Study of reinnervation process in patients with 2 type of spinal muscular atrophy: clinical experimental study

Maria Sokolova 1, Valentina Penniyaynen 2, Anna Kipenko 2,3, Nicolai Aleksandrov 1, Ekaterina Lopatina 2,3

1 North-West State Medical University named after I.I. Mechnikov, St. Petersburg, Russia; 2 Pavlov Institute of Physiology of the Russian Academy of Sciences, St. Petersburg, Russia; 3 Almazov Federal North-West Medical Research Centre, St. Petersburg, Russia.

Correspondence to: Maria G. Sokolova, Ph.D. of Medicine, North-West State Medical University named after I.I. Mechnikov, Department of Neuropathology named after S.N. Davidenkov, av. Piskarevsky 47, 195067 St. Petersburg, Russia Federation, TEL: +7(921)9137335; E-MAIL: sokolova.m08@mail.ru

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Abstract

OBJECTIVES: The aim of the research was to study the correlation between the level of NGF of blood serum and electroneuromyography data with impact of blood serum of patients with 2 type SMA on growth of dorsal root ganglion neurites of 10–12-day old chicken embryos in an organotypic tissue culture. METHODS: 10 patients with (2 type SMA) aged 8–12 years were examined. Electroneuromyography study was done to all of them. Study blood serum of the 10 patients was done in an organotypic tissue culture. Studies were performed on 500 explants sensory dorsal root ganglia of the chick embryo. The ganglia were cultured for three days on collagen supports in Petri dishes at 36.5 °C and 5% CO2 incubator (Sanyo, Japan) was used. In experimental dishes, blood serum of patients with 2 type SMA was added to the cultural medium. The growth of explants in tissue culture was controlled on vital preparations using a confocal laser scanning microscope LSM-710 (Carl Zeiss, Germany). The preparations were also visualized by a television microscope attachment Axiostar Plus (Carl Zeiss, Germany). Neurites outgrowth was quantified using the Image J program. The area index (AI) was used to estimate the neurite outgrowth. It was calculated in relative units as the ratio of total explant area to the central zone area. All values were taken as 100% in the control experiments. Level Beta-NGF in 10 patients (SMA 2 type) was determined in serum using Beta-NGF ELISA Kit (RayBiotech, Inc). RESULTS: According to electroneuromyography study in patients with 2nd type SMA reinnervation process was poor. It was found out that concentration NGF (nerve growth factor) in serum of patients with 2nd type SMA is considerably higher than in control group. We studied the influence of blood serum of patients with 2 type SMA on growth of neurites of dorsal root ganglia of 10–12 days old chicken embryos in organotypic culture. Blood serum of 12 patients with type 2 SMA was researched in a wide range of dilution (1:100–1:2). The plasma of patients in a dilution of 1:2–1:50 completely blocked the growth of dorsal root ganglion neurites. All of experimental explants cultured in the medium containing blood serum in dilution 1:70 was 25% less than the control value. CONCLUSIONS: For the first time it was shown that blood serum of patients with 2nd type SMA inhibits the growth of neurites of dorsal root ganglia in dose-dependent manner. Apparently, neurite-inhibitory effect is caused by high concentration of neurotrophins (NGF) in patient serum, and due to that the reinnervation in 2 type SMA patients is not efficient.
INTRODUCTION

Spinal muscular atrophy is autosomal recessive disorder, characterized by progressive degeneration of alpha-motor neurons of spinal cord. With an incidence of 1 in 6,000–10,000 live births and a carrier frequency of 1 in 40–50. The disease manifests itself as a weakness of proximal muscles, pareses, respiratory insufficiency and early mortality. This disease is one of motor neuron diseases, which progression results in denervation process and in mechanism of reinnervation of denervated muscular fibers (Cuppini et al 1990). Restoration of the lost function can be made by the remained nerve fibers when they start to ramify intensively, heading to denervated muscle fibers. The reinnervation process is regulated by neurotrophin, in particular – by nerve growth factor (Huang 2001; Levi-Montalchini 1987).

Now an activity of denervation-reinnervation processes is usually estimated with the help of electroneuromyography. Neurophysiological and morphological mechanisms of reinnervation process in patients with 2 type SMA are underinvestigated, which makes the study of neurotrophic factors for realization of adaptive-compensatory process in the patients with the disease so relevant. Studying the mechanism of neurotrophic regulation of denervation-reinnervation process in patients with 2 type SMA by means of organotypic tissue culture method may help to clarify pathogenesis of this disease, and, probably, will open up new differentiated treatment approaches for clinicians.

The purpose of the study was complex studying of denervation-reinnervation process in patients with 2 type SMA with the help of clinical-neurophysiological and laboratory-experimental research methods.

