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アルツハイマー病生物学的診断マーカーの確立に関する臨床研究

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研究要旨
アルツハイマー病（Alzheimer Disease, AD）の早期診断を可能にする生物学的診断マーカーの確立のためには、ADの病理学的カスケードの最上流に位置する普遍的な変化を解明することが重要である。われわれは、孤発性・家族性ADおよび関連疾患の剖検脳を用いて、酸化ストレス（Oxidative Stress, OS）がADの変性過程において早期段階の変化であることを明らかにした。続いて、OSマーカーとして尿中8-hydroxydeoxyguanosine、血清CoQ10酸化率（ubiquinolに対するubiquinoneの比）、およびserum total antioxidant status（STAS）を測定し、AD患者では尿や血清を用いてOSの増加が検出されることを明らかにした。今年度はこれらのOSマーカーの早期診断マーカーとしての有用性を検討する目的で、(1) 健常対照群(39例、平均65歳)、(2) 軽度認知障害（mild cognitive impairment, MCI)群（10例、平均72歳）、(3) AD群（33例、平均71歳）、および(4) 前頭側頭型認知症(frontotemporal dementia, FTD)群（4例、平均63歳）の4群を対象に検討した。その結果、尿中8-hydroxydeoxyguanosineはAD群のみで、血清CoQ10酸化率はMCI群とAD群で、STASはMCI群、AD群およびFTD群で、対照群に比べて有意に変化していた。以上のことから、OSマーカーの一部がADの早期診断に有用である可能性が示唆された。OSマーカーの疾患特異性については、今後さらに種々の認知症疾患を対象に検討する必要がある。

キーワード：アルツハイマー病、軽度認知障害、酸化ストレス、生物学的診断マーカー

A. 研究目的

アルツハイマー病（Alzheimer Disease, AD)の早期診断を可能にする生物学的診断マーカーの確立のためには、ADの病理学的カスケードの最上流に位置する変化を解明することが重要である。従来、ADの生物学的診断マーカーとしては、AD脳で観察される老人斑や神経原線維変化の主要構成蛋白であるアミロイドβやタウに注目が集まっていた。もしAD脳において老人斑や神経原線維変化の

形成に先行する普遍的な変化を見い出すことができれば、早期診断や発症前診断法の確立に寄与することが期待される。

近年、AD の病態に酸化ストレス（Oxidative Stress, OS）が関与していることを示唆する報告が相次いでいるが、われわれは、孤発性および家族性 AD や AD 型の脳病理が併存するレビー小体型認知症の剖検脳を用いて、神経細胞内の酸化的傷害が AD 型の変性過程において早期段階の普遍的な変化であることを明らかにしてきた（平成 13 年度および平成 14 年度の成果）。続いてわれわれは、患者の尿や血清中で測定可能な OS マーカーとして、尿中 8-hydroxydeoxyguanosine（8-OHdG）、血清 CoQ10 酸化率（ubiquinol に対する ubiquinone の比）、および serum total antioxidant status (STAS) を測定したところ、AD 患者でこれらが健常対照群に比べて有意に変化していることが明らかになった（平成 15 年度の成果）。また、これらの OS マーカーの変化は最軽度の AD でも認められた（平成 16 年度の成果）。

今年度はこれらの OS マーカーの早期診断マーカーとしての有用性および疾患特異性を検討する目的で、AD の前段階と考えられる軽度認知障害 mild cognitive impairment(MCI) や AD とは異なる変性性認知症である前頭側頭型認知症 (frontotemporal dementia, FTD)における OS マーカーの変化に注目した。

B. 研究方法

臨床的診断により、対象を以下の 4 群、すなわち、(1) 健常対照群（39 例、平均 65 歳）、(2) MCI 群（International Working Group on MCI による診断基準(Winblad ら、2004) を満たし、clinical dementia rating (CDR) が 0.5 で mini-mental state examination (MMSE) が 24 点以上である 10 例、平均 72 歳）、(3) AD 群（ICD-10 診断基準を満たし、CDR が 1 以上あるいは MMSE が 23 以下である 33 例、平均 71 歳）、および(4) FTD 群（Lund-Manchester Group による診断基準(Neary ら、1998)を満たす 4 例、平均 63 歳）に分けて検討した。各群の対象から朝食前に尿および静脈血を採取し（解析まで−70℃で保存）、以下の OS マーカーについて検討した。すなわち、OS 強度の指標として尿中 8-OHdG (ELISA 法)および血清 CoQ10 酸化率 (HPLC 法) を測定し、OS に対する防御能力の指標として STAS (比色法) を測定した。

統計解析は、ANOVA(post hoc Fisher's PLSD)を用いて 4 群間の比較を行った。

（倫理面への配慮）

旭川医科大学倫理委員会の承認(平成 15 年 1 月 17 日承認、受付番号 104)のもとに研究を行った。各対象例からの採血・採尿にあたっては、文書にて本研究の趣旨を患者および保護者（対照群は本人のみ）に説明し、研究に対する協力の同意を得た。協力の同意の有無によって、患者が臨床上、不利益を被ることがないように配慮した。

C. 研究結果

尿中 8-OHdG は、対照群、MCI 群、AD 群、FTD 群の順に、7.9(4.2)、9.3(5.2)、12.3(7.4)、9.8(6.4) ng/ml, [平均(標準偏差)]であり、対照群に比較して AD 群のみで有意に高値であったが（$p < 0.01$）。

血清 CoQ10 酸化率は、対照群、MCI 群、AD 群、FTD 群の順に、4.8(0.9)、7.8(2.4)、7.9(2.3)、5.1(1.1) [平均（標準偏差）]であり、対照群に比較して MCI 群（$p < 0.03$）あるいは AD 群（$p < 0.02$）で有意に高値であった。

STAS は、対照群、MCI 群、AD 群、FTD 群の順に、1398(129)、1223(167)、1124(101)、1220(124) μM[平均（標準偏差)]であり、対照群に比較して MCI 群（$p < 0.002$）、AD 群（$p < 0.0001$）あるいは FTD 群（$p < 0.02$）で有意に低値であった。

なお、STAS では MCI 群と AD 群との間でも有意差が認められた（$p < 0.03$）。また、尿中 8-OHdG 、血清 CoQ10 酸化率、および STAS のいずれにおいても、MCI 群あるいは AD 群と FTD 群との間に有意差は認められなかった。

D. 考察

AD および関連疾患の剖検脳における検討から、酸化的傷害は AD 型の変性過程において早期段階の普遍的な変化であることが示唆されている。したがって、OS マーカーは、AD 早期診断マーカーとして重要な候補の１つであると考えられる。平成 15 年度までの検討によって、AD 患者の尿および血清において OS 強度の増加や OS に対する防御能力の低下が認められ、AD 患者では尿や血清を用いて OS の増加が検出されることが明らかになっている。

