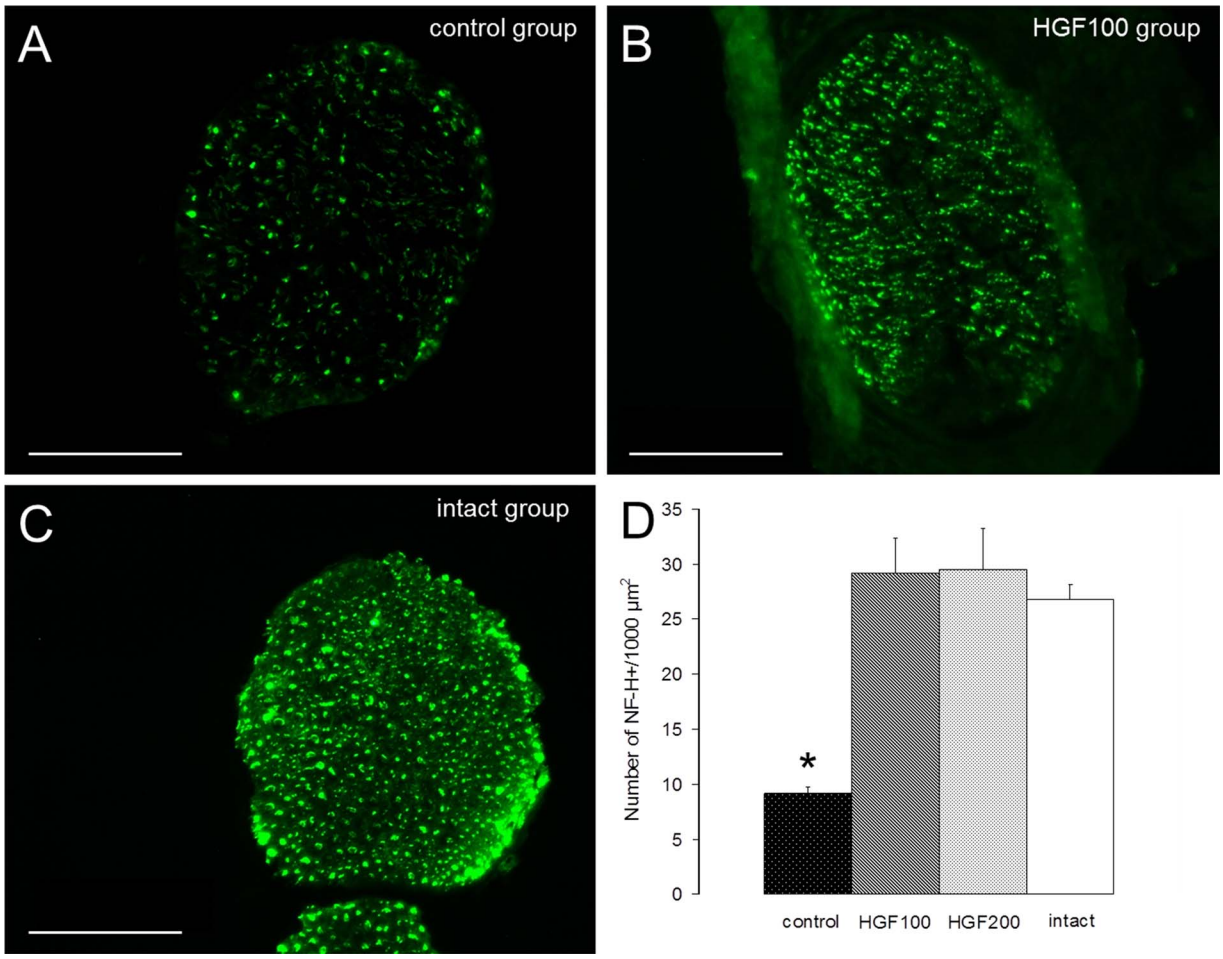

Fig. 2. HGF promotes nerve structure recovery after crush injury.
A–C: Cryosections of nerve stained with NF-H antibody 14 days after injury: A – control group (pC4W), B – HGF100 group (pC4W-hHGF, 100 µg), C – intact peroneal nerve.
D – Results of quantitative analysis of axons in peroneal nerve expressed as number of NF-H$^+$ fibers/1000 µm^2 of cross section. Crush injury followed by plasmid pC4W-hHGF injection essentially enhanced the number axons compared to control in both (100 µg and 200 µg) doses (*p < 0,001 vs. control).
Data is presented as mean ± SEM (Mann–Whitney *U*-test). Scalebar = 100 µm.

