

IDENTIFICATION OF PROTEINS IN FLUID COLLECTED FROM NERVE REGENERATION CHAMBERS

YILIN YE¹, LI LI² and QINGFENG LI³

¹Department of Medical Cosmetology, Anhui University of Traditional Chinese Medicine, Anhui Province, China

²Department of Pharmacology, Anhui University of Traditional Chinese Medicine, Anhui Province, China

³Department of Plastic Surgery, Affiliated Ninth People's Hospital of Shanghai Jiao Tong University, Shanghai 200000, China

Corresponding author: yelilin23@hotmail.com

Abstract – We examined whether there are novel neurotrophic factors (NTFs) in nerve regeneration conditioned fluid (NRCF). Nerve regeneration chamber models were established in the sciatic nerves of 25 New Zealand rabbits, and NRCF was extracted from the chambers 1 week postoperatively. Proteins in NRCF were separated by native polyacrylamide gel electrophoresis (PAGE), and Western blot and ELISA were used to identify the proteins. A novel NTF was identified in a protein fraction corresponding to 220 kDa.

Key words: Nerve regeneration conditioned fluid; neurotrophic factor; protein extraction; rabbit

INTRODUCTION

Nerve regeneration refers to the repair or regrowth of nervous cells, tissues or cell products and includes the generation of new neurons, myelin, glia, axons or synapses (Kandel et al., 2000). Biologically active proteins or chemokines in nerve regeneration conditioned fluid (NRCF), such as neurotrophic factors (NTFs), play important roles in nerve regeneration (Meek and Coert, 2002). NTF is a family of proteins that are essential for the maintenance of mature neurons and the growth of developing neurons. Research has shown that the initial growth and development of neurons is promoted by NTFs in the central nervous system (CNS) and peripheral nervous system (PNS), and that damaged neurons can regrow *in vitro* and *in vivo* in the presence of NTFs (Deister and Schmidt, 2006). Nerve growth

factor (NGF) is the first discovered member of the neurotrophin family (Ebendal, 1992). NGF is widely distributed in the adult organism and plays a role in the differentiation of sensory and sympathetic ganglia in developing chick embryos (Schenkein and Bueker, 1962). Brain-derived neurotrophic factor (BDNF) is expressed and widely distributed in the CNS where it acts as a trophic factor for dopaminergic and cholinergic neurons of the substantia nigra/ventral mesencephalon (Barde et al., 1982; Lewin and Barde, 1996). The neurotrophic factor derived from glial cells (GDNF) is a potent neurotrophic factor with an important role in the differentiation, development, growth and survival of the cells in the CNS (Oppenheim et al., 1995). Neurotrophin-3 (NT-3) also contributes to the formation of neural networks and NT-3 can be found outside the CNS, such as skeletal muscle, lymphocytes and pancreatic

beta cells (Tazi et al., 1996; Terenghi, 1999). NT-4 is a NTF and a ligand for tropomyosin-related kinase B (TrkB). Yoshizaki et al. (2008) have found that NT-4 promotes the expression of enamel matrix genes and enhances the differentiation of dental epithelial cells. Ciliary neurotrophic factor (CNTF) was originally identified as a trophic molecule for the survival of embryonic chicken ciliary neurons *in vitro* (Adler et al., 1979).

The use of nerve regeneration tubes to collect fluid for the analysis of the neurotrophic and neurotogenic agents that influence regenerative events was first described about 30 years ago (Longo et al., 1983a; Longo et al., 1984). The peripheral nerve regeneration model was established by suturing the openings of cylindrical silicone chambers to the proximal and distal stumps of transected rat sciatic nerve. Longo et al. (1983b) found that fluid collected from nerve regeneration chambers contains NTFs directed at sensory, sympathetic and spinal cord neurons. These studies confirmed that NRCF containing factors with neuronotrophic activities could be collected by establishing models of sciatic nerve regeneration.

