was increased by more than 50% in the 16,000 g supernatants of AD brains as compared with normal aged controls [39]. This increase in PKB levels corresponded to a several-fold increase in the levels of total tau and abnormally hyperphosphorylated tau. Apparently, it is difficult to explain co-localization of the phosphorylated tau with both active GSK-3 and active PKB, because active PKB is thought to inactivate GSK-3. Presumably, more complex mechanisms are involved in the neurodegenerative process in AD; a decreased activity of protein phosphatases might be able to explain these situations.

In addition to active GSK-3 and PKB, other kinases related to the protein translation system have also been reported to be active in AD brains. Eukaryotic initiation factor-2α (eIF2α) is a regulator of protein translation and a phosphorylated form of eIF2α terminates global protein translation [40]. This eIF2α is phosphorylated by several kinases including protein kinase R (PKR), double-stranded RNA (dsRNA)-activated protein kinase, that is a ubiquitously expressed serine/threonine protein kinase induced by interferon and activated by dsRNA, TNF, IL-1 and lipopolysaccharide, or viral infection [41] (fig. 3). Chang et al. [42] reported that in AD brains both phosphorylated eIF2α and PKR were observed in affected neurons, while they were rarely observed in age-matched control brains.

The ribosomal S6 protein kinase p70 S6 kinase is known for its role in modulating cell size and cell survival [43]. Activated p70 S6 kinase upregulates ribosomal biosynthesis and enhances the translational capacity of the cell. Signal transduction cascade that activates p70 S6 kinase has been investigated, and the mammalian target of rapamycin (mTOR) and tuberous sclerosis complex (TSC) were reported to be important regulators in this translational machinery [44] (fig. 3). Recent reports revealed that phosphorylation of p70 S6 kinase by mTOR induced activation of p70 S6 kinase, leading to activation of translation, and that mTOR and p70 S6 kinase were inhibited by TSC [44]. On the other hand, the inhibitory potential of TSC to mTOR was reported to be downregulated by its phosphorylation by PKB [45, 46]. Therefore, p70 S6 kinase is located down stream of the PI3K pathway. An et al. [47] reported that the levels of phosphorylated p70 S6 kinase (at Thr389 or at Thr421/Ser424) were increased in accordance with the progressive sequence of neurofibrillary changes according to Braak's criteria [47]. Both PKR and p70 S6 kinase Both eIF2α and p70 S6 kinase are working to regulate translation, but the former inhibits translation and, on the other hand, the latter enhances it. Therefore activation of PKB and p70 S6 kinase might be an auto-feedback response to inhibition of translation induced by activated eIF2α and PKR. Until now only little evidence on the involvement of translational machinery in AD pathology has been reported. On the translational system, we have previously reported that peptidyltransferase inhibitors induced the phosphorylation of tau protein and

neuronal cell death in SH-SY5Y cells; dysfunction of ribosome in AD was also reported by others [48, 49]. More investigations on this matter will be required to clarify the neurodegenerative mechanisms including AD.

Conclusion

The mechanisms of phosphorylation of tau protein are still unclear; however, the signal transduction pathway of GSK-3, a strong candidate that phosphorylates tau abnormally, was overviewed. The PI3K pathway has an important role in both the regulation of phosphorylation of tau and cell survival. In addition PKB, which participates in the PI3K pathway, also has an important role in the regulation of p70 S6 kinase activity that regulates protein translation. From the reports on the phosphorylation of p70 S6 kinase, eIF2α and PKR in AD brains, both the PI3K pathway and the protein translational machinery might be closely involved in AD pathology, and more investigations will be required to understand tau phosphorylation in 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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精神薬療研究年報　第37集　(2005) 別冊

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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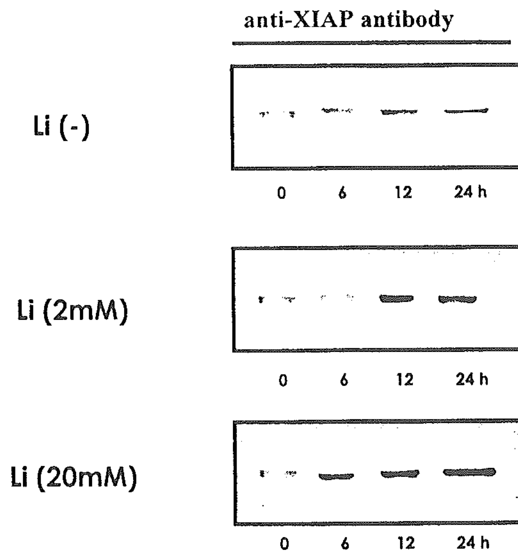

