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Research Article

Effects of cerebrolysin on motor-neuron-like NSC-34 cells

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ABSTRACT

Although the peripheral nervous system is capable of regeneration, this capability is limited. As a potential means of augmenting nerve regeneration, the effects of cerebrolysin (CL) – a proteolytic peptide fraction – were tested in vitro on the motor-neuron-like NSC-34 cell line and organotypic spinal cord cultures. Therefore, NSC-34 cells were subjected to mechanical stress by changing media and metabolic stress by oxygen glucose deprivation. Afterwards, cell survival/proliferation using MTT and BrdU-labeling (FACS) and neurite sprouting using ImageJ analysis were evaluated. Calpain-1, Src and α-spectrin protein expression were analyzed by Western blot. In organotypic cultures, the effect of CL on motor neuron survival and neurite sprouting was tested by immunohistochemistry.

CL had a temporary anti-proliferative but initially neuroprotective effect on OGD-stressed NSC-34 cells. High-dosed or repeatedly applied CL was deleterious for cell survival. CL amplified neurite reconstruction to limited extent, affected calpain-1 protein expression and influenced calpain-mediated spectrin cleavage as a function of Src expression. In organotypic spinal cord slice cultures, CL was not able to support motor neuron survival/neurite sprouting. Moreover, it hampered astroglia and microglia activities.

The data suggest that CL may have only isolated positive effects on injured spinal motor neurons. High-dosed or accumulated CL seemed to have adverse effects in treatment of spinal cord injury. Further experiments are required to optimize the conditions for a safe clinical administration of CL in spinal cord injuries.

Introduction

A partial or total loss of spinal cord motor neurons can be the direct result of a traumatic injury of the spinal cord (paraplegia) or of neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS). Furthermore, motor neurons can die as a result of their denervation after peripheral nerve injuries. After their axons have been damaged, motor neurons may in principle be able to

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change their phenotype into a regeneration-supporting state and to re-innervate denervated targets by regenerating injured axons or by the collateral branching of undamaged axons. A lack of adequate re-innervation might even be compensated for by neural plasticity [1]. Unfortunately, the reality is another matter. Often, peripheral nerve injuries induce a loss of motor neurons and result in neuropathic pain, in which case functional recovery is limited. Thus, one important aim of medical basic research is to develop possible therapeutic strategies that will be able to enhance axonal regeneration. Moreover, even in the age of gene therapy, drug treatment has not lost its importance.

Thus, we were prompted to determine the usefulness of cerebrolysin (CL) in the context of motor neuronal degeneration. CL is a proteolytic extract from the pig brain. One milliliter CL consists of approximately 75% free amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan and tyrosine) and 25% low-molecular-weight, biologically active peptides [2,3], which are able to react with antibodies against glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) [4,5]. The motor neuron protective and/or regeneration-promoting capacity of these growth factors is well established. When forming the axon growth-promoting pathway (so-called Büngner bands), Schwann cells (SC) have been demonstrated to overexpress GDNF [5,6]. CNTF was shown to be able to support neuronal survival in primary mouse spinal cord cultures [7] and was necessary for spinal cord motor neuron sprouting response [8]. In addition, IGF-1 has been shown to be synthesized locally around a peripheral nerve lesion and then transported in a retrograde manner, triggering regenerative events in motor neurons [9].

Our experiments are also based on literature findings indicating that CL protects mouse hippocampal neurons in organotypic cultures from glutamate excitotoxicity [10] and chicken cortical neurons in primary cultures from stress induced by oxygen glucose deprivation (OGD) [11]. Moreover, positive CL effects have been reported following ventral root avulsion [12]. In addition to these in vitro studies, the neuroprotective effect of CL has also been reported in a clinical setting (stroke patients; [13]). CL is currently approved in 44 countries worldwide for functional disorders following stroke and traumatic brain injury, as well as for Alzheimer's disease and vascular dementia [14].

