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Synergistic effects of NGF, CNTF and GDNF on functional recovery following sciatic nerve injury in rats

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ABSTRACT

Purpose: To investigate the synergistic effects of nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and glia cell line-derived neurotrophic factor (GDNF) on survival and growth of sensory neurons and motoneurons, as well as on the functional recovery following sciatic nerve injury in rats.

Methods: Experimental rats and neurons were randomized into 8 groups: NGF group, CNTF group, GDNF group, NGF+CNTF group, CNTF+GDNF group, NGF+GDNF group, NGF+CNTF+GDNF group and control group. Each group received local intramuscular injection of indicated NTFs according to the treatment protocol. The sciatic nerve function index (SFI), nerve conduction velocity and wet weight recovery rate of gastrocnemius muscle were tested to evaluate the functional recovery in vivo. A 2 (presence or absence of NGF) x 2 (presence or absence of CNTF) x 2 (presence or absence of GDNF) analysis of variance (ANOVA) was used to examine the main effects and interactions among NGF, CNTF and GDNF, and one-way ANOVA was calculated for multiple comparison.

Results: NGF and GDNF acted significantly on the survival of sensory neuron and motoneuron, respectively. CNTF was a dominant factor promoting cell body development, and GDNF had the most powerful effect on neurite outgrowth and elongation of sensory neurons and motoneurons. Combined administration of the three factors resulted in optimal functional recovery following sciatic nerve injury in rats.

Conclusions: It is demonstrated that differential and complementary biological effects of various neurotrophic factors contribute to synergistic promotion of nervous function recovery.

Key words: neurotrophic factor; sensory neuron, motoneuron, sciatic nerve, synergistic effect; functional recovery

INTRODUCTION

Peripheral nerve injury is a common disease with a high incidence rate. Lack of neurotrophic factors (NTFs) in axonal retrograde transport following peripheral nerve injury is one of the factors causing pathological changes and death of related segmental neurons. Since exogenous neurotrophic factors support the survival of neurons [1,2], their application has become an important method to improve therapeutic effects on nerve injury [3,4]. Increased expressions of multiple neurotrophic factors following peripheral nerve injury are locally detected [5], suggesting that peripheral nerve regeneration involves various neurotrophic factors. Previous studies mainly focused on the effect of mono factor on functional recovery of peripheral nerves [6-8] and only a few reports involved combined application of two neurotrophic factors [9-11]. Additionally, application of three factors in combination was only used in studies on neuronal differentiation [12]. Most NTFs belong to one of the three major NTF families, including the neurotrophin, neuropoietic cytokine, and glial cell line-derived neurotrophic factor families [13]. NGF is a typical target-derived neurotrophic factor, CNTF belongs to the neurocytokine family, and GDNF originates from the glia cell line-derived neurotrophic factor family. In the present study, rat dorsal root ganglion sensory neurons and ventricolumna motoneurons following isolated culture *in vitro* were used to investigate synergistic effects of NGF, CNTF and GDNF on survival and growth of neurons.

Moreover, the rat sciatic nerve injury model *in vivo* was applied to evaluate functional recovery following sciatic nerve injury in rats.

MATERIALS AND METHODS

Animals

In the present study, Sprague-Dawley rats with 15-day gestation and adult healthy Sprague-Dawley rats were provided by the Experimental Animal Center, Daping Hospital, Third Military Medical University (Chongqing, China). The animals consisted of males and females weighing 180-220 g in the same quantity. They were housed in individual cages in a temperature and light-dark controlled environment with free access to food and water. The rats were anaesthetized with 40 mg/kg Pentobarbital sodium and autonomous respiration resumed throughout the procedure. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Experimental Animal Center, Daping Hospital, Third Military Medical University. This study was approved by the ethics committee of the Third Military Medical University.