In this study, the nerve regeneration chamber model was established in the sciatic nerves of New Zealand rabbits, and NRCF was extracted from the silicone chambers at 1 week postoperatively. The proteins in the NRCF were subjected to ammonium sulfate precipitation and gel filtration, and the presence of NGF, GDNF, BDNF, NT-3, NT-4 and CNTF was examined by Western blot analysis and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Animals

A total of 25 male New Zealand white rabbits weighing 1.8-2.5 kg were recruited for the study. Rabbits were obtained from the Experimental Animal Center of Shanghai Second Medical University. Animal care and laboratory treatment were in accordance with the guidelines established by the Animal Ethics Committee of the Shanghai Ninth People's Hospital.

Establishment of nerve regeneration chamber model and extraction of NRCF

Anesthesia was administered intramuscularly using a mixture of ketamine (100 mg/kg) and sumianxin (500 mg/kg). The sciatic nerves of each rabbit were exposed bilaterally under aseptic conditions and 2 mm length of the sciatic nerve between the divided ends was removed. The proximal and distal stumps of the sciatic nerves were sutured into the openings of cylindrical silicone tubes (diameter: 2.5 mm, length: 4 cm) by 8-0 nylon.

One week after surgery, the rabbits were anesthetized and the sciatic nerves were re-exposed bilaterally. The silicone tubes were removed after cutting the proximal and distal end of the sciatic nerves. Then, 50-110 μ L NRCF in each nerve regeneration chamber was collected by microinjector. A total of 3 mL NRCF was obtained and preserved at -20°C .

Protein purification

Three ml of NRCF were diluted with an equal volume of 0.2 M PBS (pH 7.2). Saturated ammonium sulfate solution was slowly added to a final concentration of 50%. The solution was kept at 4°C overnight, and the supernatant was removed by centrifugation at 154 g for 20 min. The obtained precipitate was dissolved in 2 mL PBS and loaded in a dialysis bag to remove ammonium ions. The crudely purified NRCF was obtained after coprecipitation and dialysis twice. The concentration of protein in the NRCF was measured by ultraviolet (UV) spectrophotometry.

Two ml of NRCF was subjected to gel filtration on a 90×1.5 cm column of Sephadex-G200 (Pharmacia, Sweden) equilibrated with 0.2 M PBS (pH 7.2) (Skoog, 2006). Two ml of a protein solution (2 mg/mL) containing molecular weight protein standards, IgM (300 kDa), IgG (150 kDa), egg albumin (45 kDa) were loaded on the column and washed with 0.2 M PBS (pH 7.2). The concentration of the collected protein was measured by UV spectrophotometry in order to draw the reference curve. Two ml of the NRCF protein solution were loaded on the

column and eluted. The relative molecular masses of the proteins from the NRCF were calculated from the relative molecular mass of the protein standards.

Protein characterization

Two protein components of the were subjected to native PAGE as described by (Laemmli, 1970), and transferred to nitrocellulose filters (Towbin H, 1992). Western blotting and ELISA were performed according to the standard protocols (Capucci et al., 1996; Lelli et al., 2012), using primary antibodies against GDNF, NGF, BDNF, CNTF, NT-4 and NT-3 (1:500; SantaCruz, America). Incubation with secondary antibodies (horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000; SantaCruz, America)) was for 1 h at room temperature. All reactions were visualized with diaminobenzidine (DAB).

RESULTS AND DISCUSSION

To identify some of the proteins in NRCF, the NRCF was collected from silicone nerve regeneration chambers in rabbits, purified by saturated ammonium sulfate precipitation followed by gel filtration and examined by ELISA and Western analysis.

The relative maximal molecular masses of the major protein fractions from the NRCF obtained after chromatography were about 200 kDa and (20-40) kDa, and were designated as a and c, respectively (Fig. 1). The relative molecular masses of the protein contained in a and c were 220KDa and (20-40) KDa, respectively, according to native-PAGE (Fig. 2).

