Journal of the Neurological Sciences 365 (2016) 101–107

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Immunolocalization of Tom1 in relation to protein degradation systems in Alzheimer's disease

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ARTICLE INFO

Article history:
Received 17 January 2016
Received in revised form 18 March 2016
Accepted 21 March 2016
Available online 13 April 2016

Keywords:
Tom1 (target of Myb1)
Tollip
Myosin IV
Ubiquitin
Autophagy
Alzheimer's disease
Dystrophic neurites
Perisomatic granules

ABSTRACT

Alzheimer's disease (AD) is an age-related neurodegenerative disorder. Its pathological hallmarks are senile plaques (SPs), which contain extracellular deposits of amyloid β (Aβ) protein fibrils and dystrophic neurites (DNs), and neurofibrillary tangles (NFTs) containing hyperphosphorylated tau. Impairment of protein-degradation systems, including the ubiquitin-proteasome and the autophagy-lysosome systems, has been proposed as one of the causes of the accumulation of these aberrant proteins in AD brains. Tom1 (target of Myb1) was originally identified by the induction of its expression by the v-Myb oncogene and is a part of two major protein-degradation systems. The present study was conducted by immunohistochemical and immunofluorescent stainings to show that Tom1 was localized in DNs, perisomatic granules (PSGs), and NFTs in AD brains. Moreover, in DNs, Tom1 colocalized with ubiquitin, lysosomal proteins, and Tom1-related proteins (Tollip and myosin VI), which act in both protein-degradation systems via Tom1. These results indicate that Tom1 plays important roles in protein-degradation systems in AD pathogenesis.

1. Introduction

A normal balance between the production and degradation of cellular proteins is required for cell survival. The ubiquitin-proteasome and the autophagy-lysosome systems are important cellular systems that are responsible for the degradation of misfolded proteins [1]. The ubiquitin-proteasome system is a protein-degradation system that involves the modification of target proteins with ubiquitin, which signals the target protein for degradation by a multisubunit protease termed proteasome. In turn, the autophagy-lysosome system is mainly responsible for the nonselective degradation of proteins or for removing damaged intracellular organelles. In the processes involved in the autophagy-lysosome system, double-membrane-bound structures called autophagosomes fuse with lysosomes, which leads to the digestion of the contents of the autophagosomes by lysosomal enzymes.

The accumulation and deposition of misfolded proteins are common features of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, and polyglutamine diseases [2–5]. The regional distribution in the central nervous system and the composition of protein aggregates are different in each neurodegenerative disease [6]. Dysfunction of protein-degradation systems has been proposed as one of the causes of the accumulation of these aberrant proteins. AD is an age-related neurodegenerative disorder that is characterized by progressive dementia. Its morphological hallmarks are senile plaques (SPs), which contain extracellular deposits of amyloid β (Aβ) protein fibrils and dystrophic neurites (DNs), and neurofibrillary tangles (NFTs) containing hyperphosphorylated tau [2,7]. NFTs and DNs are ubiquitinated in AD brains [8]. Conversely, autophagic vacuoles, including autophagosomes and autolysosomes, accumulate in DNs in AD brains [9,10]. Thus, in the AD pathology, impairment of these two major protein-degradation systems has been proposed as one of the causes of the accumulation of these aberrant proteins [11].

The target of Myb1 (Tom1) was originally identified by the induction of its expression by the v-Myb oncogene and is predominantly localized in the cytosol. Tom1 belongs to the VHS (VPS-27, Hrs, and STAM)-domain-containing protein family [12], and its homologues are ancient and widely present in eukaryotic cellular components [13]. VHS-domain-containing proteins have been proposed to function as adaptor

Abbreviations: AD, Alzheimer's disease; H&E, hematoxylin and eosin; APP, amyloid precursor protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.jns.2016.03.035
0022-510X/

proteins in the intracellular trafficking and sorting of plasma membrane proteins. Recently, it was revealed that Tom1 participates in membrane trafficking as an adaptor protein via an interaction with several proteins, such as clathrin, endofin, myosinVI, and the Toll-interacting protein (Tollip) [12,14–18].

The present study reports the novel findings that Tom1 was localized in hallmarks of AD pathology. In addition, Tom1 colocalized with key proteins of the protein-degradation systems and with Tom1-related proteins, which act in protein-degradation systems. These findings suggest that Tom1 is a crucial protein in the accumulation of aberrant proteins in AD brains.