In previous experiments [15], we demonstrated that CL was able to enhance SC functionality, which is relevant to nerve regeneration, suggesting the suitability of CL for therapeutic use to enhance PNS regeneration/reconstruction. In the present study, the effects of CL on spinal cord motor neuron, an obligatory player in peripheral nerve regeneration, were investigated. Therefore, the effects of CL on motor-neuron-like NSC-34 cells in primary cultures and on motor neurons in organotypic spinal cord cultures were analyzed. NSC-34 is a hybrid cell line produced by the fusion of neuroblastoma with mouse motor neuron-enriched primary spinal cord cells [16]. These cells share several morphological and physiological characteristics with mature primary motor neurons [16–18] and thus are an accepted model for studying the pathophysiology of motor neurons. We monitored CL effects on the proliferation, stress (OGD) response, neurite sprouting and calpain-I as well as Src and α-spectrin expression of NSC-34 cells. In organotypic cultures, the effects of CL on motor neuron survival and neurite sprouting after preparation-induced stress were analyzed.

Material and methods

NSC-34 cell line

Cultivation

NSC-34 cells were stored at −80 °C in cryo tubes. Before using the cells, they were pre-cultured for 7 days (days in vitro, DIV) in 75-cm² flasks at 37 °C in humidified 5% CO_2 atmosphere (normal conditions). After this pre-culturing, cells were harvested using a cell scraper, centrifuged for 10 min at 360g, resuspended in 10 ml pyruvate-free Dulbecco's modified Eagle's Medium (DMEM; Gibco® Invitrogen, Darmstadt, Germany; containing 4.5 g/l glucose; 10% fetal calf serum (FCS), Gibco®; 0.2% Ciprobay, Gibco®; normal medium) and plated at different densities (described below) under different experimental conditions (DIV 0). For experiments under oxygen glucose deprivation (OGD), glucose-free DMEM containing 10% FCS and 0.2% Ciprobay (OGD-medium) were used. Anaerobic conditions (OGD conditions) were reached by exposing the cultures to an atmosphere composed of 5% CO_2 and 1% O_2 (using nitrogen gas to displace ambient air in an incubator C200, Labotect GmbH, Göttingen, Germany) at 37 °C. For reoxygenation the incubator atmosphere was reestablished to 5% CO_2 and 21% O_2 and glucose (4.5 mg/ml) was added. For neurite sprouting experiments, a differentiation medium (Diff-medium) based on DMEM/F12 (Gibco®) containing 1% non-essential amino acids (NEM, Gibco®), 1% Ciprobay and 1% FCS was used under normal conditions. For reference, culturing under normal conditions with normal medium was performed.

Cerebrolysin©

The CL used in these experiments was freshly prepared from 1-ml vials delivered by EVER Neuro Pharma GmbH (Unterach, Austria) with a basic concentration of 215.2 mg/ml and was tested at end concentrations in culture medium containing 0.5 mg/ml (2.3 µl/ml), 2.5 mg/ml (11.6 µl/ml) or 5.0 mg/ml (23.2 µl/ml). For each experimental condition, a control group was evaluated, which underwent whole cultivation but was not treated with CL (detailed CL application regimens are presented in the following chapters).

Assessment of cell proliferation/survival (MTT)

To determine the viability of the NSC-34 cultures, MTT tests were performed. Namely, the specific turnover of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 6 mg/ml; Sigma-Aldrich, Munich, Germany) to formazan by viable cells was analyzed using photometry.

Pre-cultured NSC-34 cells were plated in 96-well plates with a cell density of 10,000 cells per well (DIV 0). OGD was induced at DIV 1. Briefly, the medium was completely removed, and either normal medium under normal conditions or OGD medium under OGD conditions was administered. CL (0.5 mg/ml, 2.5 mg/ml, 5.0 mg/ml) was added 24 h before and again in parallel with the respective medium change (pre-treatment groups, Pre-CL), only in parallel with (Para-CL) or 8 h after OGD induction (Post-CL). The cultivation and treatment regimens for these basic MTT tests are summarized in Table 1.