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Abnormal cortical lysosomal β-hexosaminidase and β-galactosidase activity at post-synaptic sites during Alzheimer's disease progression

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ABSTRACT

A critical role of endosomal–lysosomal system alteration in neurodegeneration is supported by several studies. Dysfunction of the lysosomal compartment is a common feature also in Alzheimer's disease. Altered expression of lysosomal glycohydrolases has been demonstrated not only in the brain and peripheral tissues of Alzheimer's disease patients, but also in presymptomatic subjects before degenerative phenomenon becomes evident. Moreover, the presence of glycohydrolases associated to the plasma membrane have been widely demonstrated and their alteration in pathological conditions has been documented. In particular, lipid microdomains-associated glycohydrolases can be functional to the maintenance of the proper glycosphingolipids pattern, especially at cell surface level, where they are crucial for the function of cell types such as neurons. In this study we investigated the localization of β-hexosaminidase and β-galactosidase glycohydrolases, both involved in step by step degradation of the GM1 to GM3 gangliosides, in lipid microdomains from the cortex of both an early and advanced TgCRND8 mouse model of Alzheimer's disease. Throughout immunoprecipitation experiments of purified cortical lipid microdomains, we demonstrated for the first time that β-hexosaminidase and β-galactosidase are associated with post-synaptic vesicles and that their activities are increased at both the early and the advanced stage of Alzheimer's disease. The early increase of lipid microdomain-associated β-hexosaminidase and β-galactosidase activities could have relevant implications for the pathophysiology of the disease since their possible pharmacological manipulation could shed light on new reliable targets and biological markers of Alzheimer's disease.

1. Introduction

Alzheimer's Disease (AD) is the most common cause of dementia in the elderly characterized by memory loss and cognitive decline (Parihar and Hemnani, 2004). The progressive nature of neurodegeneration leads to synaptic failure and neuronal damage in brain cortical areas (Isacson et al., 2002). In particular, many findings support the hypothesis that the memory failure in AD results from synaptic dysfunction and loss of synapses is a key event in early cognitive decline (Gylys et al., 2004).

Mutations in the β-amyloid precursor protein (APP) or presenilin (PSN) genes have been described in patients with familial early-onset AD (Bertram and Tanzi, 2008). However, the majority of late-onset AD cases are not genetically transmitted and their

Abbreviations: CT-B, cholera toxin B subunit; flot-2, flotillin-2; Gal, β-galactosidase; GM1, Galβ1,3GalNAcβ1,4-(NeuAcα2,3)-Galβ1,4Glc-ceramide; GM2, 3GalNAcβ1,4-(NeuAcα2,3)-Galβ1,4Glc-ceramide; GM3, NeuAcα2,3Galβ1,4Glc-ceramide; GSL, glycosphingolipid; Hex, β-hexosaminidase; Man, α-mannosidase; MUGal, 4-methylumbelliferyl-β-D-galactopyranoside; MUG, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide; MUGS, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide-6-sulphate; MUMan, 4-methylumbelliferyl-α-D-mannopyranoside; PSD-95, postsynaptic density protein-95; syp, synaptophysin; TX-100, Triton X-100; Tween 20, polyethylene glycol sorbitanmonolaurate.

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pathogenesis remain unknown. Definitive clinical and neuropathological manifestations of both early- and late-onset sporadic AD forms are indistinguishable, with the exception of the first stage leading to the Aβ accumulation and oligomerization (Blennow et al., 2006; Magini et al., 2010; Selkoe, 2002).

Many neurological diseases are characterized by mutations of proteins involved in endosomal/lysosomal transport, and deregulation of endosomal trafficking is a common feature in AD. Evidence have been reported demonstrating an increase of lysosomal glycohydrolases activity in the brain and also in peripheral tissues of AD patients (Adamec et al., 2000; Cataldo et al., 1996, 1995; Emiliani et al., 2003; Mathews et al., 2002; Nixon et al., 2001). A critical role of lysosomal system alteration in neurodegeneration is supported by studies of primary lysosomal storage disorders (LSDs) (Tancini et al. 2010). GM1 and GM2 gangliosidoses are LSDs caused by defective β-galactosidase (Gal, EC 3.2.1.23) and β-hexosaminidase (Hex, EC 3.2.1.52) activity, respectively, characterized by prominent nervous system degeneration involving disruption of the internal environment of the lysosome (Mahuran, 1999; Walkley, 2009). Hex is an acidic glycohydrolase that cleaves terminal β-linked N-acetylglucosamine or N-acetylgalactosamine residues from oligosaccharides, glycolipids, glycoproteins and glycosaminoglycans (Mahuran, 1999) while Gal catalyses the hydrolysis of terminal N-linked galactosyl moiety from oligosaccharides and glycosides (Okada and O'Brien, 1968).