2. Materials and methods

2.1. Immunohistochemistry

Brain tissues were obtained from 15 autopsied subjects (average age, 79.2 years; 8 males and 7 females) who had a neuropathologically confirmed diagnosis of AD, and from seven control subjects (average age, 76.1 years; 3 males and 4 females) who died of cancer, stroke, pneumonia, or myocardial infarction with no signs of neurodegeneration (Table 1). All AD cases fulfilled the quantitative neuropathological criteria for the diagnosis of AD according to the National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines for the neuropathological assessment of AD; i.e., an Alzheimer disease neuropathological change score of A3, B3, or C3 [19]. None of the controls met the pathological criteria for AD. Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissues were immunostained with the appropriate antibodies, followed by application of the streptavidin-biotin complex (ABC) method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). For all stainings, sections were deparaffinized and subsequently immersed in 0.3% hydrogen peroxide (H_2O_2) (Doujin Laboratories, Kumamoto, Japan) in methanol for 30 min to block endogenous peroxidase activity. All sections, with the exception of the sections that were stained with anti-Aβ antibodies, were autoclaved for 10 min in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. The sections used for staining with anti-Aβ antibodies were immersed in 99% formic acid for 5 min for antigen retrieval. Immunoreactivity was visualized using 0.5 mg/mL of 3.3′-diaminobenzidine tetrachloride and 0.03% H_2O_2 (Doujin Laboratories). In this study, we used two commercially available anti-Tom1 antibodies [antibody-1 (SIGMA-ALDRICH, St Louis, MO) and antibody-2 (Proteintech Group, Chicago, IL)], an anti-pTau antibody (clone AT8; Thermo Fisher Scientific, Waltham, MA), an anti-ubiquitin antibody (clone Ubi-1; Merck Millipore, Darmstadt, Germany), an anti-microtubule-associated protein 1 light chain 3 (LC3) antibody (Medical and Biological Laboratories, Nagoya, Japan), an anti-lysosome-associated membrane protein 2 (LAMP2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-amyloid β E22P antibody (clone 11A1; IBL, Takasaki, Japan), an anti-Tollip antibody (Thermo Fisher Scientific), and an anti-myosinVI antibody (LifeSpan Bioscience, Seattle, WA). All sections were counterstained with hematoxylin. The specimens were observed under an Olympus DP72 microscope using the DP2-BSQ software (Olympus, Tokyo, Japan).

Table 1
Profiles of the subjects and immunoreactivity for anti-Tom1 antibody.

	Age (years)	Gender	PMI (hours)	DNs	NFTs	PSGs
AD1	83	F	2	+	+	+
AD2	97	F	10.5	+	+	+
AD3	94	M	1.5	+	−	+
AD4	81	M	1.5	−	−	−
AD5	79	F	8	++	+	+
AD6	102	M	2.5	+	−	+
AD7	55	F	3.5	+	−	+
AD8	65	M	11.5	+	−	+
AD9	82	M	12.5	+	−	+
AD10	63	F	1.5	+	+	−
AD11	86	F	2.5	+	+	+
AD12	62	F	4.5	++	−	+
AD13	73	M	2.5	++	+	+
AD14	85	M	1	++	−	+
AD15	81	M	9.5	+	+	+
Cont1	59	M	2	−	−	−
Cont2	80	M	1.5	−	−	−
Cont3	73	F	6.5	+	−	+
Cont4	70	F	2	−	−	−
Cont5	64	F	3	−	−	−
Cont6	96	F	12.5	−	+	−
Cont7	91	M	2	+	−	−

AD: Alzheimer's disease, Cont: control, PMI: post-mortem interval, F: female, M: male.
All AD cases: Alzheimer disease neuropathologic changes: A3, B3, C3.
All control cases: Alzheimer disease neuropathologic changes: A0 or 1, B0 or 1, C0 or 1.

2.2. Double immunofluorescence

To assess the colocalization between Tom1 and pTau, ubiquitin, or LAMP2, we performed double immunofluorescence using fluorescent secondary antibodies [20]. The primary antibodies used for double immunofluorescence were anti-pTau, anti-ubiquitin, and anti-LAMP2 antibodies. After deparaffinization and autoclaving, sections were incubated with the primary antibodies overnight at 4 °C. The secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG; H + L) (Molecular Probes-Invitrogen, Eugene, OR) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (H + L) (Molecular Probes-Invitrogen). To avoid autofluorescent signals, sections were treated with Sudan Black B for 5 min, and rinsed in 70% ethanol. Specimens were mounted with Vectashield (Vector Laboratories, Burlingame, CA), and examined under a microscope equipped with a confocal system (FV1000; Olympus). The obtained images were processed further using Adobe Photoshop CS4 Extended (Adobe, San Jose, CA).