Cell culture

Embryo dorsal root ganglions (DRGs) and ventral spinal tissues were separated from Sprague-Dawley rats with 15-day gestation under sterile condition for culture of sensory neurons and motoneurons. DRGs and ventral spinal tissues were treated with a digestion mixture of 2.5 g/L trypsinase and 5.0 g/L collagenase (Sigma) at 37℃ for 30 min, and the treatment was terminated by administration of 20% fetal bovine serum (FBS; Hyclone). Dissociated cells were washed by centrifugation (5 min at 1000 r/min) and resuspension in Neurobasal (Gibco) medium, placed on 1.0×1.0 cm^2 coverslips pre-coated with poly-L-lysine(100 g/L) and laminin (20 mg/L) at a density of 5×10^5/ml, maintained in Neurobasal medium containing 2% B27, and then stored into the 5% CO_2 incubator (37℃, saturation humidity). Cytosine arabinoside with final concentration of 10^{-5} M was added to eliminate nonneuronal cells after 24 hours, and was removed after 48-72 hours. Half of the medium was replaced every 2-3 d.

Preparation of the sciatic nerve injury model

After experimental rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), a dorsal vertical incision on left hind limb was made under sterile condition and blunt dissection was performed between biceps femoris and vastus lateralis. The sciatic nerve was transected 5mm away from the lower edge of musculus piriformis, and a segment of 5 mm was removed. The proximal and distal nerve stumps were sutured into opposite ends of a 10 mm sterile silicone tube (inner diameter, 1.6 mm; outer diameter, 2.4 mm), leaving an interstump gap of 6 mm.

Treatment protocol

Neurons in culture and experimental animals were randomized into 8 groups in regards to different means of combination treatment between NGF (Sigma), CNTF and GDNF (PeproTech EC Ltd): NGF group, CNTF group, GDNF group, NGF+CNTF group, CNTF+GDNF group, NGF+GDNF group, NGF+CNTF+GDNF group and control group. Each group included 5 samples. For cell samples, neurotrophic factors were directly added to the medium composed of neurobasal medium and 2% B27 to finalize concentrations of NGF, CNTF and GDNF to 50, 20 and 50 ng/ml, respectively. No neurotrophic factor was added to the medium for the control group. For animal experiments, 100 μl medical chitosan gel was injected intraoperatively from the distal end to the silicone tube, and 5 μl of each neurotrophic factor was added through a microinjector according to the grouping protocol. In addition, each group except control group received intramuscular injection (below biceps femoris) of neurotrophic factors 100 ng/kg once a day in the following first month post operation. The same quantity of normal saline was used in the control group.

Assessment of cell growth

Count of NSE- and ChAT- Positive neurons

After culture of neurons for 7 days, the coverslips were removed and then fixed with 4% paraformaldehyde. Dorsal root ganglion sensory neurons were labeled with anti-NSE antibodies and spinal motoneurons were stained with anti-ChAT antibodies using the immunocytochemical technique. NSE- and ChAT-positive cells were counted in 10 randomly selected fields under light microscope.

Quantitative analysis of neuron morphology

Under the inverted phase contrast microscope, 10 fields of neurons with halation and neurites were randomly selected. When images were collected, the cell body diameter (average of the longest cell diameter and shortest cell diameter) and neurite length were measured using the SPOT software, and the cell body diameter, neurite length and neurite number were calculated averaged.

Evaluation of nerve functional recovery

Determination of sciatic nerve function index of rats

A 40 cm × 8.5 cm confined walkway with a dark chamber in the end was prepared. A sheet of white paper of the same size was placed on the bottom of the track. Rats were urged to walk through the pathway after their postpedes were uniformly stained with blue-black ink, leaving footprints on the white paper. The same tests were conducted 1 d prior to, and 4, 8, 12 weeks post operation. The print length (PL), toe spread (TS) and intermediary toe spread (IT) of the injured foot (E) and the normal foot (N) were measured, respectively. The 3 variables were introduced into the following formula [14,15]: SFI=-38.3 (EPL-NPL) / NPL+109.5 (ETS-NTS) / NTS+13.3 (EIT-NIT) / NIT-8.8 to calculate the sciatic nerve function index (SFI).