Fig. 1 Increased expression of XIAP in SY5Y cells treated with lithium chloride
Human neuroblastoma SH-SY5Y cells were cultured in the presence and absence of lithium chloride (2 mM and 20 mM). Cells were collected and lysed after 0, 6, 12, and 24 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Increased expression of XIAP was observed in cells treated with lithium chloride by dose-dependent manner.

ることを報告してきた[21)22)24)]．そこで，今年度は，XIAPの発現を逆に促進する因子を検討した．

リチウムは臨床的には主に躁病に用いられる薬剤であるが，タウ蛋白をリン酸化する酵素の1つであるグリコーゲンシンターゼキナーゼ-3（Glycogen Synthase Kinase-3（GSK-3））の阻害機能があることが判明し，タウ蛋白のリン酸化制御の側面からも研究が行われている[10)12)]．また，neuroprotectionの作用があることも知られ，そのメカニズムとしてはProtein Kinase B（PKB）の活性化やBcl-2の発現促進なども報告されている[1)2)]．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．

方　　法

まず，培養細胞に対するXIAPの発現の検討を行うためSY5Y神経芽細胞腫を5％ウシ胎児血清を含むD-MEM/F-12培地にて培養し，2 mMおよび20 mMの塩化リチウムを添加し24時間まで経過を追った後，細胞を集めた．また，昨年度5 μMのA$\beta_{25\text{-}35}$によって，XIAPの発現が低下することを確認しているので，リチウムがそれにどのような影響を与えるかを見るために，SY5Y神経芽細胞腫に5 μM A$\beta_{25\text{-}35}$および5 μM A$\beta_{25\text{-}35}$と2 mM塩化リチウムを添加して24時間後，細胞を集めた．さらに，リチウムの作用機序を検討するために，市販のGSK-3阻害剤（10 μMのGSK-3 Inhibitor-Iおよび-II（Calbiochem社））を添加して3時間後に細胞を集めた．集めた細胞はバッファー（100 mM PIPES, pH6.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinine, 5 μg/ml leupeptine, 0.1％ Triton-X100）にて溶解し，そのlysatesを200K × Gにて遠心し，supernatantを得た．このsupernatantの各50 μgをポリアクリルアミドゲルの各レーンにアプライして，抗XIAP抗体（R&D社）を用いたウエスタンブロットを行いXIAPの発現を検討した．

そして，リチウムのneuroprotectionの効果を確認するために，SY5Y神経芽細胞腫に2 mMの塩化リチウムを添加して24時間培養したものとそうでないものを用意し，これにさまざまな細胞死ストレス（10 μMおよび50 μMの過酸化水素，25 μMのA$\beta_{25\text{-}35}$）を添加した．そして24時間後の細胞死のレベルについてLive and Dead Assay（Molecular Probe社）を用いて検討した．

結　　果

SY5Y神経芽細胞腫に2 mMおよび20 mMの塩化リチウムを添加したところ，時間とともにXIAPの発現量は増加した．この効果は濃度依存的であった（**Fig. 1**）．次に，同じ細胞に5 μMのA$\beta_{25\text{-}35}$を添加して24時間後のXIAPの発現は低下していたが，同時に2 mMの塩化リチウムを添加していた細胞では，逆にXIAPの発現は増加していた

