ORIGINAL ARTICLE / BRIEF REPORT

Localization of serine palmitoyltransferase in human brain with Alzheimer's disease

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Abstract

Localization of 63 kDa subunit of serine palmitoyltransferase (SPT2), a key enzyme in the first step of sphingolipid biosynthesis, was investigated by immunohistochemistry of postmortem human brain tissues of patients with Alzheimer's disease (AD) and healthy controls. SPT2 was localized in cell bodies, apical dendrites, and nuclei of neurons in the control group. Whereas, SPT2 was strongly immunolabeled in neuropil threads, dystrophic neurites in senile plaques, and intracellular neurofibrillary tangles (iNFTs) in AD brains of surviving neurons. Double immunofluorescence staining showed dual labeling of iNFTs for SPT2 and tau. Similar results were obtained in reactive astrocytes for a combination of SPT2 and glial fibrillary acidic protein. SPT2 labeling was also dense in axons stained for highly phosphorylated neurofilament protein. These suggest a role for SPT2 in the disease-specific pathology, including the formation of iNFTs, in AD.

Keywords: serine palmitoyltransferase, human brain, Alzheimer's disease, neurofibrillary tangle, reactive astrocyte, immunohistochemistry

Introduction

The pathology of Alzheimer's disease (AD) is characterized by intracellular neurofibrillary tangles (iNFTs), extracellular senile plaques (SPs), neuronal loss, and the activation of glia [1–3]. NFTs are composed of straight and paired helical filaments with an aberrantly hyperphosphorylated form of the microtube-associated protein (MAP) tau [4–6]. These abnormal filaments accumulate in the cell bodies of surviving neurons, neuropil threads, and dystrophic neurites in or around SPs. In healthy controls, tau promotes the assembly and stabilization of microtubules [7], one of the three major components of the cytoskeleton. The amyloid β-peptide (Aβ) is the principal constituent of SPs and is generated by proteolysis of an integral membrane protein, the amyloid precursor protein (APP) [8]. APP processing and Aβ generation are associated with membrane microdomains, known as lipid rafts, which are rich in cholesterol, sphingolipid, and Aβ [9]. The cellular levels of cholesterol and sphingolipids modulate the formation of lipid rafts [10], and molecules other than cellular cholesterol and sphingolipid may also be involved in modulating APP cleavage.

Sphingolipids are ubiquitous cellular membrane components. They regulate cellular homeostasis, mitogenesis, apoptosis, and proliferation [11]. Ceramide was reported to be increased in AD brains [12], and it suggests an enhancement of Aβ biogenesis by modulating APP β- or γ-cleavage [13, 14]. Serine palmitoyltransferase (SPT) is a housekeeping enzyme and is important for regulating sphingolipid levels in cells [15]; structurally, it is a heterodimer of the 53 kDa SPT1 and 63 kDa SPT2 subunits, which are both bound to the endoplasmic reticulum (ER) [16]. The catalytic and regulatory activities are mediated by SPT2 and SPT1, respectively. The enzyme catalyzes the condensation of palmitoyl-CoA with serine to form 3-ketodihydrosphingosine (KDS), which is further metabolized into ceramide [17]. Upregulation of SPT activity may play a role in apoptosis induced by certain types of

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cell stress. To characterize the signaling pathway of catalytic SPT2 in AD-affected brains, we attempted to determine the localization of SPT2 in the human brain immunohistochemically.

Materials and Methods

Human brain tissues

We autopsied 23 human brains, 10 of which came from patients without neurological disorders (7 males and 3 females; age range, 56–88 years; mean age ± SD, 67.5 ± 11.7 years) and 13 from patients with sporadic AD patients (7 males and 6 females; age range, 56–89 years; mean age ± SD, 80.3 ± 8.9 years). Further details of the cases used in the present study was previously described [18]. All the procedures in this study were performed strictly according to the local Ethics Committee Clinical Study Guidelines and were approved by the Internal Review Board of Kobe University.