3. Results

First, we examined the immunoreactivity of Tom1 using an anti-Tom1 antibody (antibody-1) in sections from 15 patients with neuropathologically proven AD and from seven control subjects. Numerous Tom1-positive DNs were observed in plaques, especially in Sommer's sector of the hippocampus, of AD brains except for only one case (case 4) (Fig. 1A, Table 1). The number of Tom1-positive DNs in Sommer's sector of the hippocampus was clearly increased in AD brains compared with control brains (Fig. 1B), and was especially increased in the CA1 and subiculum (Fig. 1C). There were fine granular stainings in some plaques (Fig. 1D), while others exhibited bulbous neurites (Fig. 1E), and a few showed granular and curvilinear patterns (Fig. 1F). In some cases, Tom1 immunoreactivity was detected in neuronal inclusions similar to NFTs (Fig. 1G, Table 1). Moreover, Tom1 immunoreactivity was detected in granular structures adjacent to pyramidal cells similar to perisomatic granules (PSGs) (Fig. 1H). The number of Tom1-positive PSGs was increased in AD brains, too (Fig. 1I). On the other hands, in control brains we cannot observe Tom1-positive structures except for a few DNs, NFTs and PSGs (Fig. 1J, Table 1).

To demonstrate the specificity of this reaction, immunohistochemistry using a different anti-Tom1 antibody (antibody-2), which recognizes other epitopes of Tom1 than antibody-1, was also applied. Antibody-2 immunolabeled the same structures (DNs, NFTs, and PSGs) in AD brains as in cases using antibody-1 (Fig. 2A–C). Therefore, it was confirmed that Tom1 is localized specifically in these structures in AD brains.

Focal deposits of Aβ, so called SPs, contain extracellular deposits of Aβ fibrils and DNs containing hyperphosphorylated tau (pTau) [21,22]. To investigate the relationship between Tom1 and the proteins that are present in SPs, we performed immunohistochemistry using anti-Tom1, anti-Aβ, and anti-pTau antibodies in serial sections. Analysis of these serial sections revealed that 85.6% of the Tom1-positive DNs in SPs were also reactive for Aβ in SPs, and 64.9% of them were reactive for