There are three major types of neural factors in NRCF: NTFs, such as NGF, BDNF and CNTF with relative molecular masses of 140 kDa, 12.3 kDa and (20-30) kDa, respectively (Varon et al., 1967; Barde et al., 1982; Gordon et al., 2003; Mouri et al., 2007); neurite-promoting factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) with relative molecular masses of 6 kDa and (15-20) kDa, respectively (Jungnickel et al., 2006), and extracellular matrix components, such as lam-

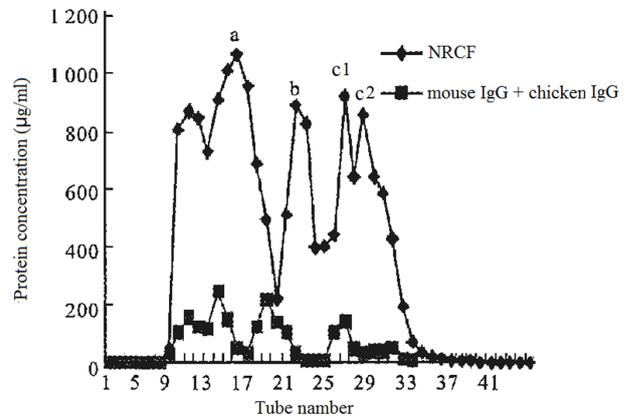


Fig. 1. Protein separation curve of NRCF after gel filtration.

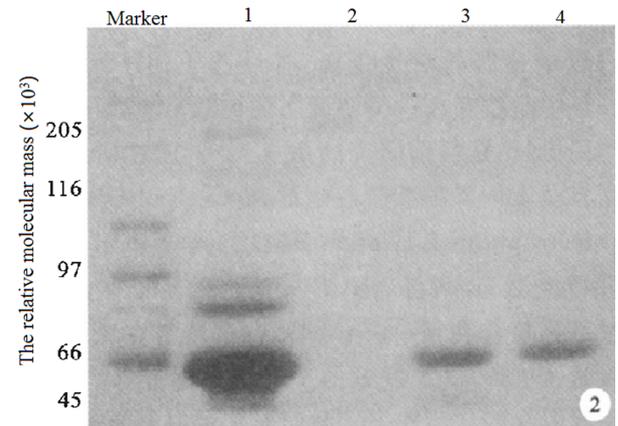


Fig. 2. Native PAGE. Unpurified NRCF (lane 1), fraction a (lane 2), fraction c1 (lane 3) and fraction c2 (lane 4).

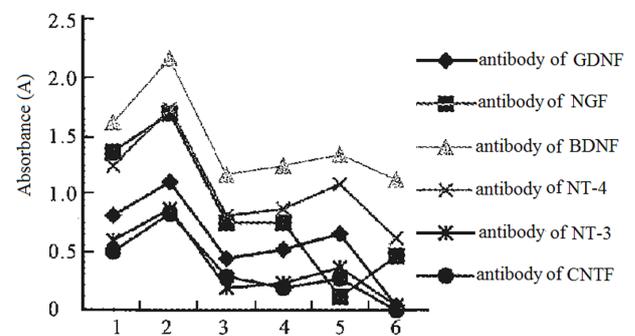


Fig. 3. Absorption values of a and c. 1 – anterior part; 2 – middle part; 3 – posterior part of c; 4 – anterior part; 5 – middle part; 6 – posterior part of a.