Immunoblotting

The temporal lobe of one AD and one control case were used for immunoblotting. The brain tissues were homogenized with a Polytron in 10 vol of ice-cold 20 mM Tris-HCl, pH 7.3, containing 5 mM EDTA, 5 mM EGTA, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and 2 mM phenylmethylsulfonyl fluoride, and mixed with SDS-PAGE sample buffer. The total brain homogenates (50 μg of protein) were electrophoresed on a gel, then transferred to polyvinylidene difluoride membranes (Merck, Darmstadt, Germany), which were incubated with a rabbit polyclonal antibody to SPT2 (Cayman Chemical, Ann Arbor, MI) diluted at 1:1,000 and then soaked in 0.01 M phosphate buffered saline (PBS), pH 7.3, containing 0.3% Triton X-100 and peroxidase-labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Immunoreactive bands were visualized by using enhanced chemiluminescence methods on a Hyperfilm ECL (GE Healthcare, Amersham, UK).

Immunohistochemistry

The fixation and processing of human brain tissues included the cerebellum, thalamus, striatum, hippocampus, entorhinal cortex, amygdale, precentral gyrus, angular gyrus, substantia nigra, caudate, and putamen. Small blocks of brain tissue were fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 4 days at 4°C and then transferred to a cold solution of 15% sucrose in 0.1 M PBS at pH 7.3. Sections were cut with a freezing microtome to a thickness of 30 μm, collected in 15% sucrose buffered solution, and then stored at 4°C. After rinsing in PBS containing 0.3% Triton X-100 (PBST), the sections were treated with 1% H_2O_2 for 30 min to inactivate endogenous peroxidase activities and then with 10% skim milk for 2 h at room temperature. After rinsing briefly, the primary antibody, anti-SPT2 diluted at 1:1,000 in PBST, and 5% newborn calf serum were placed on the sections for 72 h at 4°C. The sections were washed in PBST, and the secondary antibody, goat anti-rabbit IgG diluted at 1:1,000 in PBST, and 5% newborn calf serum were placed on the tissues for 2 h at room temperature. After rinsing, avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) was added for 1 h at room temperature. The sections were washed and treated with 0.01% 3-3'-diaminobenzidine, 0.6% nickel ammonium sulfate, 0.05 M imidazole, and 0.00015% H_2O_2 to form purple precipitates. Sections were mounted on glass slides, dehydrated through graded ethanol solutions, penetrated through xylene, and coverslipped with Entelan (Millipore, Tokyo, Japan).

Localization of SPT2-positive structures and pathological markers of AD in neurons was done using double immunofluorescence staining with SPT2 and one of the following: tau [HT7 (Innogenetics, Ghent, Belgium) diluted at 1:1,000], astrocyte filament [GFAP: glial fibrillary acidic protein (Millipore) diluted at 1:1,000], or phosphorylated neurofilament [SMI-31 (Calbiochem La Jolla, CA) diluted at 1:5,000]. After immunostaining and incubation with the appropriate secondary antibodies (similar methodology as in a previous study [18]) coupled either to fluorescein or rhodamine isothiocyanate (Chemicon, Temecula, CA), the sections were analyzed using a Fluoview FV 500 confocal scanning laser microscope (Olympus, Tokyo, Japan).

Results

For characterization of anti-SPT2 antibody, immunoblot revealed that the anti-SPT2 recognized a signal band at 63 kDa in total homogenates of the temporal lobe from both control and AD cases (**Fig. 1**). Localization of SPT2 in human brains was examined immunohistochemically with light microscopy. In the control group, SPT2 immunoreactivity was localized to neurons (**Fig. 2A**, **2C**, **2E**, and **2F**); cell bodies, apical dendrites, and nuclei in pyramidal neurons (**Fig. 2A** and **2C**), Purkinje cells (**Fig. 2E**),

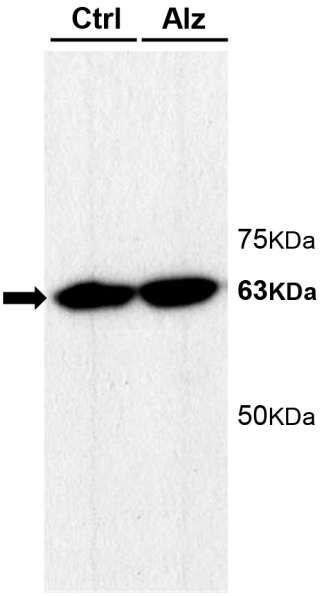

Fig. 1 Immunoblot for SPT2 in human brains. Immunoblot-membrane with samples made from human control (Ctrl) and Alzheimer (Alz) brain and labeled with SPT2 antibody. The position of the marker protein is indicated in 63 kDa (arrow), corresponding to the molecular weight of SPT2.

