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Brief communication

Presenilin 2 mutations alter cystatin C trafficking in mouse primary neurons

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Abstract

Mutations in the presenilin genes account for the majority of familial Alzheimer disease (FAD) cases. In the present report we demonstrated that the FAD-linked presenilin 2 mutations (PS2 M239I and T122R) alter cystatin C trafficking in mouse primary neurons reducing secretion of its glycosylated form. These mutations showed a different impact on cystatin C: PS2 T122R had a much stronger effect determining a dramatic intracellular accumulation of cystatin C (native and glycosylated), followed by a reduction in the secretion of both forms. Several experimental evidences suggest that cystatin C exerts a protective role in the brain and favors stem cells proliferation. Confocal imaging showed that the effect of PS2 T122R mutation was a massive recruitment of cystatin C into the neuronal processes, in the presence of an intact cytoskeletal structure. The consequent reduction in the cystatin C extracellular levels might result in a failure of neuroregeneration. Understanding the interplay of PS2 and cystatin C in the pathogenesis of AD might highlight new therapeutic prospective.

Keywords: Presenilin 2; Mutation; Familial Alzheimer disease; Cysteine protease; Protein secretion; Glycosylated cystatin C

1. Introduction

Mutations in the presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes account for the majority of familial Alzheimer disease (FAD) cases in which a genetic defect was detected [7]. Identification of these genes and associated mutations was extremely important for the understanding of the AD etiology. The majority of presenilin mutations result in increased production/aggregation of amyloid β-peptide (Aβ), the main constituent of senile plaques. Although the mechanism of neuronal damage by Aβ is uncertain, the amount of protofibrillar Aβ correlates with oxidative damage to lipids and proteins in the brain of patients, resulting in impaired synaptic plasticity and neuronal degeneration [22]. Neuropathological and molecular studies suggest a functional link between cystatin C and Aβ [15,21]. The physiological high concentration of cystatin C in the cerebrospinal fluid and its production by choroid plexus, leptomenigeal and glial cells strongly suggest that cystatin C could exert a protective function in the brain [16]. Alzheimer disease is characterized by continuous loss of neurons not replaced. Recent studies demonstrated that murine glycosylated cystatin C is an essential autocrine/paracrine cofactor for the proliferation of brain-derived stem cells [17,23].

Aim of this study was to investigate the interplay between presenilin 2 (PS2) and cystatin C taking in consideration the

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role played by the recently described presenilin 2 T122R (PS2 T122R) and M239I (PS2 M239I) [5,9] FAD mutations.

2. Materials and methods

2.1. Primary cortical neurons preparation, transfections and cell viability

Mouse primary cortical neurons preparation and trasfections were performed as described in Benussi and Ghidoni et al. [4]: after 4 days in vitro (DIV), neurons were transfected by using pcDNA3 void vector or pcDNA3 construct containing the cDNA encoding either for human PS2 wild type (PS2 wt) or PS2 T122R/M239I mutations. Forty-eight hours after transfection (6 DIV), the number of viable neurons was determined by trypan blue exclusion [24].

2.2. Media purification and cell lysates analysis

Forty-eight hours after transfection (6 DIV), conditioned media were collected and purified as reported elsewhere [2]. Media and lysates Western blot analyses were performed by using the PS2 polyclonal antibody Ab-2 PC235 (Oncogene, Merck, Darmstadt, Germany), the monoclonal anti beta-tubulin (Sigma, St. Louis, MO, USA), the polyclonal anti beta-Amyloid precursor protein (APP) antibody (Zymed Laboratories, San Francisco, CA, USA) that recognizes the C-terminal fragment of the APP protein and the cystatin C polyclonal antibody (upstate Biotechnology, Lake Placid, NY, USA) that detects the native and glycosylated forms of cystatin C (bands ranging from 14 to 17 kDa). Means of densitometric measurements normalized by the void vector were compared by *t*-test for independent samples.

2.3. Confocal imaging

Following transfection, the neurons were fixed at six DIV and immunostained as previously described [4] by using the cystatin C polyclonal antibody (1:25; upstate Biotechnology, Lake Placid, NY, USA) the monoclonal anti beta-tubulin (1:1000; Sigma, St. Louis, MO, USA) and the polyclonal anti-tau antibody (1:50; BYA 1074, Accurate, Westbury, NY, USA). Confocal imaging was performed using the BioRad 2100 confocal laser scanning system mounted on a Nikon Eclipse TE2000-S inverted microscope.

