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Increased neuronal Rab5 immunoreactive endosomes do not colocalize with TDP-43 in motor neuron disease

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ABSTRACT

Sporadic motor neuron disease (MND) is characterized by progressive degeneration of motor neurons and intraneuronal cytoplasmic translocation and deposition of the nuclear protein TDP-43. There is a paucity of data on the subcellular mechanisms of the nuclear-cytoplasmic trafficking of TDP-43, particularly about the precise role of the endosomal–lysosomal system (ELS). In the present study, using a neuron-specific morphometric approach, we examined the expression of the early endosomal marker Rab5 and lysosomal cathepsins B, D, F, and L as well as PAS-stained structures in the anterior horn cells in 11 individuals affected by sporadic MND and 5 age-matched controls. This was compared with the expression of ubiquitin, p62 and TDP-43 and its phosphorylated form. The principal finding was the increased expression of the endosomal marker Rab5 and lysosomal cathepsin D, and of PAS-positive structures in motor neurons of MND cases. Furthermore, the area-portion of Rab5 immunoreactivity correlated well with the intracellular accumulation of ubiquitin, p62 and (phosphorylated) TDP-43. However, double immunolabelling and immunogold electron microscopy excluded colocalization of phosphorylated TDP-43 with the ELS. These data contrast with observations on neuronal cytopathology in Alzheimer's or prion diseases where the disease-specific proteins are processed within endosomes, and suggest a distinct role of the ELS in MND.

Introduction

Motor neuron disease (MND) or amyotrophic lateral sclerosis is characterized by the degeneration of either or both upper and lower motor neurons. In addition to shrunken and chromatolytic neurons, intracytoplasmic, mainly ubiquitinated, protein deposits may be observed in neurons (Kato, 2008). Recent studies indicated a crucial role for the TAR DNA-binding protein (TDP-43) and its phosphorylated form in the pathogenesis of MND (Benajiba et al., 2009; Hasegawa et al., 2008). Hyperphosphorylated, ubiquitinated and N-terminally truncated TDP-43 is the pathological hallmark lesion in most familial and sporadic forms of MND (Lin and Dickson, 2008; Mori et al., 2008; Neumann et al., 2009). Genetic studies indicate that mutations in the *TARDBP* gene are associated with some familial and sporadic forms of MND (Sreedharan et al., 2008). Thus, MND, as a TDP-43 proteinopathy, shows similarity to other neurodegenerative diseases characterized by the deposition of certain proteins (Cairns et al., 2007; Geser et al., 2009). In contrast to Alzheimer's disease (AD), Parkinson's disease (PD), or Creutzfeldt-Jakob disease (CJD), where the role of the endosomal–lysosomal system (ELS) was suggested in the processing of disease-associated proteins (Kovacs et al., 2007; Nixon and Cataldo, 1995; Pan et al., 2008), little is known about the precise mechanism of the nuclear-cytoplasmic trafficking of TDP-43 and the role of the ELS, in particular in view of the enhanced capability of TDP-43 for aggregation and cellular toxicity (Johnson et al., 2009; Zhang et al., 2009).

Physiological functions of the ELS include endocytosis, trafficking, and degradation of protein molecules, and also involve transition from the early endosome to the late endosome and lysosome (Conus and Simon, 2008; Zaidi et al., 2008). The GTPase Rab5 is one of the key coordinators of vesicle traffic that regulate mainly the early steps of the endocytotic pathway (Rink et al., 2005; Stenmark, 2009). Cathepsins are lysosomal peptidases localized within lysosomes and belonging to cysteine, serine or aspartic protease classes. Cathepsins B, F, and L are cysteine proteases of the papain family, and cathepsin D is an aspartic protease (Conus and Simon, 2008). Their functions are important for normal protein turnover and were found to be involved in certain pathological conditions such as cancer or neurodegenerative disorders (Kuester et al., 2008; Nixon et al., 2008).

A previous study indicated that the ELS, namely the lysosomal cathepsin B, may play a role in the pathogenesis of MND (Kikuchi et al.,

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2003). However, there is paucity of data on other components of the ELS in the pathogenesis of MND, in particular whether these have any relation with the intraneuronal protein deposition. In the present study we examined the expression of the early endosomal marker Rab5 and lysosomal cathepsins B, F, L, and D in anterior horn cells in individuals affected by MND, in comparison with the expression of ubiquitin, p62 and TDP-43 and its phosphorylated form. Using double immunolabelling and immunogold electron microscopy, we investigated the relationship of phosphorylated TDP-43 to cell compartments. Our results indicate that an increased expression of the early endosomal marker Rab5 correlates well with intracellular protein deposition, whereas phosphorylated TDP-43 protein aggregates are located outside the ELS, suggesting no primary relation of the ELS with the translocation process of TDP-43.

Materials and methods

Tissue samples

This study included autopsy material from the spinal cord of 11 cases of adult-onset sporadic MND (age range: 48–74 years, median: 65.3 years) and 5 non-diseased control cases (age range: 46–69 years, median: 62.6 years). All control cases underwent neuropathological examination to exclude alterations in the brain and spinal cord. Brain and spinal cord were fixed for 3–4 weeks in buffered 10% formalin and then embedded into paraffin. Sections (5 μm thick) were prepared and stained with hematoxylin–eosin, Klüver–Barrera, and periodic-acid-Schiff (PAS). In the present study the cervical level of the spinal cord was evaluated. The data were analysed with respect for patients' privacy, and the local Ethics Committee of the Institute of Postgraduate Education in Medicine, Prague, Czech Republic, approved the protocol.