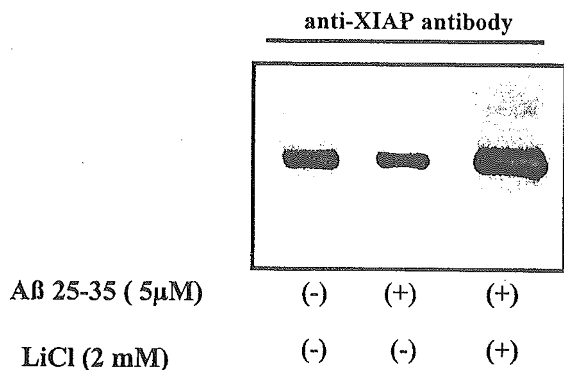

Fig. 2 Decreased expression of XIAP is reversed by lithium
SH-SY5Y cells were cultured in the presence of amyloid β25-35 or in the presence of combination of amyloid β25-35 and 2 mM lithium chloride. Cells were collected and lysed after 24 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Decreased expression of XIAP was observed in cells treated with amyloid β25-35 , however this effect was reversed by lithium chloride.

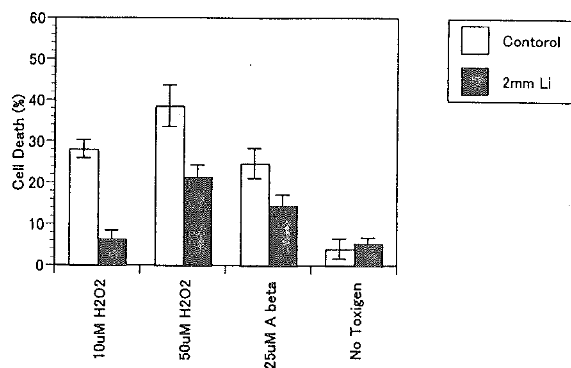

Fig. 4 Decreased cytotoxicity in SY5Y cells treated with lithium
SH-SY5Y cells were cultured in the presence and absence of 2 mM lithium chloride for 24 hours. Then the cells were treated with hydrogen peroxide (10 μM and 50 μM), and 25 μM amyloid β25-35 peptide for 24 hours. And live and dead cells were counted. Dead cells were observed in cells treated with hydrogen peroxide and amyloid β 25-35 peptide, however decreased cytotoxicities were observed when cells were pretreated with 2 mM lithium chloride.

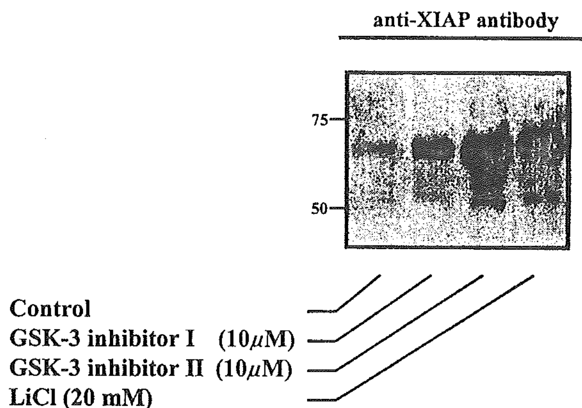

Fig. 3 Increased expression of XIAP by GSK-3 inhibitors
SH-SY5Y cells were cultured in the presence of 20 mM lithium chloride or 10 μM GSK-3 inhibitore I or II. Cells were collected and lysed after 3 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Increased expression of XIAP was observed in cells treated with GSK-3 inhibitore I or II, and also with lithium chloride.

(**Fig. 2**). さらに，GSK-3 阻害剤（10 μMの GSK-3 Inhibitor-I および -II）を添加して3時間後のXIAPの発現量を検討したところ，20 mMの塩化リチウムを添加した場合と同様にXIAPの発現は増加していた（**Fig. 3**）. 最後に，SY5Y神経芽細胞腫に2 mMの塩化リチウムを添加したものとそうでないものを24時間培養し，さまざまな細胞死ストレスを与えると，10 μMおよび50 μMの過酸化水素の場合も，25 μMの A$\beta_{25\text{-}35}$の場合も，細胞死レベルは減少していた（**Fig. 4**）. 以上のことから，リチウムはXIAPの発現を亢進させること，その機序はGSK-3の阻害であること，そしてリチウムによる 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が示唆された.

