The mode of spinal motor neurons degeneration in a model of slow glutamate excitotoxicity in vitro

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Abstract
The defective glial and/or neuronal glutamate transport may, in chronic neurotoxicity, contribute to several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) – a progressive neurodegenerative disorder of lower and upper motor neurons (MNs). To determine the detailed ultrastructural characteristics of excitotoxic motor neurons neurodegeneration we used a model of slow excitotoxicity in vitro based on selective inhibition of glutamate uptake. The study was performed on organotypic cultures of the rat lumbar spinal cord subjected to various concentrations of glutamate uptake blockers: threo-hydroxyaspartate (THA) and L-trans-pyrrolidine-2, 4-dicarboxylate (PDC). The chronic inhibition of glutamate transport resulted in a dose-dependent slow neurodegeneration of spinal MNs consisting of necrotic, apoptotic and autophagic mode of cell death. There were some MNs that shared certain characteristics of a different type of cell injury. The results showed that a different mode of cell death in excitotoxic MNs degeneration may coexist resulting in apoptosis-necrosis and apoptosis-autophagocytosis continuum.

Key words: glutamate uptake blockers, spinal cord cultures, SLA model, ultrastructure

Introduction
Glutamate in mammalian central nervous system (CNS) may act as an excitatory neurotransmitter and as a potent neurotoxin as well [24,38]. The chronic excitotoxicity has been suggested to be a common final pathway in the pathogenesis of various neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) – a progressive neurodegenerative disorder of lower and upper motor neurons (MNs) [2,15,29,34,36]. It has been suggested that the selective loss of MNs in ALS can be in part due to the defective glial or neuronal glutamate transport related to concomitant persistent exposure to synaptic glutamate. A useful model of slow glutamate excitotoxicity in vitro, based on selective inhibition of glutamate uptake with sustained elevation of extracellular glutamate in culture medium, was introduced by Rothstein et al. [35]. The main advantage of this model is the maintenance of neuron-astrocyte structural and metabolic interactions that is of particular importance in neuronal response. To date,
several subtypes of glutamate transporters have been identified, including GLAST and GLT-1 which are primarily expressed on the surface of astrocytes membranes [6,7,12,17,20,33]. Some evidence underlines the severe decrease of GLT-1 expression in the motor cortex and spinal cord of patients with ALS [36]. It has been suggested that the glial component might be involved in the progressive MNs loss in ALS through glutamatergic toxicity. The detailed morphological studies of MNs pathology important for understanding the mechanism of cell death in neurodegenerative disorders are lacking.

The aim of this study was to determine the ultrastructural characteristics of MNs changes in a model of slow neurodegeneration in vitro. The contribution of different modes of neuronal death was established in the organotypic cultures of rat lumbar spinal cord that was chronically exposed to various concentrations of specific glutamate uptake blockers: DL-threo-β-hydroxyaspartate (THA) and L-trans-pyrrolidine-2, 4-dicarboxylate (PDC).

Material and methods

Organotypic cultures were prepared from the spinal cord obtained from 8-day-old rat pups. The lumbar spinal cords were dissected under sterile conditions and cut transversely into thin slices. The explants were placed on the collagen-coated cover glasses with two drops of nutrient medium and sealed into the Maximow double assemblies. The cultures were kept at 36.6°C in a medium consisting of 25% inactivated fetal bovine serum and 75% DMEM (Dulbeco Modified Eagle's Medium) supplemented with glucose to a final concentration of 600 mg% and with antibiotics. The medium was changed twice a week. On the 10-14th day in vitro (DIV), the well-differentiated cultures were

![Fig. 1. Normal motor neuron with large nucleus, dispersed chromatin, distinct nucleolus and abundant cytoplasm containing numerous cytoorganelles. Control culture, 28 DIV. Bar = 2 µM](image1)