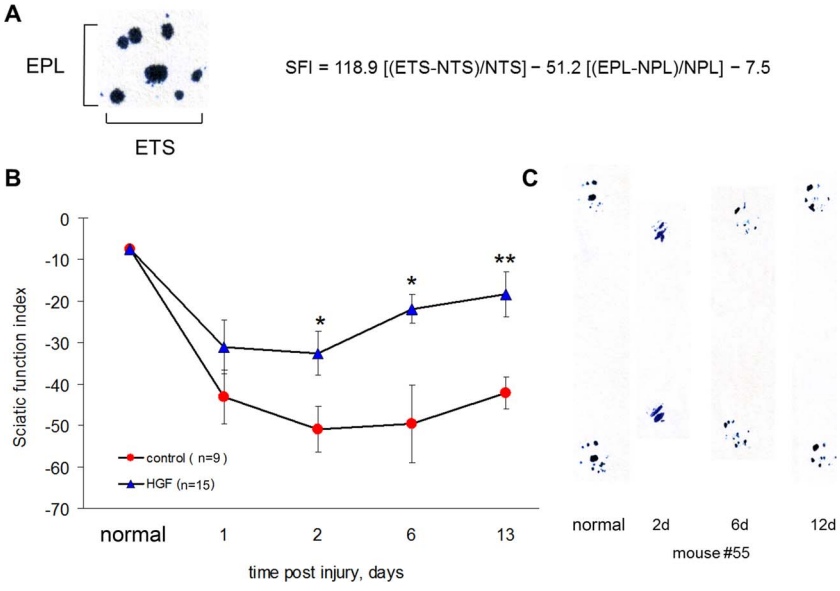

Fig. 3. Track profile analysis after HGF gene delivery reveals functional restoration of nerve after gene therapy.
A – Intact footprint and its parameters: experimental toe spread (ETS) and experimental print length (EPL). The equation proposed by Inserra to determine SFI in mice; NTS, NPL – Normal Toe Spread and Normal Print Length, respectively (intact animal).
B – Comparison of functional recovery determined by SFI analysis during 13 days after injury. Control mice showed significantly reduced recovery in comparison with HGF group at 2, 6 and 13 days post injury. Points of the curves represent group mean ± SEM, *p < 0,05; **p < 0,01, Mann–Whitney *U*-test. C – Walking tracks from the same control group mouse #55 obtained before injury and on the 2, 6 and 12 days post injury: two consecutive prints of the operated left foot presented for each time-point.