今回、AD の前段階と考えられる MCI において、OS 強度の指標である血清 CoQ10 酸化率の増加や OS に対する防御能力の指標である STAS の低下が認められたことから、これらの血清 OS マーカーが AD の早期診断に有用である可能性が示唆された。

また、STAS は FTD でも対照群に比べて変化していたが、OS マーカーの疾患特異性については、今後さらに種々の認知症疾患を対象に多数例を用いた検討が必要である。

E. 結論

血清中の OS マーカーである CoQ10 酸化率や STAS は、MCI において AD と同様に変化しており、AD の早期診断上、これらの OS マーカーが有用である可能性が示唆された。OS マーカーの疾患特異性については今後さらに種々の認知症疾患を対象にした検討が必要である。

F. 健康危険情報

なし。

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なし。

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EDITORIAL

Multiple pathogenesis of frontotemporal dementia

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Along with the recent development in basic research on neurodegenerative disorders and dementing diseases, a new classification of dementing diseases should be considered and established. Lewy body disease and frontotemporal dementia (FTD) have been clinically separated from senile dementia of the Alzheimer type and now qualify as independent clinical entities.

After many discussions on dementing disorders involving frontal or temporal lobar atrophy, the Lund and Manchester groups, pioneers of the study of FTD, have clinically assigned a generic term, frontotemporal lobar degeneration, to include FTD, semantic dementia and progressive aphasia. FTD remains a heterogeneous clinical entity that includes dementias of the Pick type, motor neuron disease type and frontal lobar degeneration type. This classification has been constructed based solely upon clinical symptoms, clinical courses and brain imaging. The point is that the characterization and classification is principally based on clinical features. In order to understand the molecular mechanism of neurodegeneration in these diseases, findings in neuropathology, neurochemistry, cell biology and molecular genetics should be taken into account.

In the case of spinocerebellar degeneration, the previous classification was based on clinical features, and it included olivopontocerebellar atrophy, late cerebellocortical atrophy, dentatate–rubro–pallido–luysian atrophy (DRPLA), myoclonus epilepsy of the degenerative type and so on. However, after the establishment of the concept of triplet repeats diseases, the classification of spinocerebellar degeneration has been reorganized using the causative genes with repeats expansion. Actually, DRPLA and myoclonus epilepsy of the degenerative type have the same causative gene *DRPLA*; however, the number of expanded repeats is different between these two diseases. Therefore, clinically differentiated diseases are grouped according to the causative gene. Likewise, FTD, which probably has several causative mechanisms of neurodegeneration, will be eventually reclassified depending on the molecular neurodegenerative mechanism.

Recently, neuropathological studies have shown that a clinically diagnosed form of FTD is characterized by intermediate filament inclusions in neurons. Neuronal intermediate filament inclusion disease (NIFID) is a novel neurological disease with clinically heterogeneous phenotypes, including progressive early-onset dementia, and pyramidal and extrapyramidal symptoms.[1] There is focal atrophy of the frontal lobes and, to a lesser degree, the temporal and parietal lobes. Microscopically, there are intraneuronal cytoplasmic neurofilament inclusions which are variably ubiquitinated, but these inclusions contain neither tau nor synuclein. In addition, accumulation of alpha-internexin, which is also one of the intermediate filaments, in NIFID has been reported.[2] The inclusions are present both in the neocortex and subcortical

nuclei and spinal cord. NIFID has many clinical symptoms, including behavioral and personality changes, memory loss, cognitive impairment, language deficit and impairment, motor weakness, extrapyramidal features, perseveration, executive dysfunction, hyperreflexia and primitive reflexes.[3]

Genetic studies have also revealed the causative genes of FTD, such as *tau*, *presenilin-1* and *VCP*. In 1998, mutation of the tau gene was found in familial FTD patients, and the term 'frontotemporal dementia with Parkinsonism linked to chromosome 17' (FTDP-17) has been proposed.[4] FTDP-17 is a familial neurodegenerative disease with links to chromosome 17q21–23. In addition, molecular genetic analyses have revealed mutations in the *tau* gene in chromosome 17q21. The mutations were found in both exons and introns, and it is speculated that either the amino acid replacement by missense mutations, or changes in the expression ratios of 3-repeats/4-repeats may affect the alternative splicing of the *tau* gene. The detailed mechanism of change in the splicing ratio is being investigated. The acceptor site for the alternative splicing of mRNA has hairpin conformation and the exon 10 is spliced out at a ratio of approximately 1:1. Furthermore, 4-repeats and 3-repeats tau proteins are translated and produced from tau mRNA with and without exon 10, respectively. Because of the mutations, the acceptor site for alternative splicing of exon 10 is not able to have the hairpin conformation. Therefore, exon 10 is not spliced out, and this results in the production of 4-repeats tau from all the mRNA containing exon 10.

The important point to note is that variable clinical and neuropathological phenotypes are observed in cases of FTDP-17. Clinical phenotypes of FTDP-17 are similar to those of Pick disease (K257T, G272V, V337M and K369I), corticobasal degeneration (P301L and S305N), pallido-ponto-nigral degeneration (N279K), multiple system atrophy (+13(A→G)), progressive subcortical gliosis (+16(C→U)) and Alzheimer's disease (R406W). This implies the possibility that one gene may trigger neuronal and/or glial dysfunction in several areas in the brain, causing different clinical and neuropathological phenotypes.

Presenilin-1 (*PS-1*) and *presenilin-2* (*PS-2*) are causative genes for familial Alzheimer's disease (AD) and they are localized in chromosome 14 and chromosome 1, respectively. More than 80 mutations have been found in *PS-1*, and eight mutations have been found in *PS-2*. The pathophysiological mechanism of PS in familial AD has been extensively investigated, and PS is considered as the major component of the gamma-secretase complex. Gamma-secretase is biologically important because it cleaves Notch, amyloid precursor protein (APP) and many other proteins at intramembrane sites, and this involves intramembranous proteolysis, which is one of the main topics of research in the field of intracellular signaling biology.

