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Cystatin C is released in association with exosomes: A new tool of neuronal communication which is unbalanced in Alzheimer's disease

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Abstract

It has recently become clear that proteins associated with neurodegenerative disorders can be selectively incorporated into intraluminal vesicles of multivesicular bodies and subsequently released within exosomes. Multiple lines of research support a neuroprotective role for cystatin C in Alzheimer's disease (AD). Herein we demonstrate that cystatin C, a protein targeted to the classical secretory pathway by its signal peptide sequence, is also secreted by mouse primary neurons in association with exosomes. Immunoproteomic analysis using SELDI-TOF MS revealed the presence in exosomes of at least 9 different cystatin C glycoforms. Moreover, the over-expression of familial AD-associated presenilin 2 mutations (PS2 M239I and PS2 T122R) resulted in reduced levels of all cystatin C forms (native and glycosylated) and of amyloid-β precursor protein (APP) metabolites within exosomes. A better understanding of the mechanisms involved in exosomal processing and release may have important implications for the fight against AD and other neurodegenerative diseases.

Keywords: SELDI-TOF MS; APP; Electron microscopy; Glycosylation; Presenilin 2 mutations; Cystatin C; Exosomes

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in humans and is characterized neuropathologically by the extracellular deposition of insoluble amyloid fibrils as amyloid plaques, primarily composed of amyloid-β peptides (Aβ) (Selkoe, 1989). Mutations in the presenilin (PS) genes account for the majority of familial Alzheimer's disease (FAD) cases (http://www.molgen.ua.ac.be/ADmutations). The majority of PS mutations result in increased production of Aβ peptides, which are derived from the larger amyloid-β precursor protein (APP). Although FAD-linked PS mutations cause increased generation of Aβ42, a large and increasing number of FAD-linked PS mutations have been shown to inhibit other PS activities. It has been recently reported that presenilins are essential for regulating neurotransmitter release (Zhang et al., 2009), suggesting that PS mutations have pathological effects beyond those caused by their abnormal proteolytic function.

Some proteins associated with AD lesions may have a role in the pathological processes leading to amyloidogenesis and neuronal degeneration and others may bind secondarily to amyloid deposits. Neuropathological and molecular studies suggest a functional link between Aβ and cystatin C (Kaeser et al., 2007; Levy et al., 2001; Mi et al., 2007; Sastre et al., 2004; Vinters et al., 1990). The physiological high concentration of cystatin C in the cerebrospinal fluid and its proliferative effect on neural rat stem cells (Taupin et al., 2000) strongly suggest that cystatin C could exert a

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trophic function in the brain. While Aβ is the major amyloid forming peptide in the brains of AD patients, the cysteine protease inhibitor, cystatin C, co-localizes with Aβ predominantly in amyloid-laden vascular walls, and in senile plaque cores of amyloid in brains of patients with amyloidoses (i.e. AD, Down syndrome, cerebral amyloid angiopathy, hereditary cerebral hemorrhage with amyloidosis Dutch type and cerebral infarction) and nondemented aged individuals (Levy et al., 2006).

New evidence shows that neurons and astrocytes have the capacity to secrete membrane proteins by incorporating them into exosomes, which are small vesicles derived from the intralumenal membranes of multivesicular bodies (Fauré et al., 2006). From their original discovery in the removal of unwanted proteins from maturing reticulocytes through their role in immune surveillance, the scope of discovered functions continues to grow (Belting and Wittrup, 2008; Février and Raposo, 2004; Schorey and Bhatnagar, 2008; Vella et al., 2008). It has been recently demonstrated that also proteins and peptides (i.e. APP, APP C-terminal fragments, APP intracellular domain, Aβ, PS) associated with AD are released in association with exosomes (Rajendran et al., 2006; Sharples et al., 2008; Vingtdeux et al., 2007). The identification of Aβ in association with exosomes is an important finding especially since other exosomal proteins, such as alix and flotillin, have been found to accumulate in the plaques of AD brains (Rajendran et al., 2006). These findings could provide potential explanation for extracellular amyloid deposition in the brain. We recently reported that FAD-linked PS2 mutations (PS2 M239I and T122R) (Binetti et al., 2003) alter cystatin C trafficking in mouse primary neurons reducing secretion of its glycosylated form (Ghidoni et al., 2007). Here we investigated the link between cystatin C and exosomes in an *in vitro* model of FAD.