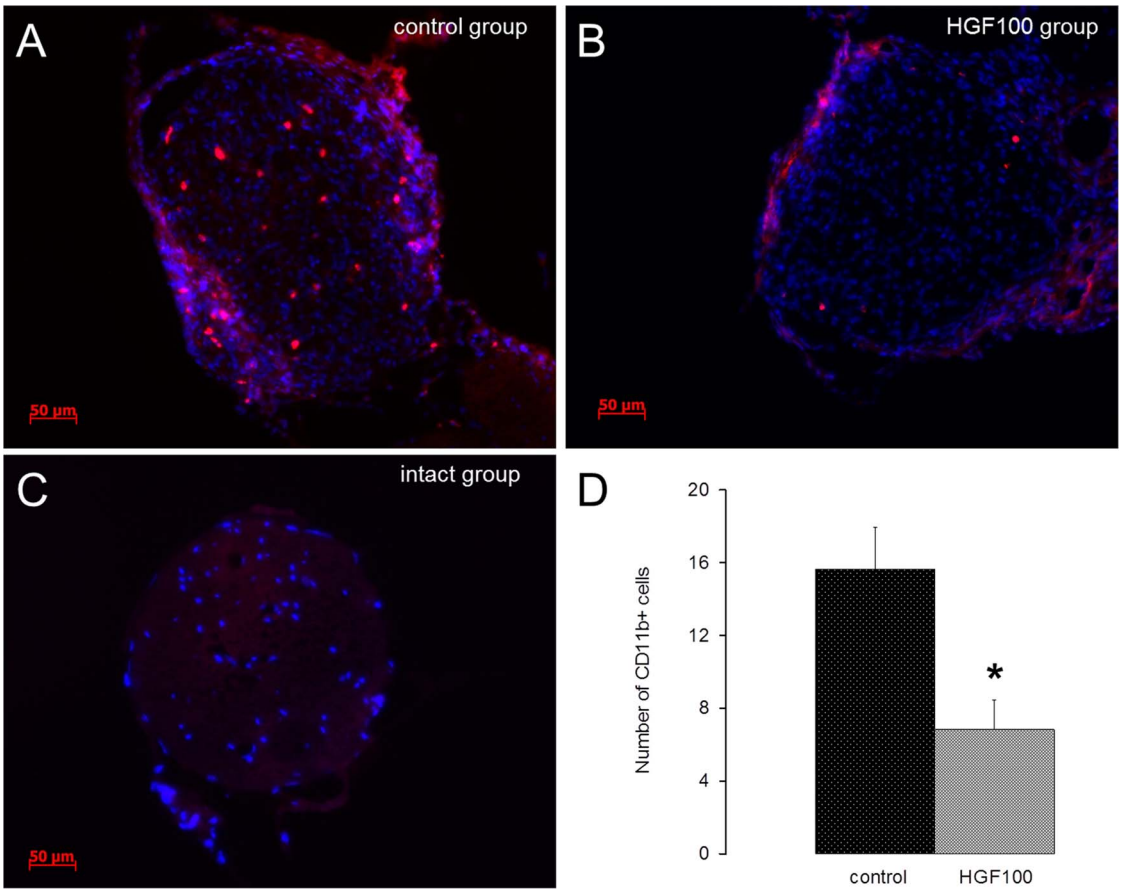

Fig. 4. HGF gene therapy reduces macrophage infiltration of*n. peroneus communis* after nerve crush injury.
Immunofluorescence staining of anti-CD11b (red) and DAPI (blue): A – control group (pC4W), B – HGF100, C – intact peroneal nerve. D – Evaluation of $CD11b^+$ macrophage counts in cross-sections of common peroneal nerve in control and hHGF100 (*p < 0,01 vs. control) groups. On the cross sections of the intact nerve $CD11b^+$cells were not detected (C). Data is presented as mean ± SEM (Mann–Whitney *U*-test). Scalebar = 50 μm.

3.5. pC4W-hHGF decrease inflammatory infiltration after nerve injury

To investigate possible mechanisms of beneficial action of HGF in peripheral nerve recovery we evaluated macrophage infiltration of injured nerve (Fig. 4). Using immunofluorescent staining against CD11b macrophage antigen protein we have shown that crush nerve injury results in significant infiltration of nerve by macrophages as in intact nerves we found no $CD11b^+$-cells. At 14 days after nerve injury and plasmid injection we observed reduced infiltration by $CD11b^+$-cells in sections of peroneal nerve from HGF-treated animals compared to empty plasmid control. Injection of 100 μg pC4W-hHGF resulted in 2-fold reduction of macrophage count in the distal nerve part by day 14. In HGF100 group (n = 9) number of $CD11b^+$ cells was 6.83 ± 1.62 vs. 15.67 ± 2.26 pcs/1000 μm^2 in pC4W-treated control (Fig. 4A vs. B, C; p < 0.01).

3.6. HGF gene delivery decreases amount of non-myelinating Schwann cells in damaged nerves

Nerve injury is accompanied by demyelination of SCs via autophagic process contributing to axonal degradation [39]. Using immunofluorescent staining of peripheral nerves against GFAP – a marker glial protein of non-myelinated SCs we have shown that crush nerve injury causes anatomical damage and increase of non-myelinated SCs number compared to intact nerve (Fig. 5). However, injection of 100 μg pC4W-hHGF resulted in 3-fold decrease of $GFAP^+$ stain area reflecting non-myelinated SCs in the distal nerve part accounting for 2.74 ± 0.29% of GFAP-positive area in HGF100 group (n = 9) vs. 9.18 ± 0.59% of GFAP stain area in section in control group (Fig. 5; *p < 0.01 vs control group).

4. Discussion

Traumatic nerve injury model used in present study closely resembles axonotomy when nerve fibers are damaged while connective tissue sheath of the nerve remains intact. This type of damage destroys nerve fibers distal to the injury site and nerve conduction is blocked but subsequent axonal re-growth over the site of injury provides gradual restoration of nerve conductivity.