Recent reports have indicated that some mutations in *PS-1* (L113P, G183V and insertion of R352) cause FTD in which no senile plaque, only tau inclusion bodies, can be found.[5,6] One report has indicated that the insertion of R352 decreases amyloid beta production; however, its neurodegenerative pathomechanism is not yet fully understood.[7]

Valosin-containing protein (VCP), a member of the AAA–ATPase superfamily, has been associated with a wide variety of essential cellular protein pathways comprising nuclear envelope reconstruction, cell cycle, postmitotic Golgi reassembly, suppression of apoptosis, DNA damage response and ubiquitin-dependent protein degradation. Recently, mutations in *VCP* have been found in patients with inclusion body myopathy associated with Paget disease of the bone and FTD.[8] Patients with this disorder display behavioral abnormality and cognitive impairment, and neuronal nuclear inclusions containing ubiquitin and VCP can be found in these patients. However, no fibrillar materials are present in neuronal or glial cells, and there is no amyloid deposition. Presumably, the pathological mechanism may involve impairment of the ubiquitin-dependent protein degradation system, but further study is required.

Other genetic loci of familial FTD have been reported in chromosome 3 and chromosome 15; however, the genes have not been identified yet. Familial FTD with a large pedigree was found in Denmark and its gene locus was linked to chromosome 3.[9] Neuropathological investigations revealed tau inclusions in the neurons and some glial cells in the frontal lobe. However, no amyloid deposit was observed. FTD linked to chromosome 15 is related to myotonic dystrophy (DM), and is called 'non-DM1, non-DM2 multisystem myotonic disorder with FTD' (DM-3).[10] Myotonic dystrophy is characterized by progressive myopathy, myotonia, posterior subcapsular cataracts, and other specific clinical features. The causative gene for this disorder has now been identified in

chromosome 19q13.3 (DM protein kinase gene with cytosine–thymine–guanine (CTG) repeats expansion) (DM-1), and in chromosome 3q21 (*ZNF9* gene with cytosine–cytosine–thymine–guanine (CCTG) repeats expansion) (DM-2). The third gene is linked to chromosome 15q21–24 (DM-3). In these cases of DM, microvacuolar and macrovacuolar spogiform degeneration have been observed in frontal, temporal and insular corticies. But tau inclusions are rare, and no amyloid deposit has been found in cases of DM-3.

The recent findings in neuropathological and genetic research have been summarized above. As indicated, further investigation of the roles of tau, neurofilaments, PS and VCP in the cellular process of neurodegeneration is necessary to understand the molecular pathogenesis of the neurodegenerative disorders described previously. As has been shown, FTD has many variations and subgroups, and one of the clinically important aspects of FTD is the management of behavioral and psychiatric symptoms of dementia (BPSD). The problem-solving approach for the management of BPSD is based on the administration of appropriate medicine and sufficient understanding of symptoms by caregivers. FTD has several variations in symptoms, and the regional pattern of neurodegeneration, rather than the type of histopathology, influences the clinical syndrome in FTD.[11] Brain imaging analysis is also important for understanding the regions of the brain that are impaired. Therefore, advances in our knowledge of biochemical and/or genetic causes of FTD may lead to a more accurate and appropriate reclassification of FTD for future pharmaceutical therapeutics, and current brain imaging analysis may provide important information for understanding the pathogenesis of clinical symptoms.

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Research report

α-Synuclein-positive structures induced in leupeptin-infused rats

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Abstract

Abnormal accumulation of α-synuclein is regarded as a key pathological step in a wide range of neurodegenerative processes, not only in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) but also in multiple-system atrophy (MSA). Nevertheless, the mechanism of α-synuclein accumulation remains unclear. Leupeptin, a protease inhibitor, has been known to cause various neuropathological changes in vivo resembling those of aging or neurodegenerative processes in the human brain, including the accumulation of neuronal processes and neuronal cytoskeletal abnormalities leading to neurofibrillary tangle (NFT)-like formations. In the present study, we administered leupeptin into the rat ventricle and found that α-synuclein-positive structures appeared widely in the neuronal tissue, mainly in neuronal processes of the fimbria and alveus. Immunoelectron microscopic study revealed that α-synuclein immunoreactivity was located in the swollen axons of the fimbria and alveus, especially in the dilated presynaptic terminals. In addition colocalization of α-synuclein with ubiquitin was rarely observed in confocal laser-scan image. This is the first report of experimentally induced in vivo accumulation of α-synuclein in non-transgenic rodent brain injected with a well-characterized protease inhibitor by an infusion pump. The present finding suggests that the local accumulation of α-synuclein might be induced by the impaired metabolism of α-synuclein, which are likely related to lysosomal or ubiquitin-independent proteasomal systems.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Parkinson's

Keywords: α-Synuclein; Leupeptin; Protease inhibitor; Immunohistochemistry; Immunoelectron microscopy

1. Introduction

Lewy bodies (LB) and Lewy neurites are universally recognized as pathological hallmarks of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Although LB can be observed microscopically with hematoxylin-eosin stain, an immunohistochemical method for detection of LB with either anti-ubiquitin or anti-α-synuclein antibody is recommended [1,6]. α-Synuclein has been proven to be one of the major components of LB in PD and DLB [9,26]; however, aggregation of α-synuclein is also demonstrated in the brains of Alzheimer's disease (AD) patients [17]. In addition, two recent cases of patients with diffuse neurofibrillary tangles disease with calcification (DNTC) have shown that neurons containing α-synuclein-positive structures are widely distributed, especially in the amygdala, hippocampus, and upper temporal gyrus [32]. Because the pure form of DLB, that is, DLB without or with very few neurofibrillary tangles or senile plaques is known [13], the process of Lewy body formation would not comprise a simple linkage of NFT formations. On the contrary, the frequent coexistence of NFT and LB in AD brains indicated that there may be a relationship between the mechanism of tau

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accumulation and that of α-synuclein accumulation in the AD and DNTC brains, although these mechanisms are so far unknown.

Leupeptin, a protease inhibitor, is known to cause (1) the accumulation of lipofuscin-like granules in the neuronal perikarya and (2) the degeneration of neurites [4,7,27]. Furthermore, we previously reported that the long-term infusion of leupeptin into the rat ventricle caused cytoskeletal changes that included the formation of abnormal bundles of paired helical filament-like filaments with 20 nm diameter and periodic constrictions at 40 nm intervals in the cortical neuron [28]. We reported that these changes had some morphological resemblance to neuropathological features of Alzheimer's brains.