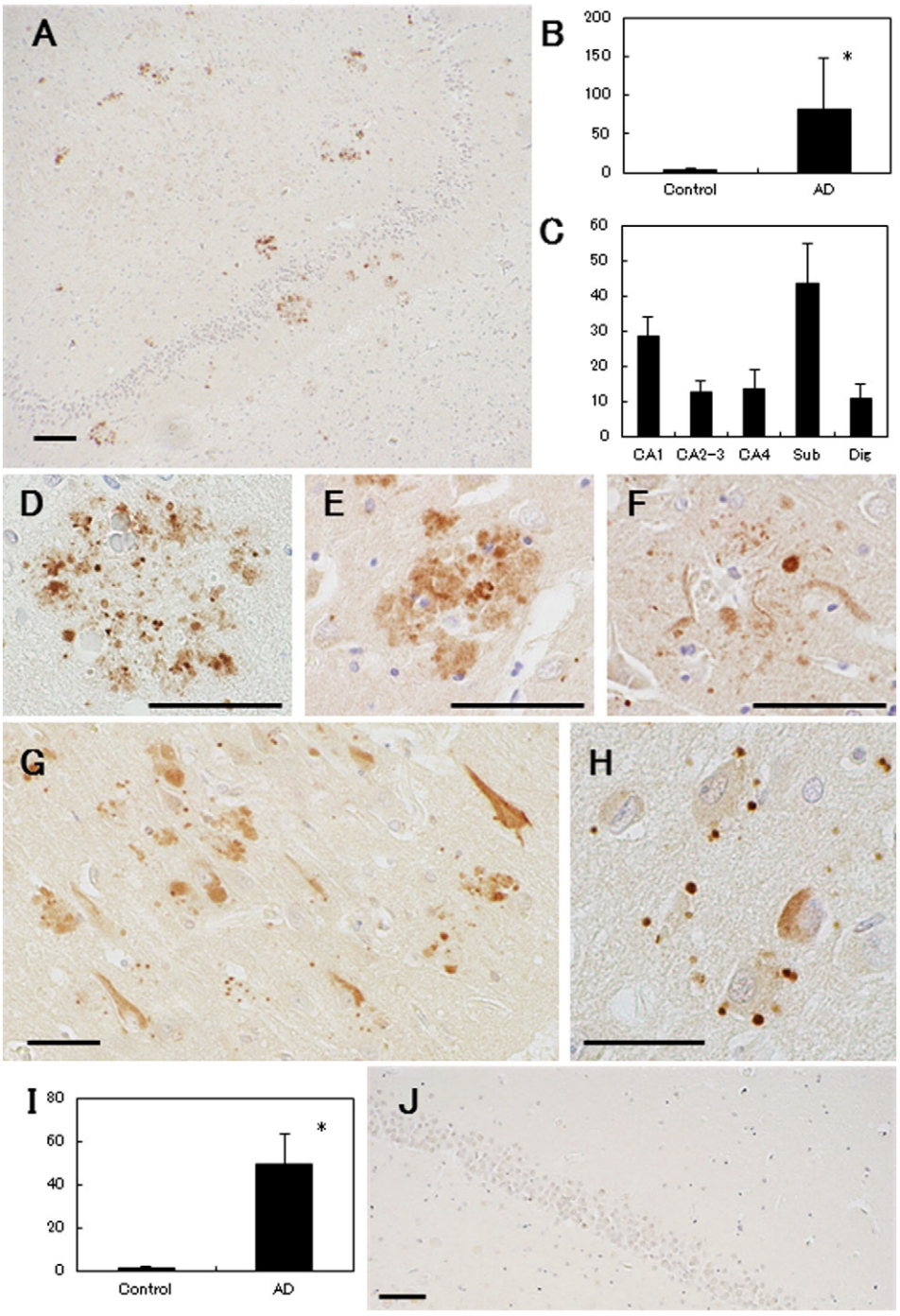

Fig. 1. Immunohistochemistry performed in AD brains using an anti-Tom1 antibody (antibody-1). A: Tom1-positive DNs in plaques were mainly observed in Sommer's sector of the hippocampus of AD brains. B: The number of Tom1-positive DNs in Sommer's sector of the hippocampus was clearly increased in AD brains compared with control brains. Student's *t*-test revealed a significant difference between control and AD brains (*$P < 0.01$). C: Tom1-positive DNs were especially increased in the CA1 and subiculum. D–F: Fine granular staining in some plaques (D) and bulbous neurites (E), and observation of granular and curvilinear patterns (F) in sections that were stained using anti-Tom1 antibodies. G, H: Tom1 immunoreactivity was detected in neuronal inclusions with characteristic NFTs (G) in some cases, and in granular structures adjacent to pyramidal cells with characteristic perisomatic granules (PSGs) (H). I: The number of Tom1-positive PSGs was clearly increased in AD brains compared with control brains. Student's *t*-test revealed a significant difference between control and AD brains (*$P < 0.01$). J: Tom1-positive DNs, NFTs and PSGs were rarely observed in Sommer's sector of the hippocampus of control brains. Immunohistochemistry performed using an anti-Tom1 antibody (antibody-1). Scale bar, 50 μm.

pTau (Fig. 3A–C). On the other hand, we could not observe Tom1-positive structures in diffuse Aβ deposits, so called diffuse plaques, and blood vessels (data not shown). To assess the colocalization of Tom1 and pTau, which is known as a main component of NFTs and DNs, we performed double immunofluorescence using fluorescent secondary antibodies [20]. As shown in Fig. 3D–I, Tom1 was colocalized with pTau in both DNs and NFTs.

To investigate the contribution to the AD pathogenesis of the two protein-degradation systems, immunofluorescence studies using antibodies against ubiquitin, autophagosome (LC3), or lysosome (LAMP2) were performed in AD brains. Tom1 colocalized with ubiquitin in DNs, NFTs, and PSGs (Fig. 4A–F, Table 2). In addition, Tom1 colocalized with LAMP2 in DNs (Fig. 4G–I, Table 2). However, autophagosomal proteins (LC3) were not localized to DNs (data not shown).