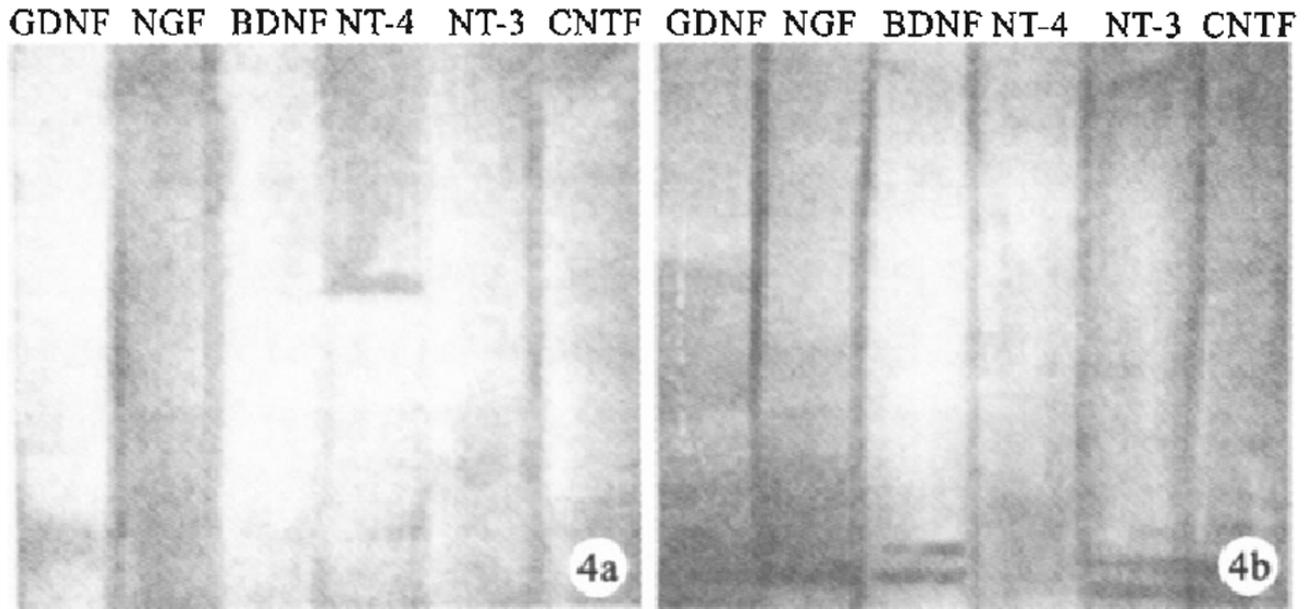


Fig.4. Western blot analysis of fractions a (a) and c (b).

inin and fibronectin (FN) with relative molecular mass of 850KDa and 440KDa, respectively (Lundborg, 2000; Meek MF, 2002; Tucker et al., 2006).

We selected NGF, BDNF, CNTF, NT-3, NT-4 and GDNF antibodies to conduct the experiment of Western blot and ELISA. ELISA revealed an increased presence of all six probed proteins in fraction c compared to fraction a (Fig. 3), notably NGF, followed by GDNF, BDNF, NT-3 and CNTF. Western blot analysis showed the presence of NT-4 in fraction a (Fig. 4a), and NGF, BDNF, CNTF and NT-3, but not of NT-4 and GDNF in fraction c from the NRCF (Fig. 4b).

An increasing number of studies of the use of non-nervous tubes (both biological and synthetic) for repairing nerve defects revealed their good clinical results (Battiston et al., 2005; Deal et al., 2012). NRCF plays an important role in the regulation of nerve regeneration (Liu et al., 1995; Midha et al., 2003).

Previously we suggested that motor nerve-derived NRCF has more neurite-promoting and neu-

robiological effects on motoneurons than on sensory nerve-derived NRCF and bFGF (Ping 2000). In the present study, protein fraction a contained only NT-4. The biological activity of NT-4 is far below those of NGF, BDNF, CNTF and NT-3 that were identified in fraction. It could be inferred that a novel NTF is present in the 220 kDa fraction a.

Acknowledgements - We thank Professor Wenxiang Guan, for his assistance with the research. The research was supported by grants from the National Natural Science Foundation of China (NO.30070776).

REFERENCES

- Adler, R., Landa, K.B., Manthorpe, M. et al. (1979). Cholinergic neuronotrophic factors: intraocular distribution of trophic activity for ciliary neurons. *Science*. **204**, 1434-1436.
- Barde, Y.A., Edgar, D. and H. Thoenen (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549-553.
- Battiston, B., Geuna, S., Ferrero, M. et al. (2005). Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair. *Microsurgery*. **25**, 258-267.