In the present study, we found, by the administration of leupeptin to the rat brain, that α-synuclein-positive structures appeared in various portions of the brain in response to the degeneration caused by leupeptin. Immunoelectron microscopic studies revealed that α-synuclein-positive materials accumulated in the swollen axons, especially in dilated presynaptic terminals and in the neuronal cell bodies. These results revealed that a disturbance of protein degradation causes not only cytoskeletal abnormality, as we reported before, but also α-synuclein accumulation in the neurons. In this study, it suggests that impairment of protein degradation might be closely related with neurodegenerative diseases including AD that is characterized by cytoskeletal abnormality with accumulation of tau, amyloid β, and α-synuclein. It was reported that decreased activities of proteasome were observed in AD brain [12], and that in cultured cells protease-dysfunction induced neuronal degeneration [10]. Therefore, we would like to raise the hypothesis that disturbed protein-degradative activities are initial causative events leading to neurodegenerative disorders, even though the cause of dysfunction of protein degradation was not clarified. Recently, in the study of polyglutamine disease, one of neurodegeneration diseases, dysfunction of proteasome was found to be an initial event in the degenerative processes, and it might support our thought.

Previously accumulation of α-synuclein was reported in transgenic-model mouse [25,30], or rodents injected with MPTP or rotenone [3,5,16]. Both of chemical compounds were not well characterized in biochemical function on the mechanisms of cytotoxicity yet, even though oxidative stress mechanisms are thought to be mediators in either compound. This is the first report of experimentally induced in vivo accumulation of α-synuclein in non-transgenic rodent brain injected with leupeptin, a biochemically well-characterized protease inhibitor, by an infusion pump.

2. Materials and methods

Twenty 8-week-old Wistar rats, weighting about 300 g, were used for the present study. Leupeptin (Peptide Institute Inc., Osaka, Japan) solution, dissolved in phosphate-buffered saline (pH 7.4) at 25 mg/ml, was infused with an osmotic minipump (Model 2002; Alzet, California, USA). The outline of the operation was described elsewhere [27]. The pump was connected by means of a Silastic tube to an intracerebroventricular cannula and implanted subcutaneously in the neck. The cannula was implanted stereotaxically (0.8 mm posterior to the bregma, 1.2 mm lateral to the midline) into the right lateral ventricle. Five rats were implanted with pumps containing only phosphate-buffered saline (PBS) and served as controls.

2.1. Immunomicroscopy

Following infusion for 14 days, the rats were anesthetized and killed by perfusion through the left ventricle with 4% buffered paraformaldehyde solution.

For immunomicroscopic study, brains were dissected and fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains, including the brain stem and the spinal cord, were cut coronally at 3 mm thickness. The slices were rinsed with PB, dehydrated through graded alcohol, and embedded in paraffin. Paraffin sections at 10 μm were made, deparaffinated, and prepared for immunostaining. Immunostaining was performed with monoclonal anti-α-synuclein antibody synuclein-1 (1:500 dilution) (Transduction Laboratories, Lexington, USA) as the primary antibody [20]. Sections were incubated overnight at 4 °C. For the immunohistochemical detection of the primary antibody, sections were incubated with a secondary antibody (1:100 biotinylated anti-mouse; Vector Laboratories, Burlingame, USA) for 2 h at room temperature. After rinsing in PB, the sections were then incubated in avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, USA) for 1 h at room temperature. After three subsequent washings in PB, sections were visualized by diaminobenzidine tetrahydrochloride.

2.2. Immunoelectron microscopy

For immunoelectron microscopic study, the rats were perfused with 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in 0.1 M PB (pH 7.4). Brains were fixed for 24 h in 4% paraformaldehyde, 7% sucrose in PB. The leupeptin-treated rat brains were cut by vibratome to make 50-μm-thick coronal slices and there were washed in PB. Sections were first incubated overnight with the anti-α-synuclein antibody (1:500 dilution) at 4 °C, then incubated for 2 h with the secondary antibody (1:100 biotinylated anti-mouse; Vector Laboratories, Burlingame, USA), and treated with the avidin–biotin–peroxidase complex for 1 h at room temperature. After three subsequent washings in PB, sections were visualized by diaminobenzidine (DAB) tetrahydrochloride. For the post-fixation for electron microscopy, sections were incubated with 4% paraformaldehyde and 0.1% glutaraldehyde in PB

(pH 7.4) for 3 days. And then, sections were osmicated with 2% OsO4 in PB and dehydrated through graded alcohol.

DAB-development was rigorously performed for 10 min, and the slices were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in PB (pH 7.4). They were slightly washed and post-fixed with 2% OsO4 in PB for 1 h at 4 °C, then flatly embedded in Spurr's resin (TAAB, Berkshire, UK) between aclar films (Nissin EM, Tokyo, Japan). Polymerization was performed overnight at 70 °C, and the specimens were pre-examined by light microscopy. The areas including positive reactions in CA4 and fimbria were trimmed, and ultra-thin sections were made with an Ultracut UCT microtome (Leica, Solms, Germany) and examined by transmission electron microscopy with a JEM 1200 EX (JEOL, Tokyo, Japan) at 80 kV.

2.3. SDS–PAGE and Western blotting

Respectively five leupeptin-infused rats and control rats were used to study the expression of α-synuclein in fimbria. Operated rats were deeply anesthetized with diethyl ether and killed by decapitation. Their brains were removed and immediately washed with ice-cold PBS. Hippocampi of the right cerebra were dissected and the fimbriae were removed. The frontal quarter of the fimbriae were cut and immersed for 3 h in 4% paraformaldehyde, 7% sucrose in PB, for overnight 20% sucrose in PB to study confocal laser-scanning microscopy. Three quarters of the remaining fimbriae were homogenized in 0.05 M Tris–HCl (pH 7.4) containing 0.1% Triton X-100 with a Potter type glass-Teflon homogenizer. Specimens were gently centrifuged (at 900 × *g* for 5 min) to remove the debris and the proteins were precipitated by the addition of 9 volumes of cold methanol. They were centrifuged at 10.000 × *g* for 10 min and re-suspended in deionized water. The protein concentrations of each extract were determined by using a spectrometer Smart Spec™ 3000 (Bio Rad, USA) and adjusted to 1 mg/ml with 0.05 M Tris–HCl buffer (pH 7.4). The samples were denatured with Laemmli's sample buffer (Laemmli, 1970), carried out SDS–PAGE in a 15% acrylamide gel, and transferred to a polyvinylidene difluoride membrane (Boehringer Mannheim, Germany). The membrane was blocked with Block Ace™ for 1 h, and then incubated with anti α-synuclein antibody and mouse anti α-tubulin (Oncogene, USA), as an internal standard, for 2 h at room temperature. After washing, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (Vector Labs, USA) for 2 h at room temperature. The signal was detected by using chemiluminescent detection system (ECL™ + Plus, Amersham Pharmacia Biotech, UK) and visualized by exposure on X-ray film (Fuji Photo Film, Japan). The signal intensities were quantified using densitometric analysis by Scion Image program (Scion Co., NIH, USA), and compared by student *t* test ($P < 0.05$).

