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Effect of controlled co-delivery of synergistic neurotrophic factors on early nerve regeneration in rats

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ABSTRACT

Present interventions to repair severed peripheral nerves provide slow and poor early axonal regeneration, which may cause unsatisfactory functional reinnervation. To improve early axonal regeneration in a 10 mm rat sciatic nerve gap model, we developed collagen nerve conduits loaded with the synergistically acting glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF). For controlling the concomitant GDNF and NGF release, the collagen tubes were cross-linked by a dehydro-thermal treatment (110 °C; 20 mbar; 5 days) prior to impregnating the tubes with GDNF and NGF and by coating drug-loaded tubes with layers of poly(lactide-*co*-glycolide). The conduits made of cross-linked collagen released low initial amounts of GDNF and NGF (2% of both during first 3 days) and enhanced significantly the early (2 weeks) nerve regeneration in terms of axonal outgrowth and Schwann cell migration in a 10 mm rat sciatic nerve gap model, as compared to the conduits made of non-cross-linked collagen releasing higher initial amounts of GDNF and NGF (12–16% within 3 days), or those releasing GDNF alone. The enhancement of early axonal regeneration using controlled co-delivery of multiple synergistic neurotrophic factors is an important requisite for eventually establishing functional connections with the target organ.

1. Introduction

Peripheral nerve injuries are steadily increasing in number, with functional recovery remaining impaired [1]. About 300,000 people are affected annually in Europe alone [2,3]. Most commonly used treatments include end-to-end suturing and autologous nerve grafting [4], although both of these procedures have their drawbacks. For example, end-to-end suturing of nerve stumps may lead to reduced stretching capacity (up to 24%), which was ascribed to changes during Wallerian degeneration, intra- and perineural fibrosis, and tissue adhesion [5]. Autologous nerve grafting is the current gold standard for the repair of nerve gaps not amenable to direct coaptation, although it is associated with morbidity at the donor site, increased scar formation at the recipient site, and sometimes unsatisfactory clinical results [6]. These disadvantages and the limited availability of donor nerves motivated the development of artificial biodegradable nerve conduits (NC).

Artificial NC should create a favorable environment for regenerating axons and support the cellular growth and migration by providing guidance and protection from the surrounding tissue [7]. Artificial NC should ideally possess several functionalities. NC must possess sufficient mechanical strength for suturing to the nerve stumps and for remaining intact for some time after implantation [8,9]. After completion of their guiding function, the NC should degrade within reasonable time [8]. Adequate mechanical and biodegradation properties are provided, for example, by collagen or aliphatic polyesters, which are common materials for NC production. Collagen also supports cellular invasion [10]. The suitability of collagen-based NC for the repair of peripheral nerve defects has been demonstrated in numerous preclinical and clinical experiments [3,4,10–14], which led to the approval by the FDA for use in humans. Nonetheless, neuroma formation occurred in a human median nerve (2 cm gap) bridged by an FDA-approved Neuragen® nerve guide [15]. Amongst the several factors that may have caused the neuroma formation, slow and poor early axonal regeneration may have played a key role. Following injury, the distal part of the nerve undergoes Wallarian degeneration (within 10–20 days), thereby forming columns of Schwann cells (bands of Bunger) that serve as guiding structure for the regenerating axons. If early axonal regeneration is delayed, the distal structures degenerate leaving the axons without guidance to the peripheral targets. Thus, there is

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a great need to enhance the rate and quality of early axonal regeneration over critical nerve gaps to improve repair of severed peripheral nerves.