- Capucci, L., Fusi, P., Lavazza, A. et al. (1996). Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. *J Virol.* **70**, 8614-8623.
- Deal, D.N., Griffin, J.W. and M.V. Hogan (2012). Nerve conduits for nerve repair or reconstruction. *J Am Acad Orthop Surg.* **20**, 63-68.
- Deister, C. and C.E. Schmidt (2006). Optimizing neurotrophic factor combinations for neurite outgrowth. *J Neural Eng.* **3**, 172-179.
- Ebendal, T. (1992). Function and evolution in the NGF family and its receptors. *J Neurosci Res.* **32**, 461-470.
- Gordon, T., Sulaiman, O. and J.G. Boyd (2003). Experimental strategies to promote functional recovery after peripheral nerve injuries. *J Peripher Nerv Syst.* **8**, 236-250.
- Jungnickel, J., Haase, K., Konitzer, J. et al. (2006). Faster nerve regeneration after sciatic nerve injury in mice over-expressing basic fibroblast growth factor. *J Neurobiol.* **66**, 940-948.
- Kandel, E.R., Schwartz, J.H. and T.M. Jessell (2000). Principles of neural science: McGraw-Hill New York.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685.
- Lelli, D., Moreno, A., Brocchi, E. et al. (2012). West Nile virus: characterization and diagnostic applications of monoclonal antibodies. *Virol J.* **9**, 81.
- Lewin, G. R. and Y.A. Barde (1996). Physiology of the neurotrophins. *Annu Rev Neurosci.* **19**, 289-317.
- Liu, H.M., Lei, H.Y. and K.P. Kao (1995). Correlation between NGF levels in wound chamber fluid and cytological localization of NGF and NGF receptor in axotomized rat sciatic nerve. *Exp Neurol.* **132**, 24-32.
- Longo, F.M., Manthorpe, M., Skaper, S.D. et al. (1983a). Neurotrophic activities accumulate in vivo within silicone nerve regeneration chambers. *Brain Res.* **261**, 109-116.
- Longo, F.M., Skaper, S.D., Manthorpe, M. et al. (1983b). Temporal changes of neuronotrophic activities accumulating in vivo within nerve regeneration chambers. *Exp Neurol.* **81**, 756-769.
- Longo, F.M., Hayman, E.G., Davis, G.E. et al. (1984). Neurite-promoting factors and extracellular matrix components accumulating in vivo within nerve regeneration chambers. *Brain Res.* **309**, 105-117.
- Lundborg, G. (2000). A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J Hand Surg Am.* **25**, 391-414.
- Meek, M. and J. Coert (2002). Clinical use of nerve conduits in peripheral-nerve repair: review of the literature. *J Reconstr Microsurg.* **18**, 097-110.
- Meek MF, C.J. (2002). Clinical use of nerve conduits in peripheral repair: review of the literature. *J Reconstr Microsurg.* **18**, 97-109.
- Midha, R., Munro, C.A., Dalton, P.D. et al. (2003). Growth factor enhancement of peripheral nerve regeneration through a novel synthetic hydrogel tube. *J Neurosurg.* **99**, 555-565.
- Mouri, A., Nomoto, H. and S. Furukawa (2007). Processing of nerve growth factor: the role of basic amino acid clusters in the pro-region. *Biochem Biophys Res Commun.* **353**, 1056-1062.
- Oppenheim, R.W., Houenou, L.J., Johnson, J.E. et al. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature.* **373**, 344-346.
- Ping P, Li, Q.F. and J.L. Gan (2000). Neurobiological effect of nerve regeneration condition fluid on motoneuron. *Chinese J Reparative and Reconstructive Surgery.* **14**, 325-327.
- Schenkein, I. and E.D. Bueker (1962). Dialyzable cofactor in nerve growth promoting protein from mouse salivary glands. *Science.* **137**, 433-434.
- Skoog, D.A. (2006). Principles of Instrumental Analysis: Thompson Brooks/Cole: Belmont.
- Tazi, A., Le Bras, S., Lamghitnia, H.O. et al. (1996). Neurotrophin-3 increases intracellular calcium in a rat insulin-secreting cell line through its action on a functional TrkC receptor. *J Biol Chem.* **271**, 10154-10160.
- Terenghi, G. (1999). Peripheral nerve regeneration and neurotrophic factors. *J Anat.* **194 (Pt 1)**, 1-14.
- Towbin H, S.T. and J. Gordon (1992). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotchnology.* **24**, 145-149.
- Tucker, B.A., Rahimtula, M. and K.M. Mearow (2006). Laminin and growth factor receptor activation stimulates differential growth responses in subpopulations of adult DRG neurons. *Eur J Neurosci.* **24**, 676-690.
- Varon, S., Nomura, J. and E.M. Shooter (1967). Subunit structure of a high-molecular-weight form of the nerve growth factor from mouse submaxillary gland. *Proc Natl Acad Sci U S A.* **57**, 1782-1789.
- Yoshizaki, K., Yamamoto, S., Yamada, A. et al. (2008). Neurotrophic factor neurotrophin-4 regulates ameloblastin expression via full-length TrkB. *J Biol Chem.* **283**, 3385-3391.

