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Co-localization of cystatin C and prosaposin in cultured neurons and in anterior horn neurons with amyotrophic lateral sclerosis

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ABSTRACT

Cystatin C (CST3) is a cysteine protease inhibitor that regulates lysosomal enzyme activity and is reported to be involved in the process of neurodegeneration. In the present study, we investigated whether CST3 interacts with other proteins and affects neurodegeneration *in vitro* and under disease conditions. We intended to identify any protein that interacts with CST3 by using a yeast two-hybrid system, and found prosaposin (PSAP) as a candidate protein. The binding of CST3 and PSAP was confirmed using an immunoprecipitation-based *in vitro* assay. An enzyme activity assay revealed that PSAP ameliorated CST3-mediated inhibition of cathepsin B activity. To investigate further, CST3 and PSAP were co-expressed in HeLa cells and in a human neuronal cell line (A1). Subsequent immunocytochemical studies demonstrated that they were co-localized mainly in the lysosomes. In spinal motor neurons of autopsy cases, both proteins showed a granular staining pattern. However, the staining intensities of CST3 and PSAP decreased in Bunina body-positive motor neurons of patients with amyotrophic lateral sclerosis (ALS). Further analysis with immunofluorescence staining revealed that CST3 was immunopositive in the inclusions of ALS motor neurons, where it was closely associated, and sometimes co-localized, with PSAP. CST3 immunoreactivity is recognized as a marker for Bunina bodies in ALS, suggesting that PSAP might also be included in Bunina bodies. The interaction of CST3 and PSAP may alter their functions, leading to motor neuron degeneration in ALS.

1. Introduction

Cystatin C (CST3) is a 13-kDa protein mainly located in the lysosome, where it acts as an inhibitor of cysteine proteases such as cathepsin B, cathepsin H, and cathepsin L [1–4]. It is also found in body fluids as a secreted protein, especially at high levels in the cerebrospinal fluid (CSF) [5]. CST3 is demonstrated to be associated with several neurological diseases including stroke, white matter lesions, and cerebral amyloid angiopathy [6–8]; nevertheless, its exact role in neurodegeneration remains obscure. In the pathophysiology of Alzheimer's disease, changes of lysosomal proteases in cortical neurons have been implicated [9], where the role of CST3 remains controversial, specifically whether it plays a neuroprotective role [10,11]. In amyotrophic lateral sclerosis (ALS), another neurodegenerative disease characterized by loss of motor neurons, cathepsin B is found to be increased in the degenerative motor neurons. CST3 expression is changed in the spinal cord in a mouse model of ALS generated by mutating the copper/zinc superoxide dismutase (SOD1) gene [12,13]. Moreover, CST3 is demonstrated to be the main protein component of Bunina bodies, the pathological hallmark of ALS. These findings suggest that CST3 and cathepsin B might have a role in the pathology of ALS.

Cathepsins are produced in an inactive form and transported to lysosomes, where the acidic environment plays a role in their activation and proteolytic function. However, like CST3, cathepsins are also demonstrated to exist in extralysosomal areas including the extracellular space and cytoplasm, where they perform important and specific functions [14]. It is conceivable that the proper balance of cathepsins and CST3 is of utmost importance for normal extracellular homeostasis, similarly to in the lysosomal environment. Indeed, accumulating evidence suggests that the disturbance of their normal balance in extralysosomal locations contributes to the processes of neurodegeneration in Alzheimer's disease, tauopathies, Parkinson's disease, and lysosomal storage diseases [15–17]. The pH inside the lysosome is very different from the cytoplasmic and extracellular environments, suggesting that

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the regulations, activities, and interactions of cathepsins and CST3 might also be different depending on the location. The interactions of CST3 and cathepsins outside of the lysosome might be important to understand the role of CST3 in neurodegeneration. Nevertheless, most of the studies regarding the regulation of the functions of CST3 and cathepsins and their interaction have focused on the lysosomal environment, and their regulation in other locations is not fully understood. For better understanding of the functions of cathepsins and CST3 outside of the lysosome, we investigated their interactions at physiological pH. We hypothesized that there may be other factors interacting with CST3 and affecting the activity of cathepsins. We mainly focused on cathepsin B activity because of its unique interaction with CST3 and its role in neurodegeneration [15,16].

Prosaposin (PSAP) is a precursor for four sphingolipid activator proteins known as saposins A-D, which are localized in lysosomes [18]. It is well known that the loss of PSAP function induces different types of neurovisceral storage diseases of multiple sphingolipids [19–24]. Besides the precursor function in lysosomes, PSAP has been identified as a multifunctional protein. There is growing *in vitro* and *in vivo* evidence showing that PSAP protects various types of neural and glial cells by inhibiting apoptotic pathways [25–27]. Moreover, it has been demonstrated that saposin C, derived from PSAP, can affect the activity of cathepsins B and D and lysosomal function [28]. As PSAP, CST3, and cathepsins are lysosomal proteins, there is a possibility that interactions between saposin C or its precursor, PSAP, with CST3 are important for the modulation of cathepsin activity and neuronal death.

In the present study, we searched for proteins that interact with CST3 by using yeast two-hybrid analysis and found PSAP as its binding partner. To investigate the interaction of CST3 and PSAP, we analyzed their localization in cultured neurons and in the spinal motor neurons of patients with ALS.