To date many reports indicate the presence of sialyltrasferases and glycohydrolases associated to the extracellular site of cell plasma membrane, where they participate to the modification of glycosphingolipids (GSLs) present at the cell surface (Aureli et al., 2011; Crespo et al., 2010; Magini et al., 2014, 2013, 2012, 2008; Mencarelli et al., 2005). GSLs and especially gangliosides are abundant and characteristic components of neuronal membranes and several studies show that impaired GSLs metabolism could be involved in AD. For instance, it has been recently demonstrated that the accumulation of GSLs promotes Aβ generation and impairs the clearance of APP C-terminal fragments. The authors suggest that accumulation of GSLs might not only underlie the pathogenesis of LSDs, but also contribute to neurodegeneration in sporadic AD (Tamboli et al., 2011). Ehehalt et al. (2003) reported evidence for lipid-raft-dependent ability of Aβ to act as a seed for fibril formation. In addition, the cholesterol-dependent sequestration of Aβ and the conformational change promoted by raft-associated GM1 have been considered to be crucial for plaque generation (Kakio et al., 2001; Mizuno et al., 1999). Therefore, the maintenance of the proper GSLs pattern, in particular at lipid microdomain levels, appears to be crucial for the correct function of neurons and to prevent the occurrence of neuro-pathological conditions (Sonnino et al., 2010).

In an effort to detect early events related to synapses loss, in this study we used a well characterized model of AD TgCRND8 (Chishti et al., 2001; Ploia et al., 2011; Sclip et al., 2014, 2011). We performed lipid microdomains purification from the cortex of both the early (3 months old) and advanced (6 months old) TgCRND8 mouse model of AD (Sclip et al., 2011) and analysed glycohydrolases Hex and Gal in lipid microdomains with respect to the AD progression. The association and behaviour of Hex and Gal glycohydrolases in the synapses of AD mouse cortex have also been investigated.

2. Materials and methods

2.1. TgCRND8 animal model

The transgenic TgCRND8 mouse exhibits disrupted spatial memory associated with amyloid plaque pathology that strongly summarizes human AD. TgCRND8 mouse expresses, under the control of the hamster Prion protein promoter, the human APP gene harbouring both the Swedish and Indiana familial AD mutations, that lead to elevated levels of $A\beta_{42}$ within the brain (Chishti et al., 2001).

Cortical tissue from both hemispheres was dissected from wild type (wt) and TgCRND8 (Tg) mice, both at 3 (early stage of AD) and 6 months of age (advanced stage of AD) to obtain the cortex which was immediately stored at −80 °C.

All the experiments were conducted in conformity with the European Communities Council Directive of November 1986 (86/609/ECC), in accordance with a protocol approved by the Animal Care and Use Committee at the University of Perugia.

2.2. Isolation of lipid microdomains

Frozen cortical tissue samples (20 mg) were thawed on ice and resuspended with 0.5 ml of 10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4 (TNE) containing 1% (v/v) Triton X-100 (TX-100). 20 μl of protease inhibitor cocktail were added. Tissue homogenization was performed by using a Dounce homogenizer (10 strokes). After 30 min on ice, the homogenate was centrifuged at 1500 × *g* for 5 min at 4 °C. The supernatant (1.5 mg of total proteins) was then mixed 1:1 with 0.5 ml of 85% (w/v) sucrose solution in TNE, transferred into a polyallomer centrifuge tube, then carefully overlaid with 2.75 ml of 35% (w/v) sucrose solution in TNE, and finally with 1.25 ml of 5% (w/v) sucrose solution in TNE. Thereafter, discontinuous sucrose-density gradient centrifugation was performed at 4 °C for 18 h at 45,000 rpm using a MLS-50 rotor and an Optima Max ultracentrifuge. Eleven fractions of equal volume (450 μl) were collected from the top to the bottom of the discontinuous sucrose-density gradient.

2.3. Determination of enzymatic activity and protein concentration

Total Hex, Hex A, Gal and α-Man activities were determined using a final concentration of 2 mM MUG (Sigma–Aldrich), 2 mM MUGS (Toronto Research Chemicals Inc.), 1 mM MUGal (Sigma–Aldrich) and 2 mM MUMan (Sigma–Aldrich), respectively, in 0.1 M citric acid/0.2 M disodium phosphate buffer, pH 4.5.

Reactions were performed in triplicate in 96-well black multiplates (Greiner, Frickenhausen, Germany) at 37 °C. At the end of the reaction period, 0.290 ml of 0.4 M glycine–NaOH buffer, pH 10.4 were added. Fluorescence of the liberated 4-methylumbelliferone was measured on the Infinite F200 fluorimeter (Tecan, Mannedorf, Switzerland) at 360 nm excitation, 450 nm emission. One enzymatic unit (U) is the amount of enzyme that hydrolyses 1 μmole of substrate/min at 37 °C.

Enzymatic activity was expressed as enzymatic units × 10^{-3} (mU). Specific activity (SA) was expressed as mU per mg of total proteins (mU/mg). Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

2.4. Western blotting and dot blot analysis

Fractions resulting from isolation of lipid microdomains were subjected to 10% SDS-PAGE under reducing conditions (Mini-Protean III, Biorad). Proteins were transferred to PVDF membrane (Biorad), blocked with 5% (w/v) skim milk in 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl containing 0.1% (v/v) Tween 20 (Sigma–Aldrich), and reacted with primary antibodies for 1 h at the following dilutions: 1:5000 for mouse monoclonal anti-flotillin-2 (BD Biosciences), 1:2000 for mouse monoclonal anti-PSD-95 (NeuroMab), 1:500 for rabbit polyclonal anti-synaptophysin (ABcam), 1:5000 for mouse monoclonal anti-actin (Sigma–Aldrich). After being washed, the blots were incubated with secondary antibodies