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Effects of cerebrolysin on rat Schwann cells *in vitro*

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ABSTRACT

Although the peripheral nervous system (PNS) is capable of regeneration, these processes are limited. As a potential means to augment PNS regeneration, the effects of cerebrolysin (CL), a proteolytic peptide fraction, were tested *in vitro* on Schwann cell (SC) proliferation, stress resistance, phagocytic and cluster-forming capacity. Primary SC/fibrocyte co-cultures were prepared from dorsal root ganglia of 5–7-day-old rats. SCs were subjected to mechanical stress by media change and metabolic stress by serum glucose deprivation (SGD). Cell survival was assessed using MTT test. SC proliferation was determined by counting BrdU-labeled cells. SC clustering was studied by ImageJ analysis of S100 immunostaining. Wallerian degeneration (WD) was evaluated by measuring acetylcholine-esterase staining within sciatic nerves *in vitro*. It was found that CL caused no effect on MTT turnover in the tested doses. CL inhibited SC proliferation in a dose-dependent manner. Media change and additional SGD stress inhibited SC clustering. CL enhanced the reorganization of SC clusters and was able to counteract SGD-induced cluster defects. Moreover, CL accelerated WD *in vitro*. CL was able to enhance the functions of SCs that are relevant to nerve regeneration. Thus, our findings suggest that CL may be suitable for therapeutic usage to enhance PNS regeneration/reconstruction.

Introduction

Although the peripheral nervous system (PNS) is able to regenerate itself to a certain extent, these mechanisms do not proceed at an optimal rate, and the PNS rarely recovers to pre-injury levels (Frostick et al., 1998).

Schwann cells (SCs) support and myelinate axons within the PNS. Immature SCs, which are derived from the neural crest through intermediate SC precursors (Mirsky and Jessen, 1996), can differentiate into both myelin-forming and non-myelin-forming cells, and these cells play a central role in the proper function and regeneration of peripheral nerves.

After a peripheral nerve is injured, the distal stump undergoes Wallerian degeneration (WD), which involves disintegration of the axonal membrane and myelin degradation. SCs and infiltrated macrophages clear cellular debris from the injury site and SCs from the distal nerve stump undergo proliferation and phenotypic changes to prepare the local environment for axonal ingrowth. Endoneurial tubes, so-called Bands of Bungner, are formed by the remaining connective tissue basement membranes (Frostick et al., 1998; Campbell, 2008), and SCs migrate from the two nerve ends to form a continuous bridge leading the sprouting axons toward and through the bands (Torigoe et al., 1996). Localized signaling by various cytokines and growth factors, as well as interactions between SCs and sprouting axons, whether they are from motor neurons or the dorsal root ganglia (DRG), are also necessary for proper nerve regeneration. For example, SCs lose their protective properties if interactions between SCs and the axon are broken (Dedkov et al., 2002), which can result in impaired regeneration of the related axon. Therefore, treatments involving neurotrophic factors and the manipulation of SCs have been suggested as potential therapies for enhancing nerve regeneration (Pollock, 1995).

Cerebrolysin (CL) is a proteolytic extract from the pig brain. One milliliter CL consists of 75% free amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, thyrosine) and 25% low molecular weight peptides (Ebewe, 1998; Hartbauer et al., 2001) which are biologically active and 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) (Chen et al., 2007). It has been shown in mice that CL can protect hippocampal neurons from damaging phenomena such as glutamate excitotoxicity (Riley et al., 2006) and that it has neuroprotective effects in cortical neurons against oxygen glucose deprivation (OGD) (Schauer et al., 2006). Positive effects have been reported for CL following avulsion injury of the ventral root (Haninec et al., 2003, 2004). Furthermore, similar positive effects have been demonstrated for certain individual

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components of CL, such as CNTF and IGF-1 (Haninec et al., 2003; Dubový et al., 2011).

The effects of CL have also been studied in a clinical setting, and it was reported that CL has neuroprotective effects in stroke patients (Ladurner et al., 2005). Today, CL is approved for use in 44 countries for treatment of dementia and stroke (Berk and Sabbagh, 2013). However, its clinical efficacy is still controversial.

For the above reasons, we aimed to test whether treatment of peripheral nerve lesions with CL can enhance the regeneration process. First, we analyzed the effects of CL on the proliferation of SCs, as the length of the G1 phase of the cell cycle can influence the differentiation process (reviewed by Salomoni and Calegari, 2010). Next, we determined whether CL treatment led to increased SC differentiation, which is important for the regeneration-promoting properties of SCs (*e.g.*, forming the bands of Bungner). We also tested the influence of CL on SC clustering under normal and stressful conditions (serum glucose deprivation) *in vitro*. Finally, as it is known that successful regeneration requires WD (Keilhoff et al., 2002), we tested the effect of CL on WD, the initial phase of regeneration, using sciatic nerves *in vitro*.

Materials and methods

All experiments were conducted in accordance with the guidelines of the German Animal Welfare Act, and the protocols were approved by a committee from Saxony-Anhalt. All animals were derived from our institute's breeding population. The rats (Wistar, inbred, Harlan–Winkelmann; Borchen, Germany) were housed under controlled laboratory conditions (light cycle of 12 h light/12 h dark with lights on at 6:00 a.m.; temperature = 20 ± 2° C; air humidity = 55–60%) with free access to water and chow.