考　　察

我々は今までに,神経細胞死とタウ蛋白異常リン酸化のメカニズムをプロテインフォスファターゼやGSK-3およびストレス関連MAPキナーゼとの関わりから詳細に検討してきた[16,17,18,19,20,23]. その中

で，アポトーシスのメカニズムに興味をいだき，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知られるIAPの1つであるXIAPについて研究を行ってきた[21)22)24)]．そして，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，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の発現が抑制されることを報告してきた．今回の研究は，XIAPの発現を逆に促進する因子としてリチウムを見出し，その検討を行ったものである．

そもそもリチウムは躁病に対する薬剤として約半世紀使用されてきたが，さまざまな生物学的作用を有し，抗躁効果の作用機序は未だ明らかではない．有力な機序としては，フォスファチジルイノシトールモノフォスファターゼ阻害作用，結果として細胞内イノシトール枯渇作用というものが知られている[8)]．しかし，抗躁効果との関連性はないようであるがリチウムの重要な薬理作用としてGlycogen Synthase Kinase-3（GSK-3）の阻害機能があることもよく知られるようになり，タウ蛋白のリン酸化制御や，詳細な機序は不明であるがAβ産生制御の側面からも研究が行われるようになった[10)12)13)]．また，リチウムには近年neuroprotection作用があることが報告されるようになり，この機序としてはProtein Kinase B（PKB）の活性化やBcl-2の発現促進などが報告されている[1)2)]．

今回の我々の研究は，XIAPの発現を促進する因子としてリチウムを見出したものであったが，今回の実験結果からはその作用機序としてはGSK-3の阻害がまず考えられる．発現量の促進に関しては，通常の転写レベルでの亢進と翻訳レベルでの亢進，そして分解速度の低下がその理由として考えられる．XIAPの発現は他の蛋白と異なり，通常の転写レベルでの制御に加えて，その遺伝子の5'-末端にIRES（Internal Ribosome Entry Sites）という配列を持つことから翻訳レベルでの制御が重要であるとされている．このIRESによる制御を介して，培地のアミノ酸除去などのストレス刺激によってmRNAから蛋白への翻訳量が増大することが知られているが，これは細胞死ストレスに対する生体反応として備わっている可能性が高い[3)9)]．今回発見した，発現亢進を誘導するリチウムは細胞死ストレスとは異なるため，神経変性疾患に対して治療的に応用されうる可能性を有している．GSK-3の機能とIRESを介した翻訳制御についてはまだ報告はないが，興味深い可能性があると思われ，さらに検討が必要と考えられる．また，リン酸化酵素が分解に関与する可能性としては，ある蛋白がリン酸化によりユビキチン化が亢進し，そのため分解が促進する例もあり，この点についてもさらに検討が必要と考えられる．

以上より，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が示唆された．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も示唆している．以前の検討から，少なくとも，アポトーシス阻害因子という細胞死の過程に逆の働きをしているものも関与している可能性が示唆されていることから，今後提起された疑問点を解明するための検討を行うことにより，さまざまな神経変性疾患におけるアポトーシス阻害因子をターゲットにした治療および診断的方法の検討が必要と考えられる．

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ABSTRACT

Study on diagnosis and therapeutics for Alzheimer disease in relation to modified-tau and inhibitor of apoptosis protein

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Amyloid β (Aβ) and tau protein are abnormally accumulated protein in Alzheimer disease (AD). Previously we reported tau protein could bind with XIAP (X chromosome-linked inhibitor of apoptosis), one of intrinsic anti-apoptotic proteins, and low dose of Aβ attenuated expression of XIAP. Here we show lithium and GSK-3 inhibitors up-regulate the expression of XIAP. This finding might support the mechanisms of neuroprotective effects of lithium, and XIAP might be one of therapeutic targets in neurodegenerative disease including AD.

