MORPHOLOGY AND PATHOMORPHOLOGY

Trophic Effects of Nootropic Peptide Preparations Cerebrolysin and Semax on Cultured Rat Pheochromocytoma

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Trophic characteristics of neuroprotectors cerebrolysin and semax were evaluated by their capacity to induce differentiation and improve survival of cultured rat pheochromocytoma (PC12) cells. Morphological signs of cell differentiation (enlargement and formation of processes) were seen 24 h after addition of cerebrolysin into culture medium. Cerebrolysin improved survival of PC12 cells in serum-free medium. In a concentration of 100 μg/ml cerebrolysin decreased the content of apoptotic cells from 32% (control) to 10%. Semax produced no trophic effect on PC12 cells. Hence, the neuroprotective effect of cerebrolysin *in vivo* probably results from trophic activity, while the protective effects of semax are mediated by other mechanisms.

Key Words: *neuroprotection; trophic factors; cerebrolysin; semax; PC12 rat pheochromocytoma cell culture*

Many neurological and mental diseases are associated with neuronal death. Neuroprotective therapy in these diseases is aimed at prevention of nerve tissue damage. Some neuroprotectors were introduced into clinical practice, but the search of new drugs is in progress. Neuroprotective preparations include glutamate receptor blockers, antioxidants, and drugs with neurotrophic and neuromodulatory effects. Studies on cultured neuronal cell provide important information about the mechanisms of neuroprotector effects.

Cerebrolysin (CL) and semax are used in the treatment of patients with memory and locomotor disorders resulting from encephalopathies of different genesis. These drugs are often used in neuroprotective therapy of ischemic stroke in the acute period and during rehabilitation [3]. Both these peptide preparations exhibit pronounced nootropic effects. CL is a protein hydrolysate of porcine brain consisting of free amino acids (85%) and peptides with molecular weights below 10 kD (15%). Neuroprotective activity of CL in ischemic stroke is determined by its capacity to improve neuronal survival during ischemia/reperfusion and protect neurons from toxic effects of glutamate [10,14]. Semax was developed at the Institute of Molecular Genetics of the Russian Academy of Sciences [5]. Semax is a synthetic heptapeptide Met-Glu-His-Phe-Pro-Gly-Pro, an analog of the adrenocorticotropic hormone fragment ($ACTH_{4-10}$). Semax improves animal survival under conditions of hypoxia [4]. Complex clinical studies demonstrated high efficiency of semax at early stages of ischemic stroke. Semax promoted recovery of impaired neurological functions and improved of electrophysiological parameters of the brain in patients with ischemic stroke [2].

Experiments on primary neuronal cultures showed that CL and semax possess trophic effects [1,8]. We investigated trophic properties of nootropic peptide preparations CL and semax on cultured rat pheochromocytoma (PC12) cells. These cells express receptors to nerve growth factor (NGF) and can differentiate into neuron-like cells in the presence of NGF [7]. This

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process is associated with the expression of neuron-specific enzymes, structural proteins, and receptors. Numerous studies show that PC12 culture can be used for modeling of neuronal differentiation and death.

MATERIALS AND METHODS

Cerebrolysin (EBEWE Arzneimittel), semax (Institute of Molecular Genetics), and murine NGF-7S (130 kD; ICN) were used in the study.

PC12 cells were cultured in DMEM supplemented with 15% fetal calf serum (BioloT), 2 mM glutamine, and 80 μg/ml gentamicin at 37°C and 5% CO_2.

For evaluation of the differentiation-stimulating capacity of the test peptides, PC12 cells were seeded in 24-well plates (Corning & Costar, 10^4 cells per well) coated with poly-L-lysine (Fluka Chemie AG) in a medium with 1% serum. Immediately after seeding CL and Semax were added to a final concentrations of 0.1, 10, and 1000 μg/ml; NGF was added to control wells to a final concentration of 100 ng/ml. The studied agents and NGF were added every 48 h for 6 days. Cell differentiation was evaluated by the shape and size of cells and number and length of their processes.

For evaluation of the capacity of peptide drugs to improve survival of cells deprived of trophic factors, PC12 cells were cultured in a serum-free medium. To this end the cells (3×10^4) were cultured on slides coated with poly-L-lysine. Adherent cells were washed with 0.15 M phosphate buffer (pH 7.2) and cultured in a serum-free medium for 18 h at 37°C and 5% CO_2. NGF served as the positive control. Apoptotic and necrotic cells were visualized with fluorescent nuclear dyes Hoechst 33258 and propidium iodide [11]. Stained cells were counted under an Axioscop 2 fluorescent microscope (Zeiss).