![Fig. 2. Completely damaged neuron (N) with total destruction of cytoorganelles and nucleus. 3 days of 100 µM THA incubation. Bar = 1 µM](image2)
incubated with medium containing selective blockers of glutamate transport: DL-threo-β-hydroxyaspartate (THA, Sigma) and L-trans-pyrrolidine-2, 4-dicarboxylate (PDC, Sigma) at concentration 100 µM and 500 µM. After 2 and 24 hours, 3, 5, 7, 14 and 28 days post treatment the cultures were processed for electron microscope. They were rinsed in cacodylate buffer (pH 7.2), fixed in a mixture containing 0.8% formaldehyde and 2.5% glutaraldehyde for 1 hour, postfixed in 1% osmium tetroxide, dehydrated in alcohols in graded concentrations and embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope.

Results

The control spinal cord cultures up to 28 DIV maintained the well-preserved large MNs and numerous normally appearing astroglial cells, mainly of protoplasmic type. The normal MNs were characterized by a large nucleus with dispersed chromatin and distinct nucleolus surrounded by an abundant cytoplasm containing well-developed granular endoplasmic reticulum, Golgi apparatus, numerous mitochondria and neurotubules (Fig. 1).

The spinal cord cultures treated with THA or PDC displayed slowly progressing MNs degeneration up to 28 days. The picture of neuronal degeneration depended on the concentration of the agent and the duration of exposure. A wide spectrum of morphological changes reflecting distinct features of apoptotic, autophagocytic and/or necrotic mode of neuronal death was identified in cultures treated both with THA and PDC. Up to 24 hours of THA exposure at concentration 100 µM, the majority of large neurons exhibited only subtle morphological abnormalities.
limited to mitochondrial swelling. At 3 days post THA treatment some MNs presented vacuolar neurodegeneration, ranging from focal accumulation of vacuoles and vesicles to severe cytoplasmic vacuolization. Only few MNs developed the features of acute necrotic damage with total destruction of cytoplasmic organelles and nucleus (Fig. 2). During the period of 3 to 7 days of THA treatment the neurons displayed various morphological changes including vacuolization of the neuronal cytoplasm or typical apoptotic features (Fig. 3). At later stages, after 7-21 days after exposure, the apoptotic and autophagic mode of neuronal damages predominated. However, the pure apoptotic changes characterized by typical condensation and margination of nuclear chromatin beneath the nuclear envelope accompanied by shrinkage of the cell body with relatively intact cytoorganelles could be seen only occasionally. In the majority of MNs the nuclear apoptotic changes were accompanied by certain characteristics of either necrotic or autophagic degeneration of the cytoplasm. Some neurons displayed aggregation of nuclear chromatin in dense masses or curved half-moon profiles associated with advanced cytoplasmic vacuolization (Fig. 4). Numerous MNs developed the peripheral aggregation of nuclear chromatin indicating early apoptotic changes, whereas their cytoplasm exhibited more or less destructed cytoorganelles and/or autophagic vacuoles (Fig. 5, 6). A subset of MNs displayed distinct cytoplasmic characteristics of typical autophagic degeneration with numerous double-membrane bound autophagosomes filled with degenerative cytoplasmic organelles (Fig. 6). Fragments of degenerated cells and membrane-bound apoptotic profiles containing fragments of condensed chromatin and cytoplasmic structures were often ingested by neighboring glial cells (Fig. 7).
The higher concentration of THA (500 µM) produced rapidly progressing MNs degeneration with more advanced acute necrotic cell death within 3 days. Completely damaged neurons with highly condensed chromatin and severely damaged cytoplasmic organelles were often seen (Fig. 8). MNs exhibiting apoptotic and/or autophagic characteristics of cell death could be seen in the later stages of observation.

PDC treatment resulted in a similar but less advanced MNs degeneration over the same time of observation. The process of neurodegeneration was characterized by different modes of cell death including necrosis, apoptosis and autophagocytosis and their combination.