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Chapter 23

Cerebrospinal fluid phosphorylated tau protein at serine 199 is a useful diagnostic biomarker in Alzheimer's disease and mild cognitive impairment

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INTRODUCTION

Our recent studies of biological markers in Alzheimer's disease (AD) have focused specifically on analysis of cerebrospinal fluid (CSF) tau protein levels and amyloid β-protein ending at amino acid 42.[1–4] Although CSF total tau (t-tau) level in AD was significantly higher than in controls, there were overlaps between AD and non-AD dementias.[1–3] One possible explanation is that the enzyme-linked immunosorbent assay (ELISA) kit we used detects not only phosphorylated but also normal tau. Therefore, we developed the sandwich ELISA system for phosphorylated tau at serine 199 (p-tau 199) in CSF[5] and examined 236 cases with AD, 206 cases with non-AD demented and non-demented disease controls, and 95 age-matched normal controls.[6]

SUBJECTS AND METHODS

Table 23.1 shows a summary of the patients' demographic data. We surveyed a total of 537 CSF samples. We also examined CSF p-tau 199

Table 23.1 Summary of patients' demographic data

	No. of patients	*Age* (years)	*Gender* (M/F)
Alzheimer's disease (AD)	235*	71 ± 9	66/172
Normal control	95	57 ± 16	51/44
Neurological disease control	122	59 ± 13	70/52
Frontotemporal dementia (FTD)	16*	63 ± 12	9/7
Progressive supranuclear palsy	21	63 ± 7	10/11
Corticobasal degeneration	15	64 ± 4	8/7
Dementia with Lewy body (DLB)	13*	63 ± 10	8/5
Vascular dementia	23	71 ± 6	16/7
Meningoencephalitis	18	51 ± 21	7/11
Creutzfeldt–Jakob disease (CJD)	11*	71 ± 6	6/5

* Two patients with AD, one patient with FTD, one patient with DLB and four patients with CJD were confirmed by autopsy

levels in a population with mild cognitive impairment (MCI). The MCI group was later subdivided into two different categories. One category was that which eventually later progressed to AD (progressive MCI). The other category was that which later did not progress to AD (non-progressive MCI). Memory complainers were patients who complained about memory disturbance, but were not demented. These constituted the control group. CSF samples were taken into polypropylene tubes by lumbar puncture after informed consent was obtained from each patient and/or family members. Bloody or traumatic CSF samples were excluded from this study. After centrifugation at 1500 rpm for 10 min, the aliquots were stored at −80°C until analysis. CSF levels of p-tau 199 were measured by a sensitive sandwich ELISA.[5,6] CSF level of t-tau protein was measured using the sandwich ELISA assay provided by the Innogenetics Company, Belgium.[7]

RESULTS

CSF p-tau 199 levels in the AD group were significantly elevated ($p < 0.001$) compared to those in all the other non-AD groups, including patients with acute neurological conditions such as meningoencephalitis and Creutzfeldt–Jakob disease (CJD) (Figure 23.1). On the other hand, CSF t-tau levels were occasionally very high in the meningoencephalitis and CJD groups,