2.4. Confocal laser-scanning microscopy

The frozen frontal quarter of the fimbriae was cut by cryostat to make 20-μm-thick slices. These sections were simultaneously incubated for 3 days with mouse anti-α-synuclein monoclonal antibody synuclein-1 (1:4000 dilution) (Transduction Laboratories, Lexington, USA) and rabbit anti-ubiquitin polyclonal antibody (1:4000 dilution) (Chemicon, USA) at 4 °C, then simultaneously incubated for 2 h with Alexa Fluor 488 donkey anti mouse IgG (1:1000 dilution) (Morecular Probes, USA) and Cy™3-conjugated donkey anti rabbit IgG (1:1000 dilution) (Jackson ImmunoResearch, USA) at room temperature. A Zeiss confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany) was used to visualize the fluorescent materials. By scanning simultaneously with two lasers (488 and 543 nm) and by using ×20 objective, we obtained a two-color image of the fimbriae.

3. Results

3.1. Immunomicroscopic findings

α-Synuclein-positive structures appeared as evenly stained sporadic granules or spheroids with clear contours. They were distributed in the alveus (Fig. 1c), fimbria (Fig. 1d), as well as in the external capsule and the deep layer of the cerebral cortex on the infused side of the leupeptin-treated rats. Qualitatively the α-synuclein structures appear to be abundant in the alveus and fimbria. Furthermore, they appear to be numerous in the external capsule than in the deep layer of the cerebral cortex (data not shown). From the present observation and our previous electron microscopic study [28], these α-synuclein-positive structures seem to correspond to the degenerated axons originating from pyramidal cells in the hippocampus.

In contrast, no α-synuclein-positive structure was observed in other regions of the central nervous system (CNS) of leupeptin-treated rats (Figs. 1e and f) or in the whole CNS of control rats (Figs. 1a and b). To evaluate specificity of the antibody, the staining procedure without application of the primary antibody was performed, and only background staining was observed in leupeptin-treated (Figs. 1g and h) and leupeptin-non-treated rat (data not shown).

3.2. Immunoelectron microscopic findings

The accumulation of dense bodies in the neuronal perikarya and in the swollen neuronal processes, which we had previously reported, was also detected in the specimens for the present experiment.

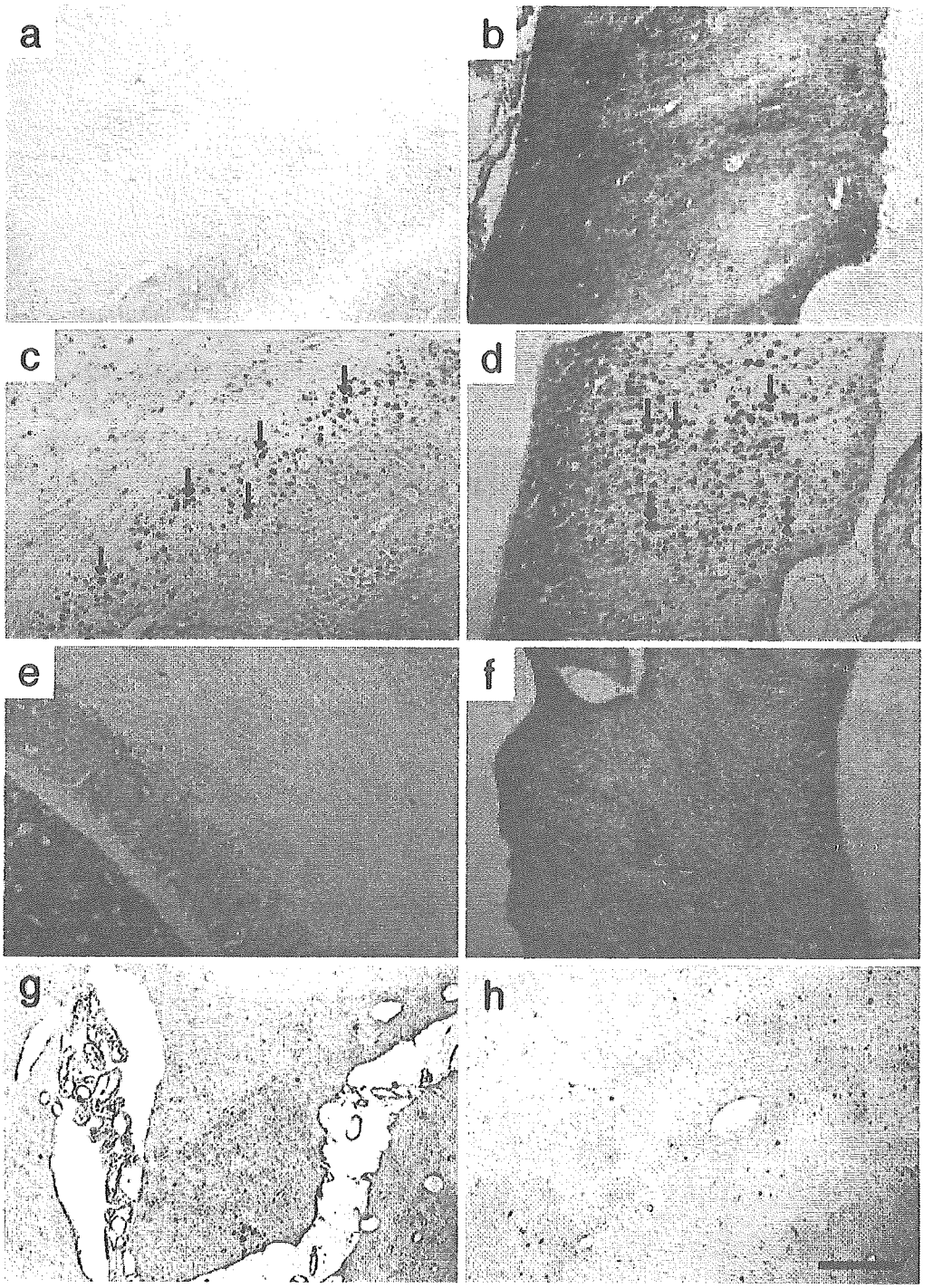

Fig. 1. (a–h) Immunohistochemical staining in rat fimbria and alveus. (a and b) Immunohistochemical staining of α-synuclein in control rat brain hippocampus. No α-synuclein-positive structure was observed in alveus (a), and fimbria (b). (c–f) Immunohistochemical staining of α-synuclein in leupeptin-treated rat brain hippocampus. (c and d) Leupeptin infused side. (e and f) Leupeptin non-infused side. α-Synuclein-positive structures (arrows) were observed in alveus (c) and fimbria (d) of the infused side. No α-synuclein-positive structures were observed in the corresponding areas of the non-infused side (e and f) (scale bar = 100 μm). (g and h) Immunohistochemical staining without primary antibody (scale bar = 200 μm).