and Betz giant cells (**Fig. 2F**) were rich in SPT2. In contrast, endoplasmic SPT2 was decreased in AD-affected neurons (**Fig. 2B**). In addition, numerous iNFTs were strongly stained for SPT2 in AD brains (**Fig. 2D**). SPT2 was also labeled in neuropil threads and dystrophic neurites within SPs in the AD brains (**Fig. 2G**). In the control hippocampus, cell bodies and apical dendrites in pyramidal neurons (**Fig. 3A**, **3C**, **3D**, **3G**, and **3H**) were rich in SPT2, while cytoplasmic SPT2 was decreased in AD-affected neurons (**Fig. 3B**, **3E**, **3F**, **3I**, and **3J**). Although axons were not stained for SPT2, mossy fiber terminals were strongly labeled (**Fig. 3G** and **3I**). In the AD hippocampus, SPT2 was localized to iNFTs, degenerative neurites within SPs, and damaged neurons. Double immunofluorescence staining showed dual labeling of iNFTs and dystrophic neurites within SPs for SPT2 and tau (**Fig. 4A** and **4B**). Similar results were obtained in cell bodies and proximal processes of astrocytes for SPT2 and GFAP (**Fig. 4C**) or SPT2 and phosphorylated neurofilament (**Fig. 4D**).