Immunohistochemistry

For immunohistochemistry 5-μm-thick sections of formalin-fixed and paraffin-embedded tissue were used with primary antibodies against the following antigens: 1) cathepsin D (1:200, rabbit polyclonal, recognizes the 34-kDa mature form of cathepsin D; Biogenex, San Ramon, CA); 2) cathepsin B (1:200, mouse monoclonal, recognizes mature cathepsin B and procathepsin B; Alexis, San Diego, CA); 3) Rab5 (1:500, rabbit polyclonal; Calbiochem, San Diego, CA); 4) cathepsin L (1:200, rabbit polyclonal, recognizes mature cathepsin L and procathepsin L; produced in the laboratory of NKJ, Ljubljana, Slovenia); 5) cathepsin F (1:100, rabbit polyclonal; recognizes mature cathepsin F and procathepsin F; produced in the laboratory of NKJ, Ljubljana, Slovenia); 6) ubiquitin (1:200, rabbit polyclonal; Dako, Glostrup, Denmark); 7) protein p62 (1:4000, guinea pig polyclonal; Progen Biotechnik GmBh, Heidelberg, Germany); 8) protein TDP-43 (1:2000, mouse monoclonal; Abnova Corp. Taipei, Taiwan; and 1:100, polyclonal; ProteinTech Group, Chicago, IL, USA), and 9) phospho-TDP-43 (1:2000, mouse monoclonal, pS409/410; Cosmo Bio Co. Ltd, Tokyo, Japan). Double immunolabelling was performed using monoclonal phospho-TDP-43, TDP-43 and polyclonal cathepsin D, L, or Rab5 antibodies. We evaluated double immunofluorescent labelling with a Zeiss LSM 510 confocal laser microscope. The fluorescence-labelled secondary antibody for TDP-43 and phospho-TDP-43 was Alexa Fluor 488 goat anti-mouse IgG (1:200; Molecular Probes, Inc., Eugene, OR, USA) and for Rab5, cathepsin D or L was Alexa Fluor 633 goat anti-rabbit IgG (1:200; Molecular Probes, Inc.).

Light microscopic morphometry

For the quantification of the volume and area-portion of Rab5, cathepsins B, D, L, and F immunoreactive structures in the anterior motor neurons in MND and control cases, we followed a previously published protocol (Kovacs et al., 2007). Briefly, we selected eight motor neurons that had the nucleolus in focus. We optically dissected the nucleus of each neuron and copied the outline of the cytoplasm into a white background image created in Adobe Photoshop 7.0. We then selected a brown immunoreactive dot representing cathepsin B, D, F, L, or Rab5 immunopositivity or a purple dot representing PAS positivity. The tolerance level was set to include both weaker and stronger immunoreactivity. Using a black background, we cut the selection and thus only black cytoplasmic dots from each neuron were present in the image. This was followed by selection and cutting the cytoplasmic area of the same neuronal images with a black background, creating a black image of the neuronal cytoplasmic area. To determine the area-portion of black dots per unit of cytoplasmic area we used analySIS software (Soft Imaging System, Münster, Germany) with similar threshold values.

For the quantification of the neuronal volume, the distance of the nucleolus to the border of the cell was measured using the software Axiovision (4.3). The volume equation was $(4^*\pi/3)^*l^3$, where l is the distance from the nucleolus to the border of the cell. This approach was considered to introduce only a small bias (Moller et al., 1990). This line from the nucleolus to the border of the neuronal cytoplasm was different for each neuron, as we changed the orientation of the lines in each image in four consequent angles. The neuronal volume estimates have not been corrected for tissue shrinkage.

In addition, we determined the proportion of neurons showing inclusions by counting all anterior horn neurons and those containing ubiquitin, p62, TDP-43, or phospho-TDP-43 immunoreactive inclusions, and neurons showing granular cytoplasmic phospho-TDP-43 immunoreactivity.

Immunoelectron microscopy

To evaluate the ultrastructural localization of phospho-TDP-43 immunoreactivities detected by light microscopical immunohistochemistry, we performed a partially modified pre-embedding immunogold-silver staining method described previously (Hacker et al., 1996). Briefly, 5-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples were incubated with the primary antibody against phospho-TDP-43 (1:1000; mouse monoclonal, pS409/410; Cosmo Bio Co. Ltd, Tokyo, Japan) in TBS containing 10% (v/v) normal goat serum (NGS, Vector Laboratories Peterborough, UK) and 0.01 g/ml glycine (Sigma-Aldrich, St-Louis, MO, USA) for 48 h at 4° C. As a second layer of labelling we used ultrasmall (0.8 nm) gold-conjugated secondary antibody (1:50, Aurion, Wageningen, The Netherlands) in TBS containing 0.1% cold-water fish gelatine (Aurion), 2.0% (v/v) NGS and 0.01 g/ml glycine for 60 min at room temperature. To visualize the bound gold particles, a silver-enhancing kit (R-GENT SE-LM, Aurion, Wageningen, The Netherlands) was applied in accordance with the instructions of the supplier. After post-fixation in 2.0% (v/v) glutaraldehyde and en block staining in 1.0% osmium tetroxide, the immunolabelled slices were dehydrated and mounted with epoxy resin for light microscopical examination. Selected areas containing immunoreactive cells were cut and re-embedded for ultrathin sectioning. Ultrathin sections were mildly counterstained with lead citrate and uranyl acetate prior to the electron microscopic investigation to analyse the ultrastructural characteristics and distribution of immunolabelling.

Statistical evaluation

We used the software SPSS 16.0 and performed one-way analysis of variance test to evaluate the significance between the neuronal area-portion values of immunoreactivities and neuronal volumes. We used the Pearson correlation test to compare each median area-portion values of immunoreactivities with the proportion of neurons containing pathological inclusions evaluated by immunostaining for TDP-43, phospho-TDP-43, p62, and ubiquitin.