Anti-α-synuclein immunoreactivity was found exclusively in the neuronal component of the leupeptin-treated rats, mainly in swollen axons of the fimbria and alveus of the hippocampus (Fig. 2a). The immunoreactivity appeared as small granules or more fuzzy electron-dense material filling the spaces among accumulated mitochondria and dense bodies observed in the so-called degenerated neurites (Fig. 2b).

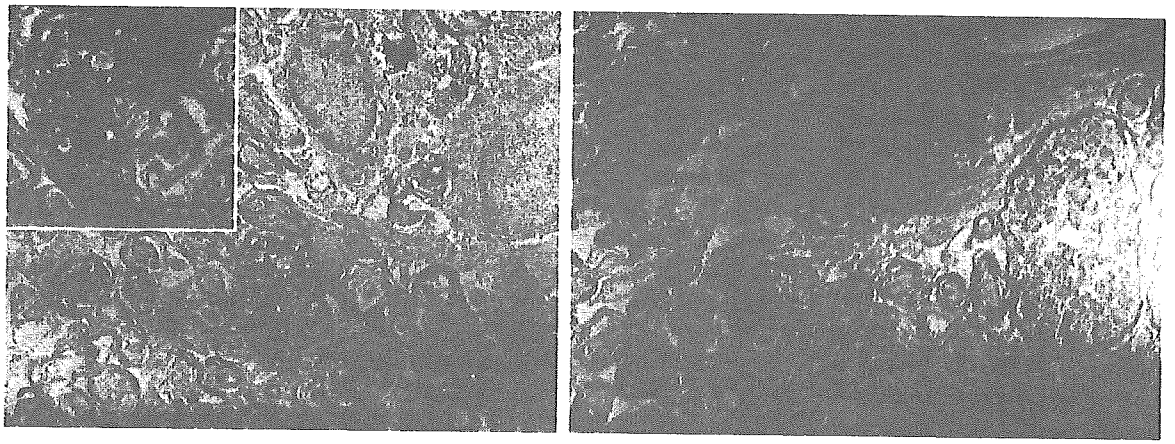

Fig. 2. (a and b) Immunoelectron microscopy for α-synuclein in leupeptin infused rat brain hippocampus. (a) A transverse section of a swollen axon in the fimbria (SP*) (scale bar = 2 μm). (b) A higher resolution immunoelectron micrograph of a swollen axon in the fimbria (scale bar = 1 μm). (c) Control rat brain hippocampus (scale bar = 2 μm).

3.3. SDS–PAGE and Western blottings

Western blot analysis showed a 19-kDa α-synuclein band in both the leupeptin-infused and the control rat hippocampal extracts (Fig. 3a); however, the densities of these reactions were obviously different. No band with higher molecular weight was observed in both experimental and control rats (Fig. 3a). This finding suggests that accumulation of monomer forms of α-synuclein was induced by leupeptin. The densities of these bands were quantified by Scion Image software (Fig. 3b). The integrated density of the leupeptin-infused specimens was 0.606 ± 0.08, and that of the control specimens was 0.421 ± 0.14. This indicates that the α-synuclein concentration in fimbria of the leupeptin-infused rats is significantly higher ($P < 0.05$) than that of the control rats.

3.4. Confocal laser-scanning microscopic findings

In the control rat fimbria (Figs. 4a–c), α-synuclein-positive structures (green) and ubiquitin-positive structures (red) were observed as diffusely distributed small granules. In the leupeptin-treated rat fimbria (Figs. 4d–f), α-synuclein-positive structures and ubiquitin-positive structures were observed as various sized and amorphous structures, and some cells were strongly labeled with anti-synuclein antibody only (arrows), or with anti-ubiquitin antibody only (arrowheads). In the merged images, colocalization of α-synuclein with ubiquitin was rarely observed.

4. Discussion

α-Synuclein, which is one of the presynaptic proteins, is composed of 140 amino acids [8] and is known to be identical to precursor (NACP) of the non-Aβ component of AD amyloid (NAC) [9]. The accumulation of α-synuclein has been demonstrated in Lewy bodies appearing in PD and DLB [1,6,26], as well as in degenerated neurons in the frontal lobe of AD [2]. In contrast to AD, in cases with multiple-system atrophy (MSA), these immunoreactivities were detected predominantly in glial cytoplasmic inclusions [31]. Moreover, neurons containing α-synuclein immunoreactive structures have been reported to be widely distributed in the amygdala, hippocampus, and superior temporal gyrus in DNTC [32]. These findings on the neurodegenerative process in general suggest that overproduction and disturbance of degradation of α-synuclein might play important roles in neuronal degeneration.

In the present experiment, leupeptin, a potent thiol protease inhibitor, was infused into the rat lateral ventricle. For two decades, leupeptin has been known to inhibit cathepsins and neural calcium-dependent protease and to cause various neuronal changes similar to those seen in the aging process or neuronal degeneration. By using the same experimental model, we have reported the accumulation of lipofuscin-like dense granules in the neuronal cell bodies, widespread degeneration of neuronal processes in the neuropil of the rat cerebral cortex, and formation of intraneuronal inclusions consisting of abnormal fibrillar structures, and we proposed that disturbances of protein turnover induced by leupeptin may have some pathogenic mechanisms in common with neuronal degeneration [27]. In this study we focus on α-synuclein. On the normal distribution of α-synuclein, the highest concentrations of α-synuclein mRNA are in the substantia nigra, the dentate granule cells and CA3 regions of the hippocampal formation, and the deep layers of the cortex [21]. Our immunohistochemical analysis in normal control revealed no obvious staining in DAB staining and dotlike structures in fluorescent staining. In the leupeptin-treated rat, immunolabeling of α-synuclein was observed in the neuropil of the fimbria, alveus. In stained neuronal cell bodies, the intense staining was distributed in whole cells, not as small punctate structures. Ultrastructurally, in normal rat brain, α-synuclein was present in synaptic boutons; however, in the leupeptin-