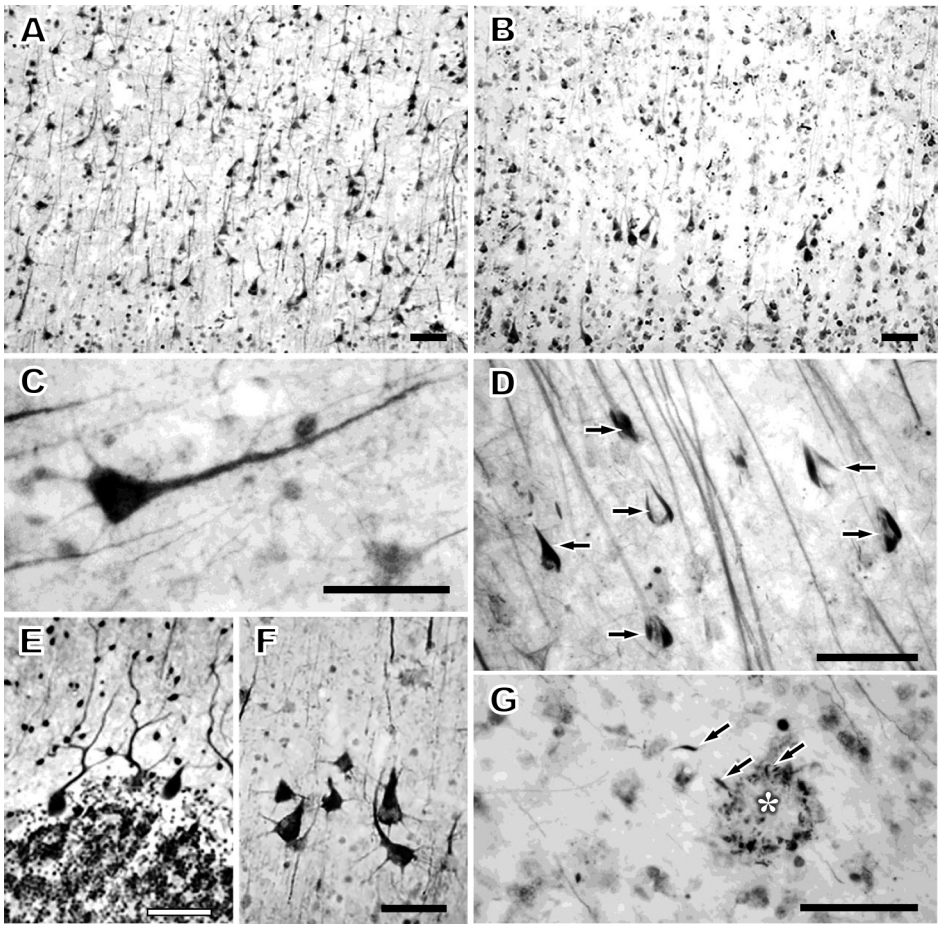

Fig. 2 Immunohistochemistry for SPT2 in human neurons. In the control group brain samples (A, C, E and F), strong immunostaining was seen in pyramidal neurons of the cerebral cortex (A), basal and apical dendrites in the middle frontal gyrus (C), Purkinje cells and granular cells of the cerebellum (E), and some Betz giant cells in the central gyrus (F). In the AD brains (B, D and G), SPT2 was expressed more weakly than in the control group neurons in the cerebral cortex (B). NFTs (arrows) around SPs (asterisk) were strongly immunolabeled by anti-SPT2 antibody in the middle frontal gyrus (D and G). Scale bars, 50 μm

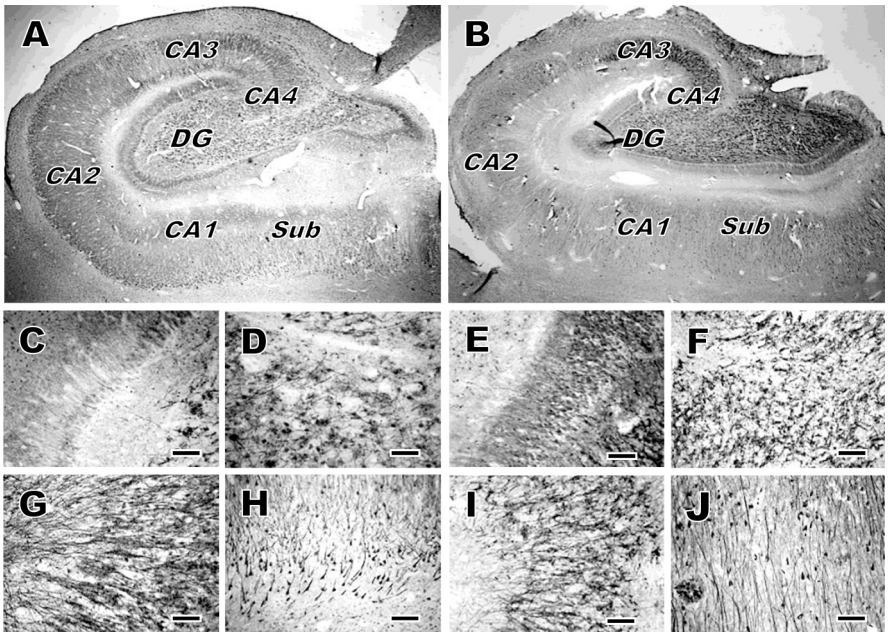

Fig. 3 SPT2 distribution in control and AD hippocampi. The granule cells in the dentate gyrus (DG, C and E) and area CA4 (D and F) and the mossy fibers in area CA3 (G and I) of the hippocampus were immunolabeled for SPT2. Neurons in the control hippocampus were rich in SPT2 (A, C, D, G and H). Cell bodies and dendrites were densely labeled in the pyramidal neurons of CA1 and subiculum (Sub) fields (H). In contrast, SPT2-positive neurons were decreased in CA1/Sub (J) in the AD hippocampus (B, E, F, I and J). Note the strong immunolabeling of intracellular NFTs and dystrophic neurites within extracellular SP for SPT2 (J). Scale bars, 50 μm.