2. Materials and methods

2.1. Subjects

Autopsied spinal cords of three patients with sporadic ALS and three control subjects were used in this study. All the patients with ALS were definitively diagnosed with ALS based on clinical and light microscopic findings of the spinal cord. The profiles of the three patients with ALS are: [1] a 68-year-old woman with a disease duration of 1 year; [2] an 83-year-old man with a disease duration of 8 months; and [3] a 67-year-old woman with a disease duration of 15 months. The profiles of the three control subjects are: [1] a 78-year-old man with pancreatic cancer; [2] a 68-year-old man with dilated cardiomyopathy; and [3] a 71-year-old woman with Parkinson's disease. All experiments using autopsied tissue were performed after acquiring informed consent and were approved by the Ethical Committee of Shimane University School of Medicine.

2.2. Cell culture

A HeLa cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (GIBCO; Invitrogen), and incubated at 37 °C in 5% CO_2. An A1 neuronal hybrid cell line was generated by fusion of human fetal cerebral neurons and human neuroblastoma cells [29], and was cultured in DMEM containing 5% FBS (Invitrogen) and 1% penicillin/streptomycin (GIBCO; Invitrogen), and incubated at 37 °C in 5% CO_2[29].

2.3. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using Matchmaker GAL4 Two-Hybrid System 3 and human brain cDNA library constructed in Y187 yeast strain (Clontech, Mountain View, CA, USA) according to the manufacturer's protocols. Briefly, CST3 cDNA was inserted into a pGBKT7 vector at the sites of *Bam*HI and *Eco*RI as bait. An AH109 yeast strain transfected with the pGBKT7-CST3 plasmid was fused with a Y187 yeast strain already transfected with the pGADT7-rec-human brain cDNA library. Fused yeast was plated on SD medium lacking His/Ade/Leu/Trp and containing X-αGal. After incubation for 10–20 days at 30 °C, a few hundred positive colonies were obtained. The plasmids pGADT7-rec-(CST3-targeted) cDNA were rescued from the obtained positive colonies and analyzed by nucleic acid sequencing.

2.4. Immunoprecipitation and Western blotting analysis

DNA fragments containing the entire coding region of CST3 were inserted into a pQE32 expression vector (Qiagen, Hilden, Germany). *Escherichia coli* (JM109; Promega, Madison, WI, USA) bacteria were transformed by introducing the CST3-pQE32 vector. The recombinant proteins were expressed in *E. coli* (JM109) with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified *via* affinity chromatography using Ni-NTA Superflow columns (Qiagen). PSAP was purchased from Abnova (Taipei, Taiwan).

For the immunoprecipitation assay, 10 pmol each of recombinant CST3 and recombinant PSAP were mixed in 50 mM Tris-HCl (pH 7.5), 0.05% TritonX-100, and 300 mM NaCl and incubated for 3 h at 4 °C. Protein G Agarose (Invitrogen) was incubated with primary IgG (anti-CST3-rabbit, anti-PSAP-rabbit, or control rabbit-IgG) for 1 h at room temperature. After 3–4 washes with Tris buffered saline (TBS buffer), the above-mentioned mixture was added to the IgG Protein G Agarose complex. Then, the mixture was incubated at 4 °C overnight with gentle mixing. The pellet was washed 5 times with TBS buffer, and the immune complex was eluted by adding 20 μl of sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) sample buffer and boiling for 5 min.

For Western blot analysis, the proteins were separated *via* SDS-PAGE gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with Blocking One® buffer (Nacalai, Kyoto, Japan), the membrane was incubated with mouse anti-PSAP IgG (1:200; Abnova) or mouse anti-CST3 IgG (1:250; R&D Systems, Minneapolis, MN, USA) IgG at 4 °C overnight. Next, the membrane was incubated with IR dye-conjugated secondary antibodies (1:5000; LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. The immunoreactions were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

2.5. Cathepsin B activity

Cathepsin B activity was measured following a previously described [30]. Briefly, cathepsin B was added to a reaction buffer (20 mM L-cysteine and 0.4 mM sodium acetate), samples (CST3 and/or PSAP) were mixed in, and the mixture was incubated for 5 min at 37°C. Then, 20 μM of substrate (*Z*-Arg-Arg-MCA; Peptide Institute, Osaka, Japan) were added to the reaction mixtures and incubated for 1 h at 37 °C. The reactions were terminated with 0.1 M monochloroacetate buffer (pH 4.3), and the 7-amino-4-methylcoumarin was measured with a multimode microplate reader (DTX880; Beckman coulter, Brea, CA, USA), with excitation and emission settings of 370 nm and 465 nm, respectively.

2.6. Co-transfection of CST3 and PSAP genes into HeLa cells

To construct *PSAP* expression vectors, cDNA was prepared from the total RNA isolated from a human microglial cell line, HMO6, and full-length ORF of human PSAP and CST3 were amplified using polymerase chain reaction (PCR). The primer sequences used for PCR were, PSAP: 5′-ATGCGGTACCCCACCATGGCATACGCCCTCTTCCTCC-3′ and 5′-ATG CTCTAGAACAGTTCCACACATGGCGTTTGCAATG-3′; CST3: 5′-ATGCG