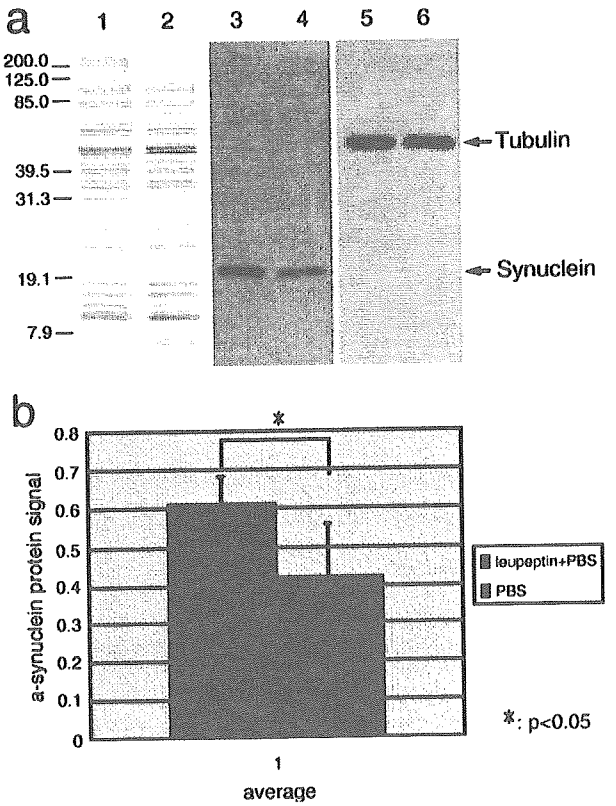

Fig. 3. (a and b) Western blotting analysis shows an increase of α-synuclein in leupeptin-infused rat fimbria. (a) Western blot analysis of α-synuclein shows an increase of α-synuclein in leupeptin-infused (lanes 1, 3, and 5) rat fimbria compared with PBS-infused (lanes 2, 4, and 6) rat fimbria. Total protein stained with Coomassie blue (lanes 1 and 2) and α-tubulin used as internal standard (lanes 5 and 6) indicates the same amount of applied sample proteins. A band of α-synuclein is detected in both leupeptin- and PBS-infused rats (lanes 3 and 4); however, the reaction density of the antibody is much more intense in lane 3 than in lane 4. Results are representative of five similar experiments. The numbers on the left indicate the molecular weight markers. The presented Western blot represents a typical result from one control and one leupeptin-treated rat brain. (b) Densitometric analysis of the Western blot is expressed as mean ± SD from five sets of experiments ($^*P < 0.05$).

treated rat brain, α-synuclein immunoreactivity was observed especially in the swollen axons in the fimbria and alveus, and in the enlarged presynaptic axon terminals as fine granules filling the spaces among the dense bodies and other organelle.

Western blot analysis showed a 19-kDa α-synuclein bands in both of the hippocampal extracts from leupeptin-infused and the control rat; however, the intensities of these bands were obviously different. No other band with higher molecular weight was observed. Scion Image software revealed that the intensity of the band in the leupeptin-infused rats was significantly stronger than that of control rats. This finding suggests that accumulation of monomer forms of α-synuclein proteins was induced by leupeptin in the neuronal cell body by the infusion of leupeptin and this event might be a trigger to the formation of α-syn accumulation previously described in this model.

In confocal laser-scan imaging, both of the α-synuclein-positive labeling and the ubiquitin-positive labeling were visualized in cell bodies in leupeptin-treated rats, while they were visualized as dotlike structures in cells in control rats. In the merged images of double labeling, colocalization of α-synuclein with ubiquitin was rarely observed. These findings suggest that α-synuclein accumulation might not be related with ubiquitin-proteasome pathway. It is possible that α-synuclein accumulation might be caused by lysosome or ubiquitin-independent proteasome [29]. Leupeptin is known lysosomal protease inhibitor; however, biochemical study previously showed that leupeptin binds not only with lysosomal proteases, but also with the non-lysosomal degradation system protease, namely the proteasome [24], suggesting that it might affect the proteosome activity, even though inhibitory effects of leupeptin to proteasome were not confirmed yet [19]. In this study, ubiquitin-positive labeling seemed to be accumulated as well as α-synuclein-positive labeling in the fimbria in confocal laser scanning. This finding suggests that leupeptin, an inhibitor to several kinds of proteases, can affect metabolic processes of neuronal proteins, and that α-synuclein and ubiquitin might be influenced with different process by leupeptin, as the α-synuclein-positive labeling and ubiquitin-positive labeling were not co-localized.

As we did not find any fibrillar structures that are the usual components of typical Lewy bodies, the accumulation of α-synuclein cannot be directly connected with the mechanism of Lewy body formation. In addition, it remains unclear whether a disturbance of degradation of α-synuclein is one of the early steps in the generation of Lewy bodies prior to nitration or other modification of α-synuclein, or not. To clarify these questions, further experiments are required.

Normal α-synuclein immunoreactivity often forms deposits or small granules, but not fibrous structures. Lewy bodies from postmortem PD brains are largely composed of the insoluble form of α-synuclein which has fibrous structures. Misfolded or damaged proteins are potentially toxic and are generally degraded in an ubiquitin-dependent manner by the proteasome, Lewy bodies are ubiquitin-positive structures and α-synuclein, one of its component, is reported to be degraded by proteasome [2]. Several studies have suggested a link between ubiquitin-proteasome proteolysis system malfunction and an increase in ubiquitinated complexes [11,23]. α-Synuclein was aggregated and formed fibrous structures, when it accumulates up to a certain concentration [15,16]. Therefore at this moment the most plausible hypothesis on the pathological pathway is that down-regulated proteolysis causes increased α-synuclein and other misfolded or damaged proteins, and that completely insoluble forms α-synuclein attenuate ubiquitin-proteasome proteolysis sys-