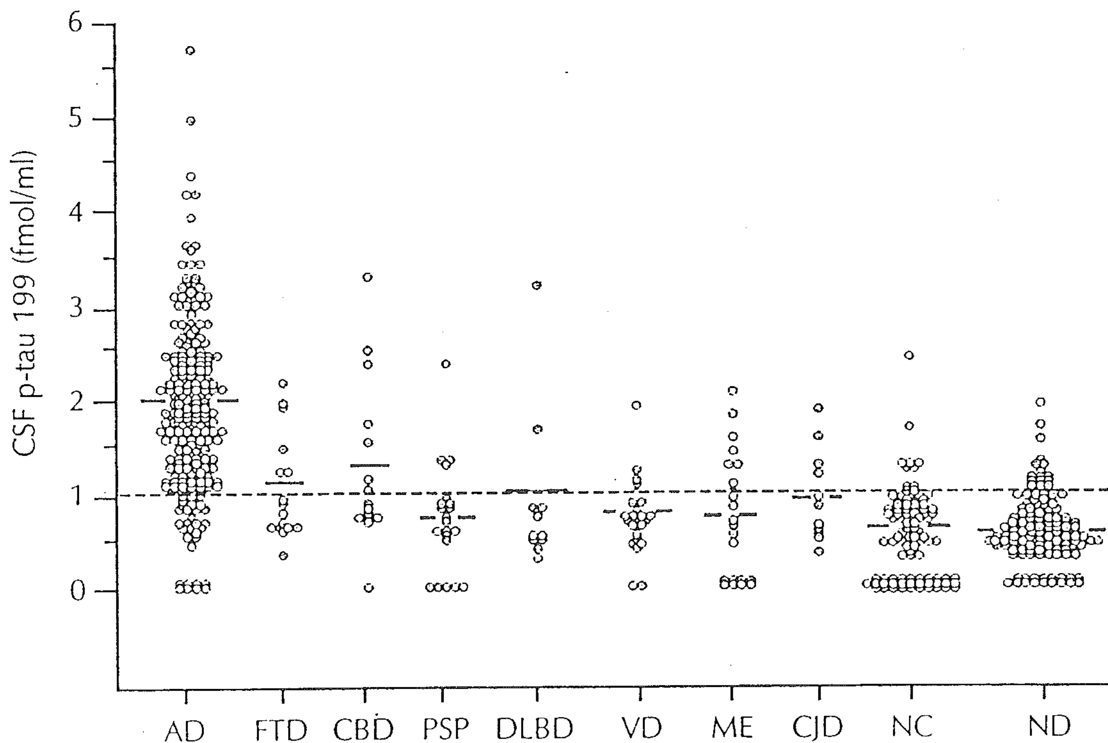

Figure 23.1 The results of cerebrospinal fluid (CSF) phosphorylated tau at serine 199 (p-tau 199) levels, among groups with Alzheimer's disease (AD), frontotemporal dementia (FTD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), dementia with Lewy body disease (DLBD), vascular dementia (VD), meningoencephalitis (ME), Creutzfeldt–Jakob disease (CJD), normal controls (NC) and neurological disease controls (ND)

although most CSF t-tau levels were significantly increased in the AD group compared to normal control groups (Figure 23.2).

A receiver operating characteristics (ROC) curve analysis demonstrated that CSF p-tau 199 was more amenable than CSF t-tau to differentiating between AD and non-AD subjects (Table 23.2).

The results of CSF p-tau 199 levels in the progressive MCI group were significantly