Peripheral nerve injury (PNI) leads to rapid and robust expression and secretion of neurotrophic and growth factors to guide and support regenerating axons. Expression of HGF in situ was chosen as a method to enhance natural response by additional growth factors supply at the earliest stages of regeneration. In our study we expressed HGF in skeletal muscle by direct intramuscular injection of a plasmid bearing human HGF. The rationale behind this is data of numerous studies indicating HGF to exert neurotrophic effects in motor, sensory, and parasympathetic neurons in addition to well-known mitogenic, morphogenic, angiogenic, antiapoptotic, antifibrotic and anti-inflammatory effects in various tissues and cells [40–44]. Due to this pleiotropic activity HGF is involved in many biological functions, such as organ regeneration and angiogenesis [25].

First part of our study demonstrated that single intramuscular injection of pC4W-hHGF was an effective way to express human HGF in murine skeletal muscle. We detected significant amount of human HGF in explanted muscle samples isolated as early as 72 h after electroporation-supported pDNA delivery which is in accordance with out previous data. This early expression of HGF is a feasible way to support

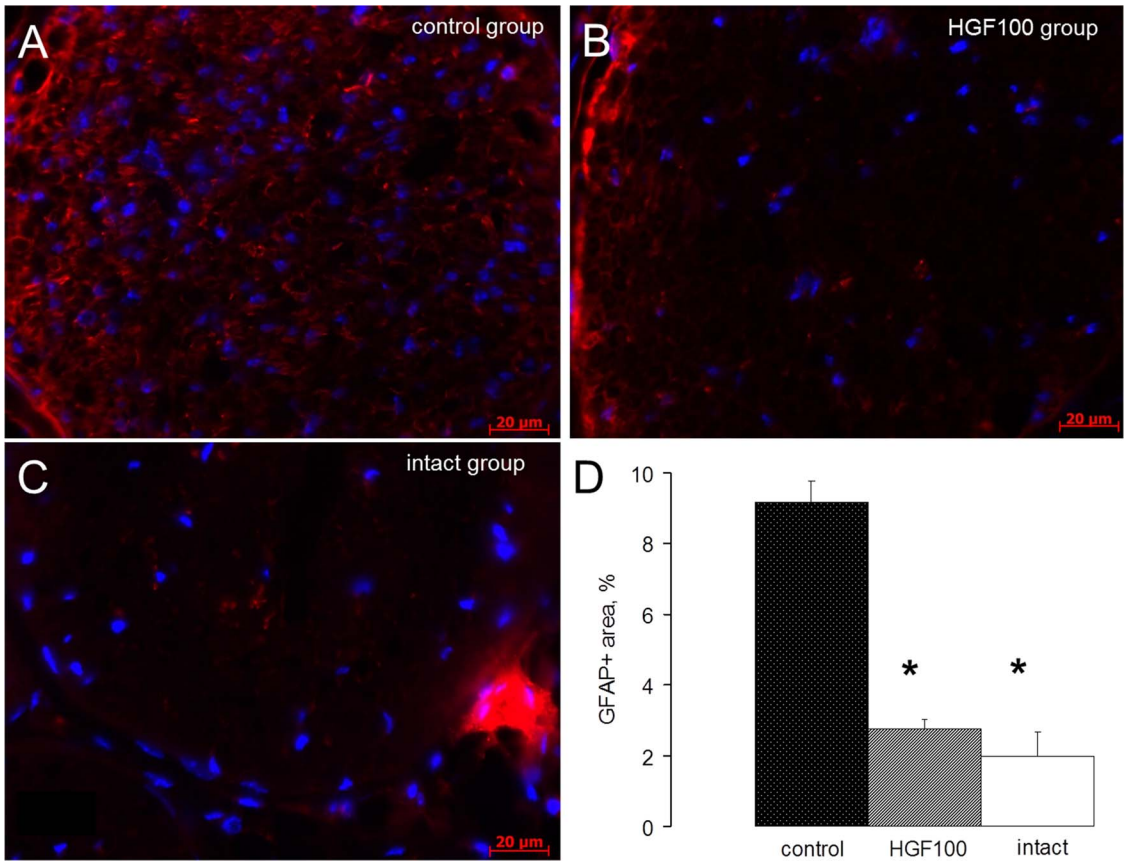

Fig. 5. Gene therapy by HGF reduces prevalence of GFAP$^+$ cells in sections of *n. peroneus communis* after crush injury.
Representative cross sections of common peroneal nerve immunostained against GFAP (red); nuclei are counterstained by DAPI (blue): A – control group (pC4W), B – HGF100 group, C – intact peroneal nerve. D – Quantitative analysis of myelinating cells using relative area of GFAP$^+$ as a percentage from total area of nerve cross-section, *$p < 0.01$ vs. control). Data is presented as mean ± SEM (Mann–Whitney *U*-test). Scalebar = 20 μm.

increase of HGF production naturally occurring after injury and we expected HGF to induce substantial improvement of axonal regeneration. HGF expression in skeletal muscle of murine hind limb stimulates of functional repair of crushed nerve and acceleration of its structural recovery.