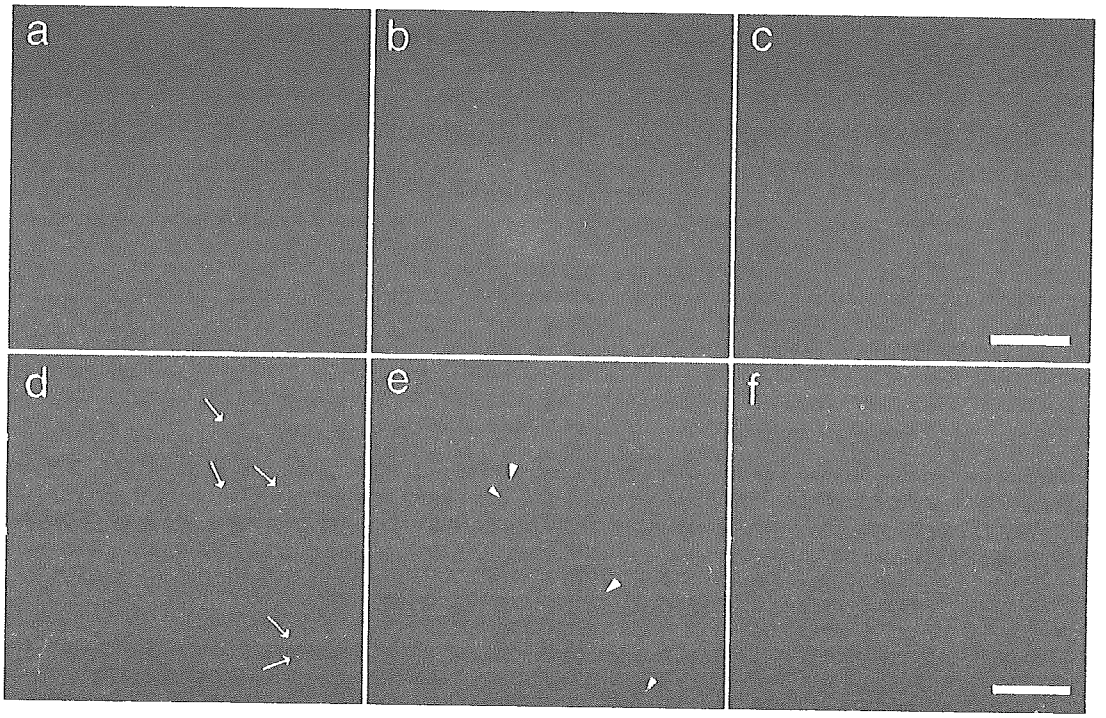

Fig. 4. (a–f) Confocal scanning images of the fimbria of rats at anti-α-synuclein (a and d; green), the anti-ubiquitin (b and e; red), and the merged images of double labeling(c and f). (a–c) The same section of fimbria in the PBS-infused rat. (d–f) The same section of fimbria in the leupeptin-treated rat. Some cells were strongly labeled with anti-synuclein antibody only (arrows), or with anti-ubiquitin antibody only (arrowheads) (scale bar = 50 μm).

tem, leading to vicious cycle. Our results also support this hypothesis because accumulation of α-synuclein in neuronal cells was induced by leupeptin, one of well-known lysosome inhibitors, which was reported to bind with proteasome also [24]. However it is still unclear which kind of protease is predominantly involved in the accumulation of α-synuclein and formation of fibrous structures, unfortunately.

In addition to the disturbance of degradation of α-synuclein, the disturbance of axonal flow caused by leupeptin, which was demonstrated in the previous studies [28], should be taken into account as another possible mechanism in the formation of α-synuclein-positive structures.

Recently, point mutations of the α-synuclein gene at position 53 (Ala53Thr) [22] position 30 (Ala30Pro) [14] and position 46 (E46K) [33] have been detected in the families of autosomal dominant Parkinson disease patients, which may be a cause of disturbed α-synuclein metabolism. On the other hand, a marked decrease of proteasomal function by 34–42% has been reported in sporadic PD cases [18], and the degradation of mutant α-synuclein as well as of wild-type α-synuclein through the ubiquitin-proteasome system was inhibited by a selective proteasomal inhibitor, β-lactone [2]. Moreover, proteasomal activities are decreased in the *para*-hippocampal gyrus, upper-middle temporal gyri, and frontal cortex in AD cases [12]. These findings indicated that dysfunction of the ubiquitin-proteasome system may cause the accumulation of α-synuclein; however, our findings suggested that dysfunction of lysosome system or ubiquitin-independent proteasome pathway might influence the accumulation of α-synuclein [29].

Experimental α-synuclein accumulation has previously been reported in vivo [3,25]; however, previously accumulation of α-synuclein was reported in transgenic-model mouse [5,16], or rodents injected with MPTP or rotenone [3,25,30]. Both of chemical compounds were not well characterized in biochemical function on the mechanisms of cytotoxicity yet, even though oxidative stress mechanisms are thought to be mediators in either compound. This is the first report of experimentally induced in vivo accumulation of α-synuclein in non-transgenic rodent brain injected with leupeptin, a biochemically well-characterized protease inhibitor, by an infusion pump. The leupeptin infusion rat model is thought to be a useful material for studying several neuronal changes resembling the aging or degeneration of the central nervous tissue. The accumulation of α-synuclein protein in the rat brain is a finding of new interest for research on the pathogenic mechanism of neurodegeneration.

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タウ蛋白修飾とアポトーシス阻害因子に関連するアルツハイマー病診断法と治療法の開発

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抄録：アルツハイマー病（AD）脳の異常蓄積成分としてタウ蛋白とアミロイドβ蛋白（Aβ）がある．我々はタウ蛋白がアポトーシス阻害蛋白であるX-chromosome-linked inhibitor of apoptosis（XIAP）と結合すること，低濃度のAβによってXIAPの発現が抑制されることを報告してきた．今回我々は，リチウムによるXIAPの発現の影響を検討し，リチウムおよびGSK-3阻害剤はXIAPの発現を亢進させることを見いだした．このことは，リチウムによるneuroprotectionのメカニズムの1つとして，アポトーシス阻害機能を有するXIAPの発現の亢進があることを示唆している．

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Key words：Alzheimer disease, tau protein, lithium, XIAP (X-chromosome linked inhibitor of apoptosis protein), apoptosis

はじめに

アルツハイマー病（AD）の神経病理学的特徴としては神経原線維変化と老人斑の存在が知られており，前者の構成成分は異常リン酸化タウ蛋白であり，後者の構成成分はアミロイドβ蛋白（Aβ）であることが知られている[5)6)7)11)]．これら異常な蓄積物の構成分子が，ADにおける神経変性メカニズムにどのように関与するかについては難しい問題である．細胞死のメカニズムとしてアポトーシスが非常に深く研究されており，このアポトーシスがADに関与するという報告は少なくないが，これに反する報告も少なからずある[4)14)15)23)25)]．我々はこのような事情を説明するための仮説として，アポトーシスを誘導するような細胞死ストレスと，それに拮抗する何らかの因子が同時に存在している可能性を想定して研究を行った．内因性に細胞内に存在しカスパーゼを抑制する蛋白として，IAP（Inhibitor of Apoptosis）というものが知られており，その中でもXIAPは多くの組織に発現して最もアポトーシスを抑制する因子とされている．昨年までに，我々はこのXIAPが過剰発現する細胞ではアポトーシスを誘導するような細胞死ストレスのもとでその細胞死を抑制すると同時に，アポトーシスに伴うタウ蛋白の脱リン酸化を抑制すること，およびN末端のMetの除かれたタウ蛋白と特異的に結合しその機能を抑制する可能性があること，そしてAβによってXIAPの発現が抑制され

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