To improve the biological performance of synthetic NC, several additional functionalities have been introduced into the devices, which can be grouped into topographical and trophical support. Especially trophical elements such as neurotrophic factors (NTFs) and Schwann cells have frequently been combined with NC. Embedment of NTFs into NC has not generally improved adequately the functional outcome of nerve regeneration, which was ascribed to aberrant axonal growth resulting in mismatched connections between the nerve cells and their peripheral targets [16]. The limited success of such combination products may be attributed to a variety of formulation factors, amongst which: (i) inadequate NTF doses; (ii) inadequate release kinetics, in particular a high initial release, (iii) inadequate location of NTF delivery, (iv) use of single growth factors rather than multiple factors occurring naturally. In general, NTFs support neuronal survival and axonal elongation [17–19], although different NTFs exert distinctive activities. For example, nerve growth factor (NGF) promotes primarily survival and axonal outgrowth of sensory neurons both *in vitro* and *in vivo* [20], while glial cell line-derived neurotrophic factor (GDNF) is one of the most protective factors for motor neurons [21]. Fine and co-workers reported that single factor treatment of GDNF or NGF supports sciatic nerve regeneration [22]. Single factor treatment may, however, not be optimal as nerves generally comprise different neuronal subpopulations that depend on different NTFs. For example, dorsal root ganglions of the rat contain different subpopulations of neurons, which depend on either GDNF (65%) or NGF (35%) alone, or on both factors (9%) [23]. This emphasizes the need to evaluate not only individual growth factors, but also combination of growth factors for improving the repair of peripheral nerves. We have recently observed the synergistic effect of GDNF and NGF on axonal outgrowth from chicken embryonic dorsal root ganglions [24]. This elicited our interest in co-delivery of GDNF and NGF from a NC for bridging an experimental nerve gap in the rat and promoting nerve regeneration. For this objective, we developed PLGA-coated collagen NC for the controlled release of GDNF alone or the concomitant release of GDNF and NGF. Release kinetics was controlled by physical cross-linking of collagen and integrating poly(lactide-*co*-glycolide) as release controlling barrier. The present study evaluated the controlled *co*-delivery effect of GDNF and NGF on early peripheral nerve regeneration in a 10 mm rat sciatic nerve gap model.

2. Materials and methods

2.1. Materials

Insoluble bovine collagen (Microfibrillar Collagen Hemostat, Avitene) was purchased from BARD (Oberrieden, Switzerland), and poly(lactide-*co*-glycolide) (PLGA, Resomer RG 503) from Boehringer-Ingelheim (Ingelheim, Germany). Human recombinant glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) were kindly provided by Amgen (Thousand Oaks, CA, USA) and Genentech (South San Francisco, CA, USA), respectively. Fertilized white chicken eggs were purchased from Eier Hungerbühler (Flawil, Switzerland). Buffer salts, solvents, and polysorbate 20 (Tween 20) were from Fluka (Buchs, Switzerland). Antibodies and reagents for ELISA were from R&D Systems (Minneapolis, MN, USA). Unless specified otherwise, all reagents for cell culture were from Invitrogen (Basel, Switzerland). Prolene® 6–0 suture thread was from Ethicon (Norderstedt, Germany).

2.2. Preparation of collagen nerve conduits loaded with neurotrophic factors

Collagen tubes, which constituted the main support structure of the NC, were fabricated by spinning mandrel technology, as illustrated previously [25]. Insoluble collagen (2.5%, w/w) was swollen in 1 M acetic acid and homogenized with a high-speed mixer at 10,000 rpm (Polytron®, Kinematica, Lucerne, Switzerland) for 1 min. The homogenous collagen dispersion was applied via a syringe onto a spinning gold-coated mandrel (diameter of 1.5 mm), installed in a sideways-reciprocating apparatus, and the solvent was dried off under laminar air flow. The resulting tubes were neutralized by incubation in 0.1 M di-sodium hydrogen phosphate (pH of 7.4) for 1 h. The tubes were finally cut into 14 mm long specimens [26]. Some of the specimens were cross-linked by physical means, i.e. by subjecting the collagen tubes to a dehydro-thermal treatment (DHT) at 110 °C and 20 mbar for 5 days [27].

Solutions of GDNF alone or of GDNF plus NGF in citrate buffer (pH 5.5) were carefully pipetted onto the distal part of the 14 mm long collagen tubes (at 3 mm from distal end of NC), previously cross-linked or not by DHT. The total amount of uploaded growth factors was 80 ng, i.e., 80 of GDNF alone, or 40 ng of NGF + 40 ng of GDNF. Water was removed by air-drying under laminar air flow. The tubes were finally coated with layers of PLGA to prevent the release of GDNF and NGF towards the exterior environment of the NC. For the coating, 4.8 ml of PLGA50:50 in ethyl acetate (RG 503, 5%, w/w) were sprayed over a total length of 90 mm of pre-cut collagen tube re-installed on a spinning and sideways-reciprocating Teflon-coated mandrel; the spraying was performed with an ultrasonic spray nozzle at a rate of 7.5 ml/h, and an ultrasonic energy of 1.6 W (USI, Lechler, Metzingen, Germany). The solvent was evaporated under controlled conditions (RT, 40% relative humidity, laminar air flow of 0.4 m/s). In the final step, the NC specimens were swollen in 50% ethanol, re-cut into 14 mm long specimens, as defined previously, removed from the mandrel, and subjected to final drying for 12 h under laminar air flow. According to the treatment of the collagen NC (C–NC) by DHT and their loading with NTFs, the NC will henceforth be referred to as C–NC, C–NC(GDNF), C_{dht}–NC(GDNF), C–NC (GDNF + NGF), C_{dht}–NC(GDNF + NGF).