MATERIALS AND METHODS

Clinical-neurologic and neurophysiological examination of 10 patients with 2 type SMA was carried out. Electrophysiological study included stimulation electroneuromyography (ENMG) and needle electromyography (EMG). The studies were carried out according to standard protocol.

Level Beta-NGF in 10 patients (SMA 2 type) was determined in serum using Beta-NGF ELISA Kit (Ray-Biotech, Inc).

The experiments were performed in the organotypic culture of a 10–11 day old chicken embryos dorsal root ganglion. Study blood serum in an organotypic tissue culture method may help to clarify pathogenesis of this disease, and, probably, will open up new differentiated treatment approaches for clinicians.

The explants in tissue culture was controlled on vital preparations using a confocal laser scanning microscope LSM-710 (Carl Zeiss, Germany). The preparations were also visualized by a television microscope attachment Axiostar Plus (Carl Zeiss, Germany). The explants growth was quantified using the ImageJ program. The area index (AI) was used to estimate the explants growth. It was calculated in relative units as the ratio of total explant area to the central zone area. AI value was taken as 100% in the control experiments. The data were processed with Student’s t-test at p=0.05. Data are expressed as means ± SE.

The work was based on the following methods of statistical analysis: determination of numerical characteristics of variables; estimation of conformity of empirical law of distribution of quantitative variables to theoretical law of Gaussian distribution according to Shapiro-Wilk test; an estimation of influence of qualitative factor on a dispersion of quantity indicator using ANOVA dispersion method, an estimation of a force and direction of linear relationship between the quantity indicators using parametrical Pearson correlation coefficient, nonlinear relationship – using Spearman’s correlation coefficient. Description of quantitative signs was carried out using arithmetic mean value and standard deviation. Zero statistical hypothesis was rejected at significance value p<0.05. The statistical analysis was carried out using STATISTICA 8.0 package (StatSoft®, Inc., USA).

RESULTS AND DISCUSSION

10 patients with 2 type spinal muscular atrophy were examined, among them: 4 girls and 6 boys at the age from 8 up to 12 years old. All patients with 2 type SMA have been under medical observation for 3 years, during this period of time the disease was progressing. Motor deficit was manifested since birth. Genetic defect was identified at the long arm of 5th chromosome (within the interval between D5S629 and D5S557). Clinical-neurologic picture includes flaccid pareses of hands and feet with prevalence of the process in the proximal parts, active movements were only in distal parts of the hands, neck muscles, mimic and respiratory muscles. There were generalized fibrillations and fasciculations of the muscles, intense diffuse hypomyotonia. 85% of children had intense atrophies of intercostal muscles with respiratory insufficiency and minor bulbar disorders. Changes of osteoarticular system were manifested as intense contractures of large joints of extremities and kyphoscoliosis. Functions of pelvic bodies were normal. There were no sensitivity and cognitive disorders.

EMG-study of muscles of patients has shown plural potentials of fibrillations, positive sharp waves. Decrease in recruitment of motor units and increase in impulse frequency is a sign of decrease in the number of functioning motor-neurons of anterior horns of spinal cord. At the maximum muscle strain the impulse frequency...
of motor units (MU) has sharply increased up to tens of Hz (up to 40–50 Hz for some muscles). There were no normal potentials of motor units (MUP), only potentials of decreased amplitude and duration were registered. Stimulation ENMG has shown that conduction velocities of the motor fibers of the nerves were within the reference range or were slightly decreased. Moderate to sharp decrease in the amplitude of M-responses in patients with 2 type SMA reflects a degree of reduction of the number of functioning MU. Amplitude of the action potential of the nerves and conduction velocity of sensitive fibers of the nerves were normal. It is known that in parallel with α-motoneuron loss process the patients with 2 type SMA show reinnervation processes which lead to change of MUP parameters: potentials of high duration and amplitude are formed (reinnervation MUP) (Sokolova et al 2013). Absence of reliable changes in reinnervation potentials of the examined patients suggests that the reinnervation process of the patients is not so active.

We have suggested that reinnervation process in patients with this pathology is not effective enough due to low synthesis of nerve growth factor. It is known that nerve growth factor is necessary for survival and differentiation of neurons; it stimulates growth and branching of axons (Yamashita 2007). According to enzyme immunoassay a concentration of the nerve growth factor in blood serum of patients with 2 type SMA was statistically significantly ($p<0.001$) higher than in the control group. Concentration of the nerve growth factor in the blood plasma in the control group was within the range from 110 to 2237 pg/ml, in patients with 2 type SMA – from 1387 to 5411 pg/ml. Thus, the obtained data testify that in patients with 2 type SMA the low reinnervation is not related to deficiency of the nerve growth factor.