Schwann cell/fibrocyte co-culture

After a peripheral nerve lesion, SCs come into contact with fibroblasts. In this way, fibroblasts influence the behavior of SCs *in vivo*, *i.e.* by stimulating the SC-SC adhesion, suggesting an important role of fibrocytes in peripheral nerve regeneration (Parrinello et al., 2010). To simulate exactly this environment *in vitro* we established a SC/fibrocyte co-culture. Therefore, 5–7-day-old rat pups were sacrificed by decapitation. The DRG were removed and incubated for 3 h at 37 °C in Dulbecco's modified Eagle's Medium (DMEM, Gibco® Invitrogen, Darmstadt, Germany) containing 6 g/l glucose, collagenase (0.05%), dispase (1.25 U/ml), hyaluronidase (0.1%) and streptomycin/penicillin (1%). Following dissociation of the DRG, the suspension was centrifuged at 360 g for 5 min. The supernatant was carefully removed, and the pellet was resuspended in DMEM containing 6 g/l glucose, streptomycin (50 μg/ml), penicillin (50 U/ml) and 10% fetal calf serum (FCS); this solution was also used for normal culturing (normal medium). The cells were counted using a Neubauer counting chamber, and 2×10^5 cells were plated in 2 ml media on laminin-coated coverslips (25 mm; Sigma–Aldrich, Munich, Germany) placed in 35-mm Petri dishes (DIV 0). For the MTT tests (see below), SCs were plated on laminin-coated 96-well plates at a density of 10,000 cells/well in 100 μl normal medium. The culture media was changed every two days.

Cerebrolysin

The CL used in these experiments was freshly prepared from 1-ml vials (EVER Neuro Pharma GmbH (Unterach, Austria) with a basic concentration of 215.2 mg/ml and was tested at end-concentrations in culture medium of 0.5 mg/ml (2.3 μl/ml) or 2.5 mg/ml (11.6 μl/ml). For the cell proliferation and WD experiments, CL was added immediately after plating and after every media change over the course of the experimental procedures. For the stress experiments (see below), CL was added 24 h before (pretreatment groups, Pre-CL) or in parallel with stress induction (Para-CL). For each experimental condition a control group was evaluated which underwent whole cultivation, but was not treated with CL. Stress was induced at DIV 6. Briefly, the media were completely removed and either normal medium or serum/glucose-free (SGD) medium was added back. Afterwards, CL was added (for the

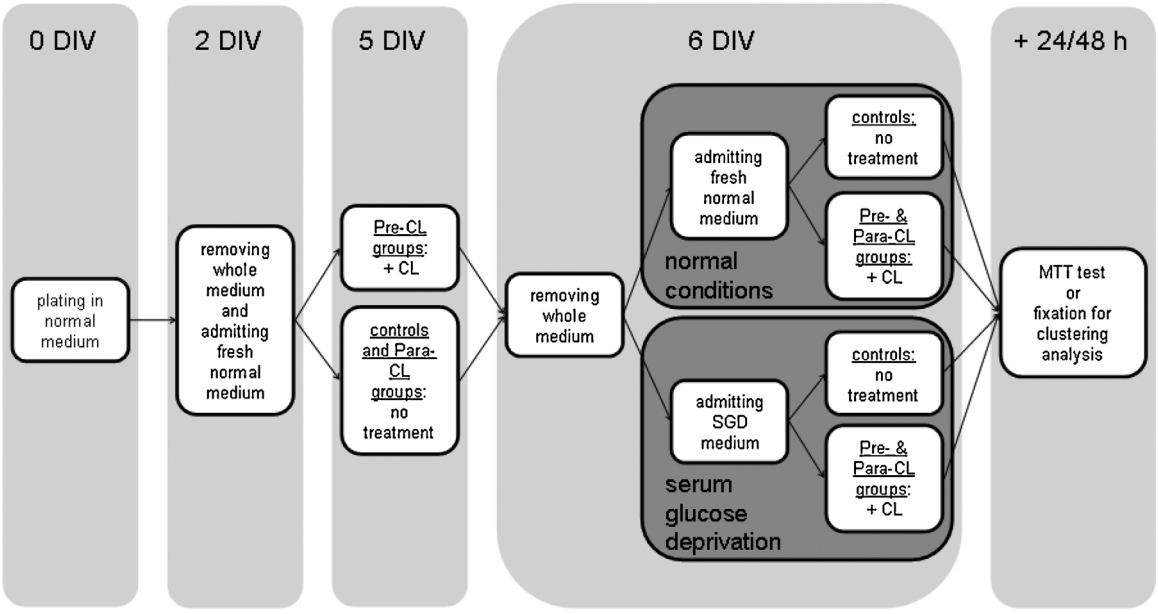

Fig. 1. Cultivation and treatment regime of SC/fibrocyte co-cultures. Cultivation/treatment regime of SC/fibrocyte co-cultures carried out for MTT tests and analysis of SC clustering. CL, cerebrolysin; DIV, day in culture; Para-CL, CL was added in parallel with SGD; Pre-CL, CL was added 24 h before SGD; SC, Schwann cell; SGD, serum glucose deprivation.