2.3. Experimental groups and surgical implantation of nerve conduits

All *in vivo* protocols were approved by the local veterinary commission of the canton of Vaud (Lausanne, Switzerland) and carried out in accordance with the European Community Council directive 86/69/ECC for the care and use of laboratory animals. Twenty male Sprague Dawley rats weighing 250–300 g (Elevage Janvier, France) were housed under standard temperature and light conditions, with food and water being provided *ad libitum*. To create an artificial 10 mm gap of the sciatic nerve, rats were anesthetized with isoflurane (Baxter, Arovet, Zollikon, Switzerland). The rats' backs and left hind limbs were shaved and locally disinfected with a 25% betadine/0.9% NaCl solution. All surgical procedures were performed under a surgical microscope (Zeiss, Gottingen, Germany). The left sciatic nerve was exposed through a gluteal muscle-splitting incision and transected to create the 10 mm nerve gap. The proximal and distal nerve stumps were inserted with a length of 2 mm into the 14 mm long conduit and secured by a single epineural suture (9/0 Prolen, Ethicon), thus producing the 10 mm nerve gap. Muscles and fascia layers were closed with single stitches (4/0 Softcat, Braun), followed by continuous running suture (4/0 Prolen, Ethicon) to close the skin. Prior to implantation, the NC were hydrated and disinfected by successive incubation in 0.9% NaCl for 15 min, 70% aqueous ethanol for 30 s, and 0.9% NaCl for 15 min. For treatment, the animals were randomized into four groups of 5 animals/group: (1) animals treated with NTF-free C–NC to bridge the artificial 10 mm sciatic nerve gap (control group); (2) animals treated with C–NC(GDNF); (3) animals treated with C–NC(GDNF + NGF); (4) animals treated with C_{dht}–NC(GDNF + NGF).

2.4. Explantation of nerve conduits and tissue preparation

Animals were sacrificed at two weeks post surgery by CO_2-chamber euthanasia. Implants were retrieved with proximal and distal nerve stumps. Harvested conduits were fixed for 1 h in 4% paraformaldehyde/1% glutaraldehyde solution and subsequently transferred into phosphate-buffered saline. The specimens were then embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) for cryosectioning.

2.5. Immunohistochemistry and microscopy

Longitudinal sections of 18 μm thickness were obtained by cryosectioning the explant (Cryostat, Leica CM 1850, Meyer Instruments, Houston, TX, USA) on glass slides (Superfrost® plus, Menzel-Gläser, Braunschweig, Germany). Serial sections were collected, and every 2nd section was processed for single staining of either PGP9.5, a marker for regenerating axons, or S100, a marker for migrating Schwann cells. First, tissue sections were washed thrice with PBS and incubated with 0.5% casein blocking solution for 1 h at room temperature. The specimens were then incubated with primary antibody (anti-PGP9.5 and anti-S100 IgG from rabbit; diluted at 1:500 with blocking solution) at 4 °C overnight. The sections were washed again thrice with PBS and incubated at room temperature for 40 min with Alexa Fluor 488-labelled goat anti-rabbit IgG (diluted at 1:500 in blocking solution). Washes were repeated as before, and slides mounted with Vectashield DAPI for microscopic analysis (Vectorlaboratories, Servion, Switzerland).

Transverse cross-sections of 4 μm thickness were obtained by cryosectioning, as described before, and serial sections were taken at 4 mm distance from the proximal nerve stump. Sections from each specimen were stained for NF200, a marker for axons, and MBP, a marker for myelinated axons. Incidentally, in this experiment, the staining for NF200 produced a more distinctive pattern than the PGP9.5-staining used above in the single marker staining of the longitudinal sections. First, tissue sections