MNs degeneration was accompanied by distinct astroglial changes, some of which proceeded the massive neuronal damage. Protoplasmic astrocytes exhibited swelling of peripheral parts of the cytoplasm already after 24 hours of THA and PDC incubation and the presence of irregular vacuoles in the cytoplasm during the later period of experiment.

Discussion

MNs death in ALS is linked to several pathogenic factors including aberrant excitatory neurotransmission [9,15,36,46], increased formation of reactive oxygen species [40] and abnormalities of neurofilaments function [1]. The cellular mechanism by which motor neurons slowly degenerate after chronic inhibition of glutamate uptake is not fully understood, although it appears to be mediated via non-NMDA receptors [37]. The evidence of both, the glutamate deficiency in brain and spinal cord [29] and the elevated level of glutamate in serum and CSF in ALS patients [21,34] has been shown.

Tissue culture represents a useful experimental system for the characterization of the mode of
neuronal degeneration. Until now, several models of MNs injury in vitro, reproducing the in vivo conditions, have been established [41]. The model of chronic glutamate excitotoxicity, originally developed by Rothstein et al. [35] on organotypic spinal cord slices, seems to be particularly useful for the study of MNs degeneration. This model was based on slow glutamate-receptor mediated neuronal death induced by incubation with the specific glutamate transporter inhibitors. Both, NMDA and non-NMDA subtypes of glutamate receptors, by which glutamate toxicity can be mediated, have been evidenced on spinal MNs [39]. Glutamate transporters play an important role in the maintenance of extracellular concentration of glutamate below neurotoxic level and harmful receptor overstimulation [13,28,42,43,44]. So far several subtypes of excitatory amino acid transporters (EAATs) have been cloned from mammalian tissue [3,10,18,42]. Two glutamate uptake blockers: threo-hydroxyaspartate (THA) and L-trans-pyrrolidine-2, 4-dicarboxylate (PDC) were identified as a potent inhibitor of EAAT 1-5 [14,32]. There is a growing evidence that reactive oxygen species (ROS) are involved in the pathogenesis of MNs degeneration in ALS [5,40]. Neurodegeneration may be a consequence of various mode of cell death induced and regulated by variety of intracellular and extracellular insults. There is a considerable controversy whether neurons injured via excitotoxic mechanism die by apoptosis or necrosis [8,31]. Some reports suggested that MNs death in ALS is mainly apoptotic [25,45]. It has been documented that caspases, essential for apoptotic cell death, are involved in MNs degeneration in patients with ALS [23]. The apoptotic mode of spinal MNs death has been also shown after neonatal nerve cell injury [19]. In vitro model of MNs toxicity caused by the chronic inhibition of mitochondrial electron transport led to a dose-dependent apoptotic death in cultured isolated motor neurons sensitive to oxidative stress [16]. On the other hand both modes of neuronal death such as necrosis and apoptosis have been evidenced in different preparation. The current belief is that cell death does not always represent a uniform event but is often a continuum of apoptotic and/or necrotic mode of death [11,31].

This morphological study of MNs degeneration in a model of ALS in vitro revealed ultrastructural features typical of a different type of cell injury. THA and PDC produced MNs degeneration in a dose dependent fashion. Ultrastructurally, MNs displayed either the pure and complete characteristics of necrotic, apoptotic and autophagocytic cell death or most often a mixture of different types of cell injury resulted in apoptotic-necrotic or apoptotic-autophagic continuum. The variation of ultrastructural features suggests that MNs death may occur along a complete or exclusive apoptotic, necrotic or autophagocytic pathway or as concurrently appearing different mode of cell death. It is well known that different toxic stimuli start the apoptotic program of cell death, which may be either completed or may be interrupted and progress a different way of cell damage. Increasing evidence shows that different modes of neuronal degeneration may coexist in various pathological conditions [4,11,26].

The present results suggest that MNs can die in different ways, including apoptosis, necrosis and autophagocytic degeneration, and that the continuum between apoptosis and necrosis or apoptosis and autophagocytosis in neurodegenerative conditions in vitro may exist.

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13