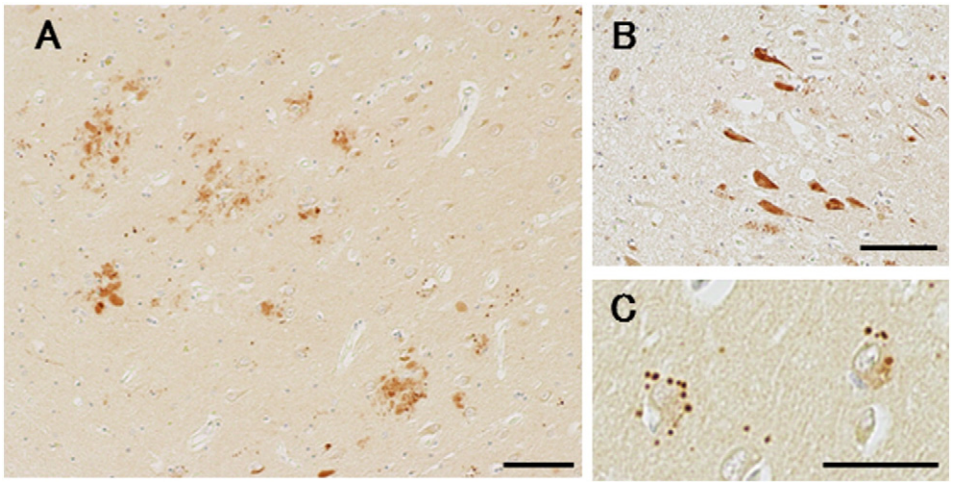

Fig. 2. Sections stained using an anti-Tom1 antibody (antibody-2). Antibody-2 immunolabeled DNs (A), NFTs (B), and PSGs (C). Scale bar, 50 μm.