The following series of experiments was aimed at investigation of an influence of blood plasma of patients with 2 type SMA on growth of neurons of dorsal root ganglions of 10–12-day chicken embryos. After three days of culturing in the control and experimental explants of dorsal root ganglions there are two zones: central, consisting of not erratic differentiated neuroblasts and peripheral, so-called growth zone. The growth zone of explants of dorsal root ganglions contains mostly growth of neurites (processes of nervous cells), to a lesser degree – migrating and proliferating fibroblast-like and glial cells.

Blood serum of 5 patients with type 2 SMA was researched in a wide range of dilution (1:100–1:2). In dilutions 1:2, 1:10, 1:50 the serum of patients blocked completely the growth of dorsal root ganglion neurites. When adding in cultural medium blood serum in dilution 1:70, a positive neurite inhibitory effect was observed. Area index of the studied explants was below the control value on the average by 25 % and has made $75.5\pm7.4$ % (Figure 1). Further dilution of blood serum practically did not affect the growth of

![Fig. 1.](image1) **Fig. 1.** Estimation of Area index in studied blood serum dilutions of patients (arithmetic mean values are shown, error bars – 95 % confidence interval of mathematical expectation of Area index parameter).

![Fig. 2.](image2) **Fig. 2.** Graphic assessment of relation between concentration of the nerve growth factor in blood serum of patients with Spinal Muscular Atrophy and the Area index.

![Fig. 3.](image3) **Fig. 3.** Graphic estimation of relationship between concentration of the nerve growth factor in the blood serum of patients with Spinal Muscular Atrophy within the range from 0 to 150 pg/ml and the Area index.
experiments. The carried out studies have shown that blood serum of patients with 2 type SMA dose-dependently inhibits growth of neurites of sensory neurons of spinal ganglia.

Results of estimation of the Area index for studied dilutions were analyzed using dispersion analysis. It is shown that blood serum dilution factor statistically significantly (F-test F=489.2; \( p<0.001 \)) influences the area index value registered in the study.

In order to estimate a type of statistical relationship between the nerve growth factor concentration in blood serum in patients with Spinal Muscular Atrophy and the Area index the scatter diagram for the specified parameters was plotted (Figure 2). The diagram analysis is indicative of presence of nonlinear negative correlation relationship between the signs.

Correlation relationship between concentration of the nerve growth factor in blood serum of patients with 2 type SMA and the Area index was established by means of nonparametric correlation analysis with calculation of Spearman rank-order correlation coefficient (Spearman Rank Order Correlations) and estimation of its statistical significance. Presence of statistically significant \( (p<0.001) \) strong correlation feedback is shown (Spearman \( R = -0.90 \)).

The analysis of the scatter diagram has helped to suggest a presence of linear site of dependence between the quantity of nerve growth factor in blood serum in patients with 2 type SMA and the Area index within the range of concentration from 0 to 150 pg/ml, which was confirmed by plotting of additional scatter diagram for this interval of nerve growth factor concentration (Figure 3).

The quantitative estimation of linear relationship has shown a presence of statistically significant \( (p<0.001) \) strong correlation feedback (Pearson correlation coefficient \( r = -0.81 \)) between the amount of the nerve growth factor in blood serum of patients with 2 type SMA and the Area index within the range of concentration from 0 to 150 pg/ml. Thus, it is proved that degree of inhibition of the growth of neurites of dorsal root ganglia of chicken embryos depends on concentration of the nerve growth factor in the Serum of patients with 2 type SMA.

Now various biologically active substances are known which can lead to inhibition of growth of neurites (Benson 2005; Goldberg 2004; Williams 2008; Yongwoo 2012). Most studies of recent years were aimed at studying the mechanisms of axon neogenesis after traumatic damage (Hannila 2008; Miyashita 2009). It was shown that oligodendrocytes synthesize the following proteins: myelin glucoprotein and myelin-associated glucoprotein, possessing neurit-inhibitory properties (Li 2004; Shen 2009). Traumatic damage of brain tissue promptly results in development of reactive gliosis or to formation of glial cicatrix with no axon neogenesis process and remielinization, because its component cells and extracellular components synthesize many proteins which inhibit the growth of axons (Monnier 2003; Wanner 2008). 2 type SMA does not result in damage of nervous fibers, which is confirmed by the results of ENMG: sensory conduction velocity of the nervous fibers in patients with 2 type SMA is normal. Thus, growth inhibition of neurits of dorsal root ganglia in organotypic culture of the tissue is not related to negative influence of myelin on this process. Apparently, neurit-inhibitory effect of blood serum in patients with 2 type SMA is caused by high concentration of the nerve growth factor.

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**References**