The results were statistically processed using Sigma Plot 2.01 software.

RESULTS

Undifferentiated PC12 cells had round shape (Fig. 1, *a*). Culturing in the presence of 10 and 1000 μg/ml CL led to cell enlargement after 24 h and appearance of neurite-like processes after 48 h (Fig. 1, *b*). In the presence of low CL concentration (0.1 μg/ml) morphological changes were seen only after 48 h. It should be noted that cells cultured in the presence of CL morphologically differed from cells stimulated with NGF (Fig. 1, *c*). In the presence of NGF practically all cells acquired a neuron-like shape, were elongated, and had more numerous processes. This degree of differentiation of PC12 cells was not attained even after 6-day culturing with CL. These differences were not caused by low content of trophic factors in CL, because the effect manifested even at a concentration of 0.1 μg/ml. Semax in concentrations of 0.1-1000 μg/ml caused no changes in PC12 cell morphology after 6-days culturing.

CL exerted a trophic effect on NGF-dependent neurons. Elongation of neurites in the presence of CL was observed in cultures of sympathetic neurons and

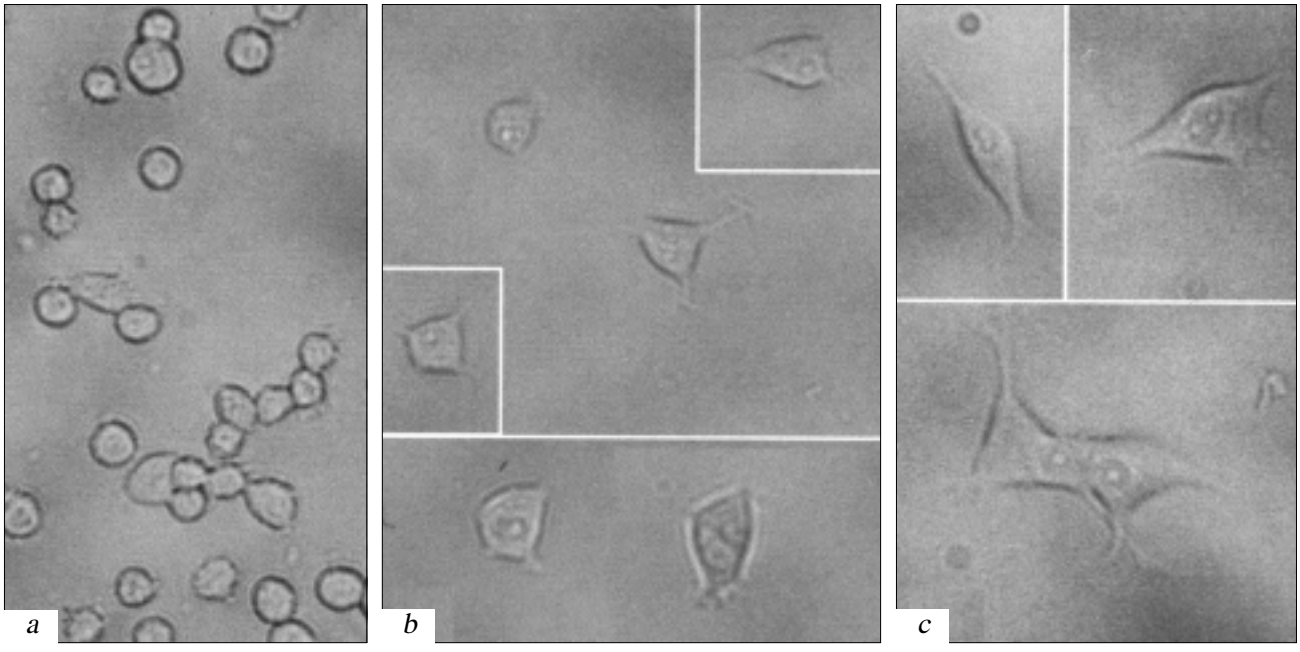

Fig. 1. Morphology of PC12 cells after 54-h culturing in a medium with 1% serum (*a*), and cerebrolysin (10 μg/ml, *b*), and nerve growth factor (100 ng/ml, *c*), ×200.