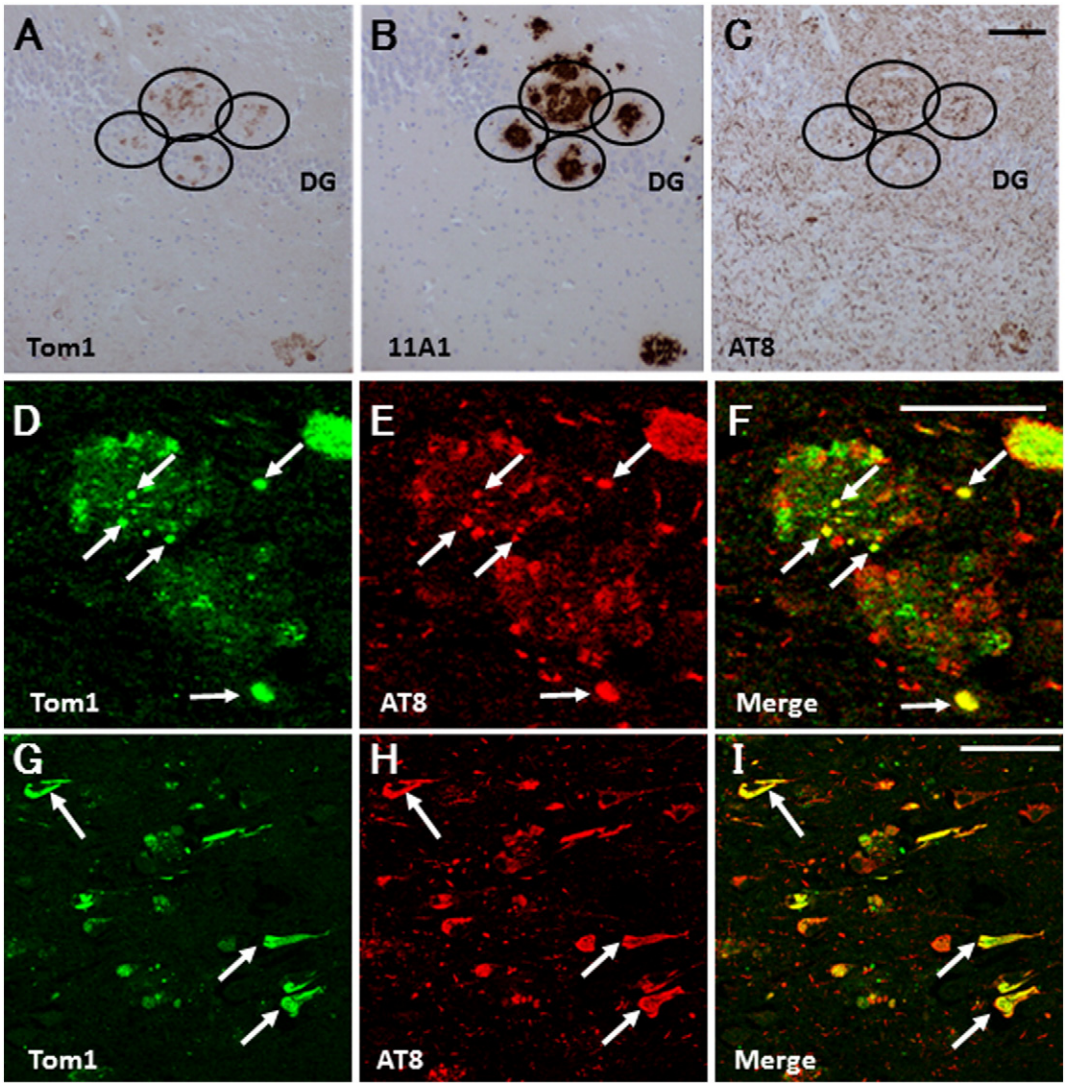

Fig. 3. A–C: Immunohistochemistry performed using anti-Tom1 (A), anti-Aβ (B), and anti-pTau (C) antibodies in serial sections. Many Tom1-positive DNs (A) were also positive for Aβ (B) and pTau (C) in SPs. Scale bar, 100 μm. D–I: Double immunofluorescence for Tom1 (green) and pTau (red) (D–I). Tom1 colocalized with pTau in DNs (D–F; arrows) and NFTs (G–I; arrows). Scale bar, 20 μm.

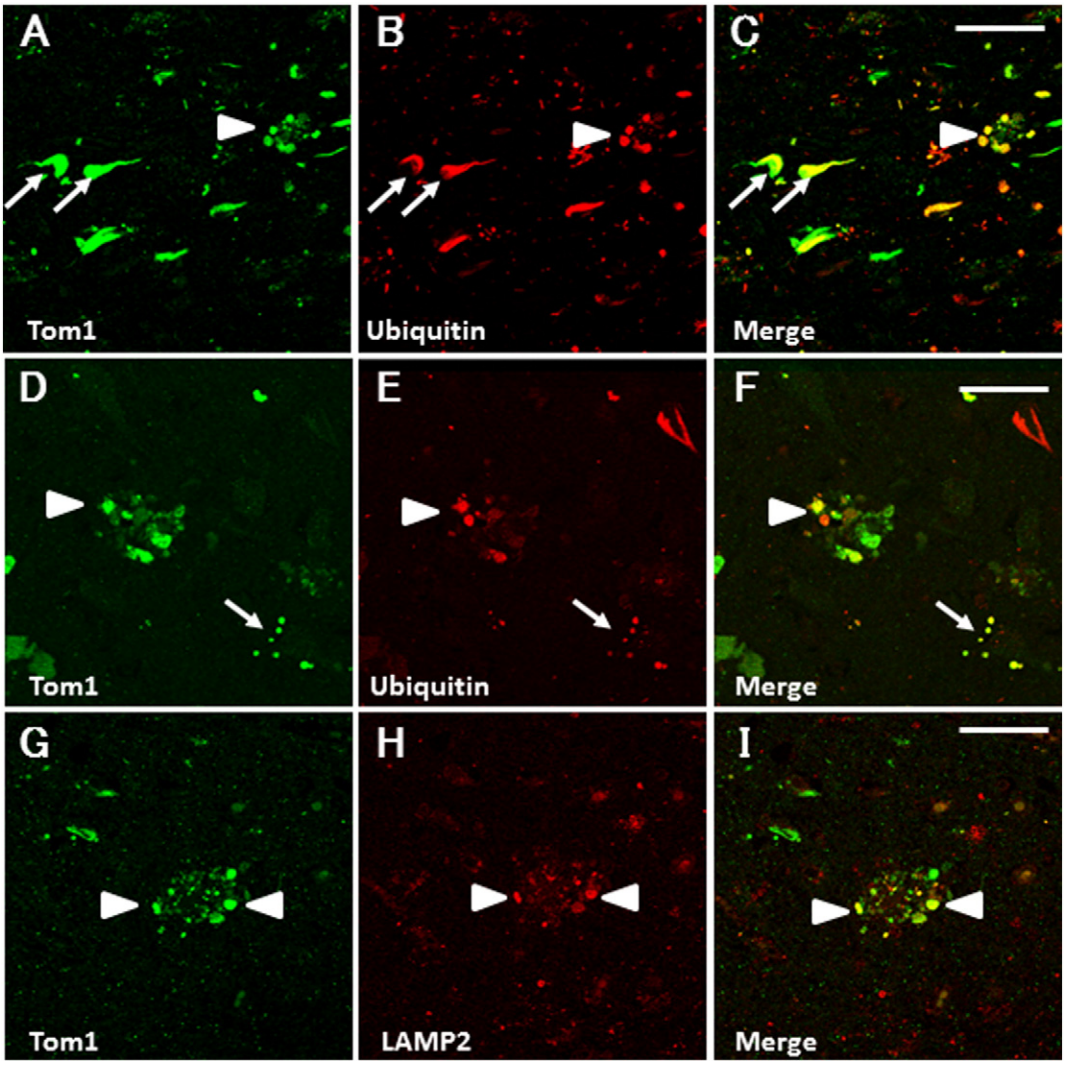

Fig. 4. A–F: Double immunofluorescence for Tom1 (green) and ubiquitin (red) (A–F). Tom1 is colocalized with ubiquitin-positive structures, including DNs (A–F; arrowheads), NFTs (A–C; arrows), and PSGs (D–F; arrows). G–I: Double immunofluorescence for Tom1 (green) and LAMP2 (red) (G–I). Tom1 colocalizes with LAMP2 in DNs (G–I; arrowheads). Scale bar, 20 μm.