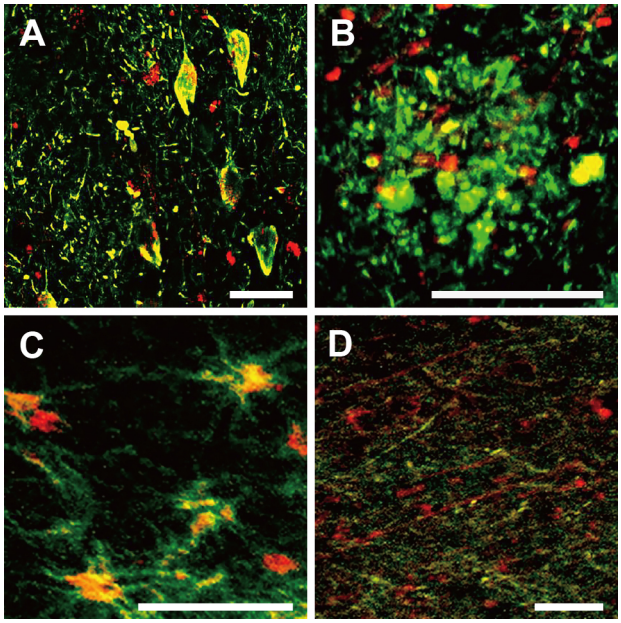

Fig. 4 Double immunofluorescence staining with SPT2 and pathological markers of AD. Double immunolabeling analysis of the CA1 of the hippocampus (A and B) and middle temporal gyrus (C and D). (A) SPT2 (red) colocalized with HT-7-positive tau (green) in intracellular NFTs (yellow). (B) The SPs were double-labeled for SPT2 and HT7-positive phosphorylated tau (green). (C) Glial filaments (yellow) in some astrocytes were double immunolabeled with anti-SPT2 (red) and anti-GFAP antibodies (green). (D) SPT2 (red) was colocalized with phosphorylated neurofilaments (green) in axons (yellow). Scale bars, 50 μm.

Discussion

A previous study reported that SPT2 is normally localized in the neuronal cytoplasm and Purkinje cells of the cerebellum [19]. In this study, SPT2 was also localized to cell bodies, apical dendrites, and nuclei in pyramidal neurons (**Fig. 2A** and **2C**), Purkinje cells (**Fig. 2E**), and Betz giant cells (**Fig. 2F**) in the control group brain samples. While strong SPT2 expression is observed in CA1 and subicular pyramidal neurons in the control hippocampus, these neurons rich in SPT2 were markedly decreased in the AD hippocampus and SPT2-positive iNFTs were observed. SPT2 is bound to the ER, which may be particularly important in neuronal death in AD [20]. The cellular reduction and redistribution of SPT2 may be involved in the aberrant phosphorylation or misfolding of the tau protein, which leads to the iNFT formation, abnormal regulation of intermediate filaments, and impairment in axonal transport.

We have detected numerous SPT2-positive iNFTs and dystrophic neurites within SPs, but also reactive astrocytes, in almost all affected areas of every AD case examined. The major neuropathological hallmarks of AD are the intracellular accumulation of hyperphosphoryla-

ted tau protein in NFTs and extracellular deposits of Aβ-forming SPs [2]. In addition, AD is also characterized by neuroinflammation [3] and altered lipid and sphingolipid metabolism [21], among others. Several studies in brain samples from patients with neurodegenerative diseases including AD have observed changes in genes related to enzymes involved in sphingolipid/ceramide metabolism [22]. For example, in the de novo pathway, the activity of SPT2 regulating de novo ceramide was reported in AD brains [12]. The expression of SPT2 is also upregulated in severe AD [23]. Thus, SPT2 may be involved in the tau pathology, neuroinflammation, and dysfunctions of various organelles in degenerating AD neurons. However, the intracellular fine distribution of SPT2 remains to be elucidated. Further histochemical studies of SPT2 in neurodegenerative tauopathies including AD need immunoelectron microscopy.

Conclusion

SPT2 in human AD brains was colocalized with tau protein and phosphorylated neurofilaments protein in neurons and with GFAP in astrocytes. The present study showed that SPT2 may regulate the functions of these proteins.

Acknowledgment

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There are no conflicts of interest to declare.

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アルツハイマー型認知症脳における SPT2 の組織化学的局在

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要旨

スフィンゴ脂質の生合成の第 1 段階の重要な酵素であるセリン・パルミトイル転移酵素のサブユニット（SPT2）のヒト脳局在について，健常高齢者とアルツハイマー型認知症（AD）患者剖検例を用いて免疫組織化学染色にて定性評価した．健常コントロール脳では，SPT2 はニューロンの細胞質および核に局在していた．AD 脳では，SPT2 はニューロピル（ニューロン間網状組織部），老人斑の変性神経突起，細胞内神経原線維変化（iNFT）などの特徴的病理像の部位へと主な局在が変化していた．二重免疫蛍光染色によって，NFT において SPT2 とタウタンパク質（HT-7）の共存が示された．さらに，反応性アストロサイトにおいて SPT2 とグリア線維性酸性蛋白，ならびに軸索において SPT2 とリン酸化ニューロフィラメントタンパク質の共存性が示された．本研究結果は，AD 患者の NFT 形成などの疾患特異的病理変化における SPT2 の関与を示唆している．