Another important point for us was the dose of plasmid DNA to be administered. We finally adopted 100 μg delivery as the optimal dose due to the following reasons. First, in muscle explants we found no significant difference in HGF production after delivery of 100 or 200 μg. Second, we found no dose-dependent enhancement of beneficial effects in electrophysiological evaluations (Fig. 1). Finally, axonal counts after delivery of 100 or 200 μg of HGF-coding DNA were similar (Fig. 2) indicating lack of dose-dependent effect on structural recovery of nerve fiber after crush. Thus, following the idea of toxicity reduction (DNA is known to induce macrophage and other immune cells via toll-like receptor activation) we chose 100μg in the rest of experiments. However, our conclusion regarding the dose is not exhaustive as far as we performed a proof-of-concept study and optimal dosage and administration regimen are yet to be defined in a pre-clinical study. Plasmid DNA has an advantage compared to viral vectors that it can be administered several times without risk of inactivation of virus by immune system or induction of immunotoxicity.

Observed therapeutic effects may be attributed to neurotrophic actions of HGF exerted in different neuron types and Schwann cells (SCs). The latter are known to have a critical role in peripheral nerve regeneration [45] and after peripheral nerve injury sequential reactive changes occur in SCs phenotype, including de-differentiation, proliferation and migration. This intricate phenotypic conversion of SCs into repair-type cells is essential for regenerative potential of peripheral nerves. Axonal regrowth is to large extent stimulated by neurotrophic factors and surface proteins released by activated SCs.

Interestingly, injury-induced change of SCs behavior and their migratory and proliferative potential resemble epithelial-mesenchymal transition (EMT) occurring in tumors [46,47]. Recently it has been shown that cooperative influence of HGF and neuregulin induces SCs migration and proliferation during peripheral nerve repair [48]. Migratory features of activated SCs depend on expression of a scaffolding oncoprotein Grb-2-associated binder-2 (GAB2) whose injury-induced activation via tyrosine phosphorylation is regulated by an EMT signal, namely the HGF/c-Met signaling axis [48].

SCs are also known to regulate the immune response after peripheral nerves injury that leads to macrophage recruitment after glial release of various chemoattractants such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) [49–51]. Recent studies have shown that interaction of fibroblasts with SCs and behavior of macrophages and endothelium at the nerve injury site play a critical role for successful nerve repair [52–54]. Taking pleiotropic effects of HGF into consideration one can suggest that increase of HGF production in skeletal muscle innervated by injured nerve can promote axon regeneration by different ways. They can include direct SCs activation by HGF binding to c-met and subsequent activation of signaling cascade leading to upregulation of c-Jun expression in SCs inducing their phenotype conversion [45,55–57]. Moreover, HGF can counterbalance macrophage accumulation by suppression of MCP-1 expression in glia and endothelium preventing excessive inflammation. Finally, HGF directly stimulates angiogenesis which is crucial for proper nutrition of regenerating nerves and muscle suffering from lack of stimuli after conduction block [57].

Up to date in clinical settings plasmid-based HGF gene therapy has found its niche in patients with critical limb ischemia [25,26] or painful

diabetic neuropathy [21] confirming long-term safety and efficacy of this therapy. However, we believe that this field of application does not limit potential use of HGF as a well-known pleiotropic growth factor with numerous modes of action in different tissues and cell types. Main obstacle remaining for gene therapy is lack of effective and safe viral delivery methods thus we chose plasmid delivery as the safest gene transfer method. Results of present study provide evidence for use of plasmid-based HGF gene therapy to treat traumatic injury of peripheral nerve. Taking into account that plasmid-based gene therapy has a long story of clinical use and a good safety profile we may expect translation of this method for treatment of a disabling lesion with no effective pharmacological cure in sight.

Acknowledgement

This study was supported by Russian Science Foundation (RSF Grant no. 16-45-03007).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.02.138.

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