2. Methods

2.1. Neuronal cultures and transfections

Mouse primary cortical neuronal preparation and transfections were performed as previously described (Benussi et al., 2005): after 4 days in culture, neurons were transfected using pcDNA3 void vector or pcDNA3 constructs containing human PS2 wt, PS2 M239I or T122R mutation cDNAs. Forty-eight hours after transfection, cells were lysed and conditioned media collected.

2.2. Isolation of exosomes from cell culture media

Exosomes from 3 to 4×10^7 neurons were prepared as described elsewhere (Théry et al., 2006). Briefly, conditioned media were collected and spun at $300 \times g$ for 10 min. The supernatants were then sequentially centrifuged at $2000 \times g$ for 10 min and $10\,000 \times g$ for 30 min to remove cellular debris, followed by ultracentrifugation at $100\,000 \times g$ for 70 min (T-8100 rotor) (Sorvall). The $100\,000 \times g$ pellet was then resuspended in PBS and centrifuged for 70 min at $100\,000 \times g$. Exosomes were loaded into a continuous 0.25–2 M sucrose gradient and after 16 h of centrifugation at $200\,000 \times g$ (TH-641 rotor) (Sorvall), fractions were collected from the top of the gradient, diluted with PBS and spun at $100\,000 \times g$ for 70 min. All centrifugations were performed at 4 °C. The pelleted fractions were then analyzed. For the enzymatic deglycosylation of cystatin C, 1 μl of endo-O-glycosidase (Roche) was added to each sample and incubated overnight at 37 °C. Sample aliquots incubated without the deglycosylation enzyme were used as controls.

2.3. Western blot analysis

Western blot analyses were performed using the PS2 polyclonal antibody Ab-2 PC235 (Calbiochem) that recognizes the PS2 C-terminal fragment, the cystatin C polyclonal antibody (Upstate Biotechnology), the TSG101 monoclonal antibody (4A10) (Abcam), the Flotillin-2/ESA antibody (BD Biosciences), and the APP monoclonal antibody (22C11) that detects APP N-terminus (Chemicon). Means of normalized densitometric measurements were compared by Student's *t*-test for independent samples.

2.4. Immunoelectron microscopy

Five microliters of resuspended 2% PFA (Merk) fixed exosomes were put on glow discharged formvar-carbon coated nickel grids. After washing with PBS, the grids were incubated with 50 mM glycine/PBS for 10 min. The grids were blocked with 1% coldwater fish skin gelatin (Sigma–Aldrich Inc.) for 10 min, and then incubated with primary antibodies in blocking solution for 1 h at RT. After washing with PBS, Nanogold-labeled Fab' anti-rabbit or anti-mouse (Nanoprobes) were applied in the blocking buffer for 1 h. The grids were washed with PBS, fixed in 1% glutaraldehyde for 5 min, thoroughly washed with distilled water, and then washed with 0.02 M sodium citrate (pH 7.0) in order to reduce the background. Silver enhancement (HQ Silver enhancement kit) (Nanoprobes) was performed for 10 min at room temperature in the dark. After washing with distilled water, the grids were contrasted and embedded in a mixture of 3% uranyl acetate and 2% methylcellulose in a ratio of 1–9. Stained grids were examined under Philips CM-12 electron microscope and photographed with a Gatan (1k × 1k) digital camera.

2.5. Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)

Two microliters of the specific monoclonal antibody (1 mg/ml) (4G8) (SIGNET) against Aβ epitope 17–24 or the cystatin C polyclonal antibody (1 mg/ml) (Upstate Biotechnology) were incubated in a humidity chamber for 2 h at RT to allow covalent binding to the PS20 ProteinChip Array (Bio-Rad Laboratories. Inc.). Unreacted sites were blocked