Moreover, because it is known that Tom1 acts synergistically with many proteins, such as Tollip and myosin VI, immunohistochemistry using antibodies against Tom1-related proteins was also performed. In AD brains, both Tom1 and Tollip immunoreactivities were observed in DNs and PSGs; however, Tollip immunoreactivity was not detected in NFTs (Fig. 5A, B, Table 2). Myosin VI immunoreactivity was observed in DNs (Fig. 5C, Table 2). On the other hand, immunoreactivities of Tollip and myosin VI were rarely observed in control brains (Fig. 5D, E). Analysis of serial sections using anti-Tom1, anti-Tollip, and anti-myosin VI antibodies showed that Tom1-positive DNs were also positive for Tollip and myosin VI (Fig. 5F–H). Finally, 89.3% of Tom1-positive DNs were also positive for Tollip and 47.6% of that were positive for myosin VI.

Table 2
The entire list of immunoreactivity in pathological findings of Alzheimer's disease.

	DNs	NFTs	PSGs
Tom1	+	+	+
Tollip	+	−	+
Myosin VI	+	−	−
Ubiquitin	+	+	+
LAMP2	+	−	−
LC3	−	+	−

DNs: dystrophic neurites, NFTs: neurofibrillary tangles, PSGs: perisomatic granules.

4. Discussion

The present study demonstrated that Tom1 is localized in DNs, which are major components of SPs in AD brains. In addition, Tom1-related proteins (Tollip and myosin VI) are also localized in DNs. Tom1 and Tom1-related proteins are the novel members among an expanding list of DN-associated proteins, such as ubiquitin, pTau, the amyloid precursor protein, neurofilaments, the growth-associated protein 43, protein kinase C, brain sectin, synaptophysin, chromogranin, lysosomal proteins, and apoptosis-related proteins [23–31]. In addition to DNs, Tom1 was also localized in NFTs, which are another hallmark of AD pathology. Following ubiquitin, pTau, caspase3, caspase9, fragment of presenilin1, calcineurin, and p105, Tom1 is a novel protein that localizes in both DNs and NFTs [32–35]. The observation of positive immunoreactivities for Tom1 in these two important AD hallmarks indicates that Tom1 is an important protein involved in the AD pathogenesis.

Tom1 colocalized with proteins related to two protein-degradation systems and Tom1-related proteins such as Tollip and myosin VI in