3. Results

The aim of the present investigation was to define whether cystatin C metabolism is altered in neurons in the presence of the FAD-linked T122R and M239I PS2 mutations. For this purpose, cystatin C levels were monitored both in the intracellular and extracellular compartments following transfection with PS2 wt and PS2 T122R/M239I constructs (Fig. 1): the over-expression of PS2 proteins was verified by Western blot (Fig. 1A). In the intracellular and extracellular compartments, both native and glycosylated cystatin C were detected (Fig. 1B and E). The over-expression of PS2 T122R resulted in an accumulation of native cystatin C in the intracellular compartment (Fig. 1B): this effect was not observed in neurons transfected with PS2 M239I constructs (% of intracellular native cystatin C level ± S.E.M. PS2 wt: 207.5 ± 46.1; PS2 M239I: 177.9 ± 39.6, $p = 0.639$ with respect to PS2 wt; PS2 T122R: 804.4 ± 227.1; $p = 0.033$ with respect to PS2 wt, $p = 0.026$ with respect to PS2 M239I; mean values normalized with respect to the void vector; $n = 5$; Student's *t*-test) (Fig. 1F). A similar effect was observed for the glycosylated form of the protein (% of intracellular glycosylated cystatin C level ± S.E.M. PS2 wt: 90.6 ± 12.6; PS2 M239I: 127.5 ± 10.3, $p = 0.064$ with respect to PS2 wt; PS2 T122R: 162.0 ± 23.9, $p = 0.039$ with respect to PS2 wt, $p = 0.233$ with respect to PS2 M239I; mean values normalized with respect to the void vector; $n = 4$; Student's *t*-test) (Fig. 1F). Intracellularly, the accumulation of cystatin C, induced by the PS2 T122R mutation, was accompanied by a significant reduction in the level of C-terminal APP fragment; a marginal effect on APP metabolism was observed also for PS2 M239I, but this reduction did not reach the statistical significance (% of intracellular C-terminal APP ± S.E.M. PS2 wt: 317.6 ± 27.3; PS2 M239I: 272.9 ± 38.1, $p = 0.3$ with respect to PS2 wt; PS2 T122R: 215.0 ± 9.4, $p = 0.01$ with respect to PS2 wt; mean values normalized with respect to the void vector; $n = 4$; Student's *t*-test). Considering the extracellular compartment, both PS2 mutations resulted in a reduced secretion of the glycosylated cystatin C (Fig. 1E) (% of secreted glycosylated cystatin C ± S.E.M. PS2 wt: 95.3 ± 4.4; PS2 M239I: 66.9 ± 4.8, $p = 0.005$ with respect to PS2 wt; PS2 T122R: 35.1 ± 13.8; $p = 0.006$ with respect to PS2 wt, $p = 0.072$ with respect to PS2 M239I; mean values normalized with respect to the void vector; $n = 4$; Student's *t*-test): in addition, differently from PS2 M239I, the PS2 T122R mutation determined a reduced secretion of native cystatin C (% of secreted native cystatin C ± S.E.M. PS2 wt: 117.3 ± 24.9; PS2 M239I: 83.9 ± 2.6, $p = 0.231$ with respect to PS2 wt; PS2 T122R: 39.4 ± 13.1; $p = 0.032$ with respect to PS2 wt, $p = 0.016$ with respect to PS2 M239I; mean values normalized with respect to the void vector; $n = 4$; Student's *t*-test) (Fig. 1G).

In order to evaluate the impact of PS2 proteins on neuronal survival, cell viability was measured by trypan blue exclusion. Over-expression of both wild type and mutated PS2 proteins did not differentially affect cell viability (% of viable cells ± S.E.M. Void vector: 77.6 ± 2.3; PS2 wt: 80.2 ± 0.98; PS2 M239I: 83.3 ± 1.7; PS2 T122R: 80.3 ± 1.6; $p = 0.188$, $n = 4$, one-way ANOVA).

We further characterized the effects of PS2 mutations on cystatin C analyzing the intracellular distribution of the protein by confocal imaging. The over-expression of PS2 wt resulted in a slight increase of cystatin C immunoreactivity