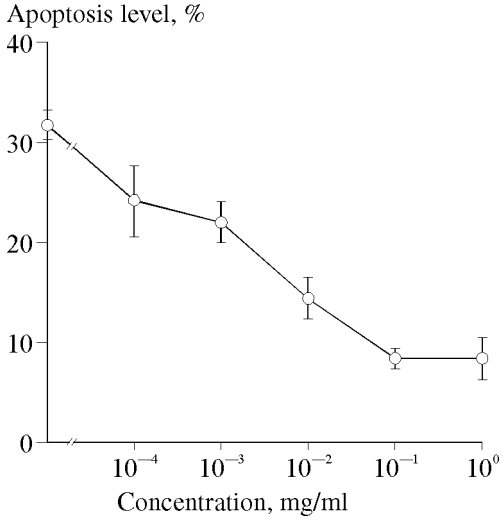

Fig. 2. Effect of cerebrolysin on the intensity of apoptosis in PC12 cell culture.

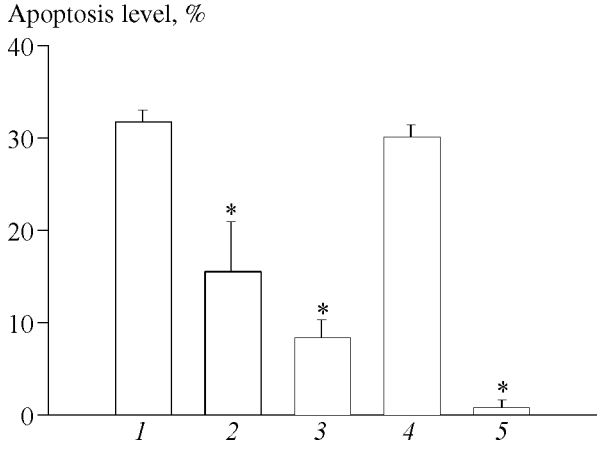

Fig. 3. Effects of cerebrolysin and semax on PC12 cell death caused by the absence of trophic factors. *1-4*) serum-free medium: *1*) control; *2*) nerve growth factor (100 ng/ml); *3*) cerebrolysin (100 g/ml); *4*) semax (100 μg/ml); *5*) medium with 15% serum. *$p<0.01$ compared to the control.

spinal ganglion neurons from chick embryos (*i. e.*, NGF-dependent neurons), but not in ciliary ganglion neurons (sensitive to ciliary neurotrophic factor) [12]. However the effect of CL on NGF-dependent cells was less expressed than the effect of NGF. The differentiation-stimulating effect of CL on PC12 cells has never been studied.

Cultures maintained in a serum-free medium for 18 h contained about 35% dead cells (3-5% necrotic cells, others underwent apoptosis, Fig. 2). CL decreased the percentage of apoptotic cells in a dose-dependent manner. In the presence of 0.1 and 1 mg/ml CL the content of apoptotic cells did not surpass 10% (Fig. 2). CL in these concentration had a more pronounced effect on cell survival than NGF in a concentration of 100 ng/ml (Fig. 3). In contrast to CL, semax in concentrations of 0.1-1000 μg/ml had no effect on the death of PC12 cells (Fig. 3).

The effect of CL on cell death under conditions of trophic factor deficiency was described for culture of cortical neurons from chick embryos [9]. The authors observed a decrease in the number of apoptotic cells and improvement of cell viability after addition of CL to the culture medium. On day 4 of culturing with CL (0.4 mg/ml) the number of apoptotic cells was 46% *vs.* 56% in the control (culturing in a medium with 2% serum). Similarly to our experiments the effect of CL manifests at concentrations of 0.1-1 mg/ml, but in our model the effect of CL was more pronounced. We hypothesized that trophic and cytoprotective effects of CL on PC12 can be due to the presence of fragments of trophic factors retaining their activity.

Hence, semax produces no trophic effect on PC12 cells and seems to be unable to interact with NGF receptors. Our previous findings indicate that the neuroprotective action of this drug is mediated via different mechanisms. Semax several-fold increased the synthesis of trophic factor mRNA in a glial cell culture from rat brain [13]. It was reported that semax significantly reduced the content of NO in rat cerebral cortex in incomplete global ischemia [6]. This probably indicates that semax modulates NO-mediated oxidative stress in ischemic stroke.

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