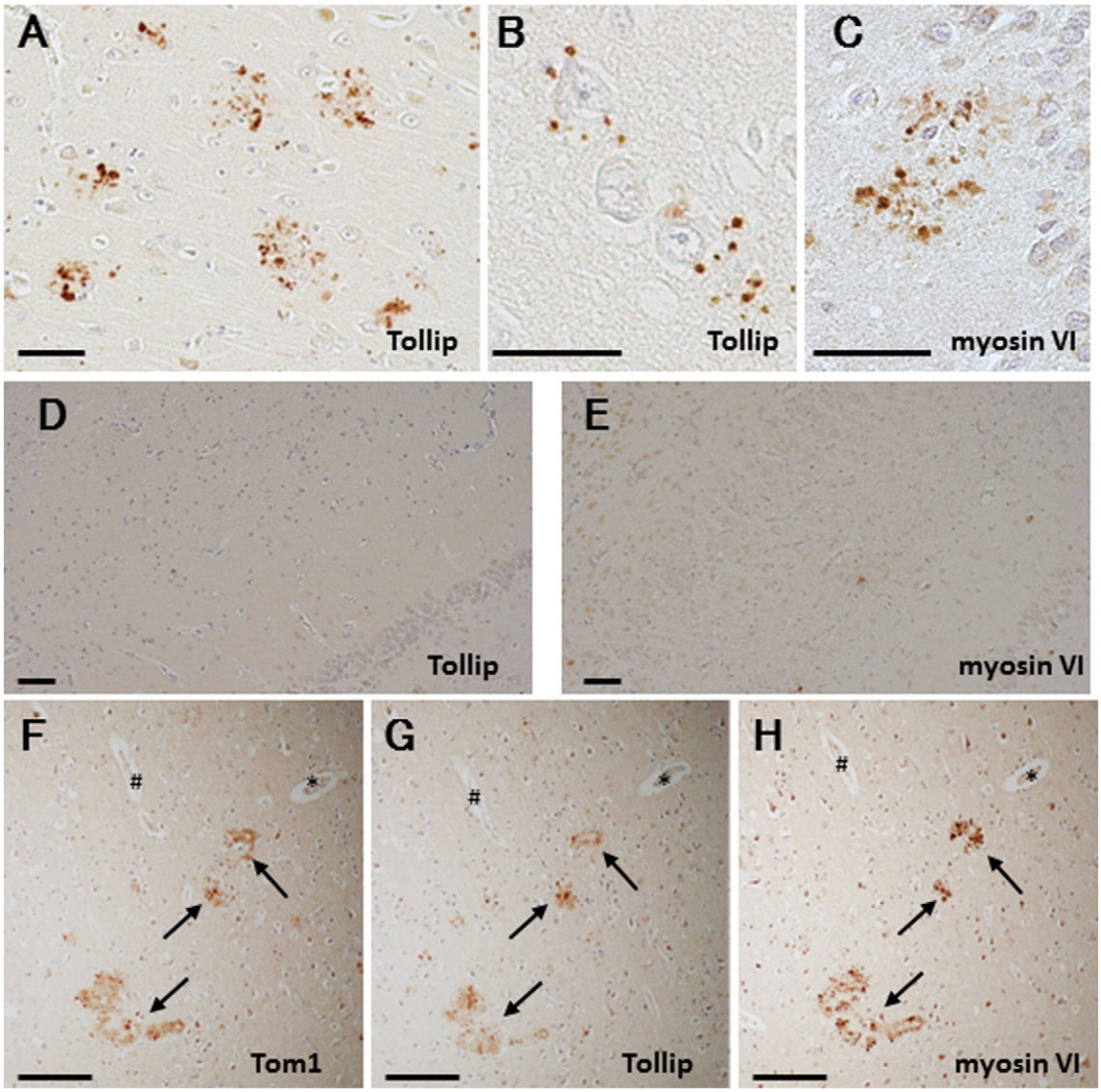

Fig. 5. Tom1-related proteins were localized in Tom1-positive structures (A–C). Tollip was localized in DNs (A) and PSGs (B), and myosin VI was localized in DNs (C). Tollip (D) and myosin VI (E)-positive structures were rarely observed in control brains. F–H: Analysis of serial sections revealed that Tom1 (F) was colocalized with Tollip (G) and myosin VI (H) in DNs. Scale bar, 50 μm.

DNs (Table 2). Tom1 is one of the E3 ubiquitin ligases and has the ability to interact with ubiquitinated proteins via Tollip [12,16]. Tom1 has also been identified in endosomes as a myosin VI-binding partner that promotes autophagosome maturation via myosin VI [36,37]. Colocalization of Tom1 with these proteins indicates that Tom1 plays important roles in protein-degradation systems in AD pathology. Previously, the ubiquitin-proteasome and the autophagy-lysosome systems were regarded as independent systems; however, extensive crosstalk between the two systems has been proposed [38–40]. Because polyubiquitinated protein aggregates or misfolded proteins are recruited into the autophagy machinery for selective autophagy, ubiquitin ligases, which are involved in the ubiquitination of misfolded substrates, can also regulate autophagy. In fact, many E3 ubiquitin ligases are associated with autophagy, and some of these E3 ubiquitin ligases are involved in neurodegeneration [20,41]. Therefore, accumulation of Tom1 and Tom1-related proteins in AD brains might be caused by dysfunction of the ubiquitin-proteasome system, the autophagy-lysosome system, or crosstalk between these protein-degradation systems. In addition, only Tom1 localized in both of DNs and NFTs. This indicates that Tom1 might play central roles in these proteins.

In summary, the present study demonstrated that Tom1 was localized in the hallmarks of AD pathology. In addition, Tom1 colocalized with Tom1-related proteins and proteins that can act in protein-degradation systems. These results indicate that Tom1 plays important roles related to protein-degradation systems in the AD pathogenesis.

Acknowledgments

This study was supported by a Health and Labor Sciences Research Grant for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan, and Grants-in-Aid for Scientific Research (C) 126461304 (to T.Y.) and 6K09665 (to Y.I.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, as well as Grants-in-Aid from the Research Committee for Ataxic Disease (Mizusawa H), the Ministry of Health, Labour and Welfare, Japan (to Y.I.).

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