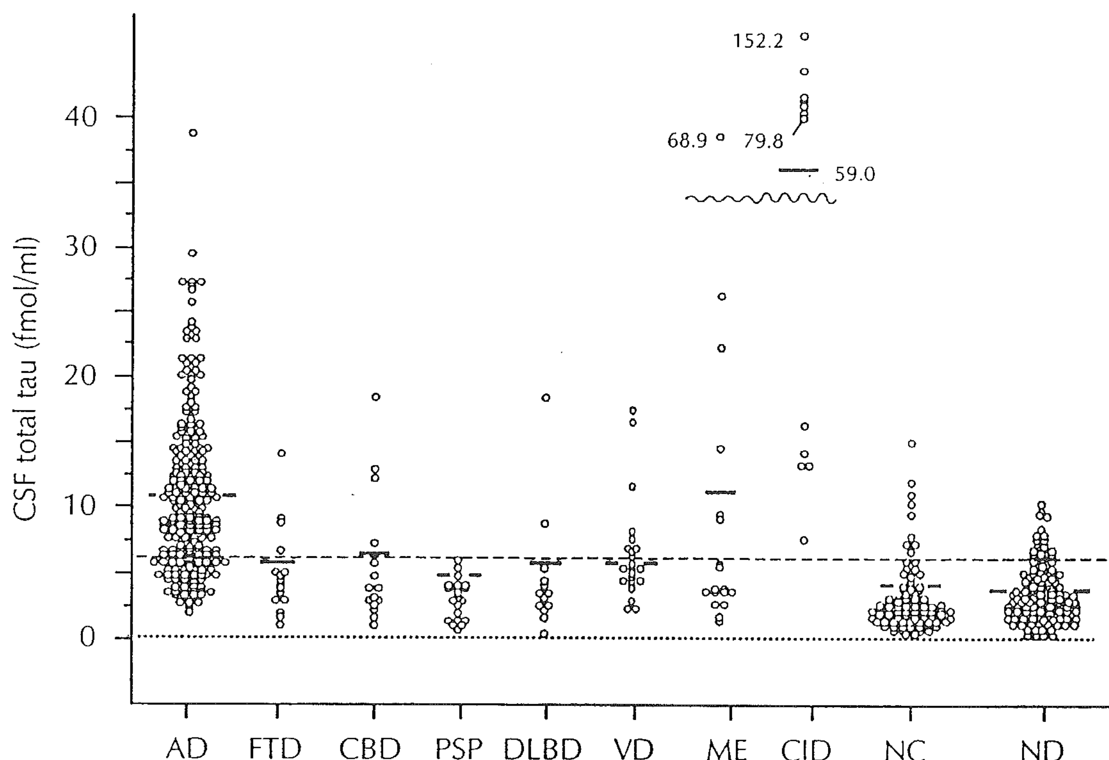

Figure 23.2 The results of cerebrospinal fluid (CSF) total tau levels, among groups with Alzheimer's disease (AD), frontotemporal dementia (FTD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), dementia with Lewy body disease (DLBD), vascular dementia (VD), meningoencephalitis (ME), Creutzfeldt–Jakob disease (CJD), normal controls (NC) and neurological disease controls (ND)

Table 23.2 Receiver operating curve analysis

	Cut-off level (fmol/ml)	*Sensitivity* (%)	*Specificity* (%)
Alzheimer's disease vs. neurological disease controls and normal controls			
Total tau	4.8	82.7	82.0
p-tau 199	0.96	87.3	87.4
Alzheimer's disease vs. others			
Total tau	6.0	77.1	77.6
p-tau 199	1.05	85.2	85.0

p-tau 199, phosphorylated tau at serine 199

elevated ($p < 0.001$) compared to those in the non-progressive MCI and the control groups (Figure 23.3). We thus propose that CSF p-tau 199 may also be useful for the diagnosis of MCI as it is for AD.

DISCUSSION

In the present study, we examined CSF p-tau 199 levels in a total of 570 living ($n = 562$) or autopsy-confirmed ($n = 8$) subjects with AD and other dementing disorders that resemble AD, as well as normal and neurological diseased controls. A combination of HT-7 (phosphorylation-independent monoclonal antibody; Innogenetics) and the anti-p-tau 199 antibody anti-PS199 allowed us to detect and quantitate CSF levels of the p-tau 199 by a newly constructed sandwich ELISA.[5,6] We reported p-tau 199 to be elevated in AD using different diagnostic antibodies that uniquely recognize specific phosphorylation epitopes of tau. We also monitored the CSF t-tau levels side by side in the same patients to assess and compare the sensitivity and specificity by ROC. Here, it should be noted that CSF p-tau 199 is not only the first biomarker that exceeds (over 85%) both sensitivity and specificity as a sole biomarker of AD, but also meets many other recommended criteria as an ideal biomarker.[8] The improvement of the diagnostic accuracy using CSF p-tau 199 seems to be accomplished not only by enhancing the lowest detection limit but also by eliminating a subset of non-AD patients with high CSF t-tau levels. Indeed, it is noteworthy that a subset of CJD patients with extremely high CSF t-tau levels showed only a mild elevation or an elevation under the cut-off level of CSF p-tau 199.

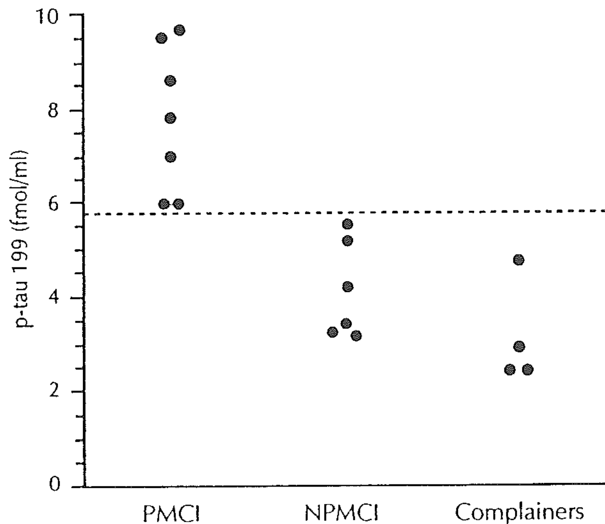

Figure 23.3 The results of cerebrospinal fluid (CSF) phosphorylated tau at serine 199 (p-tau 199) in mild cognitive impairment (MCI). PMCI, progressive MCI (Alzheimer's disease; AD); NPMCI, non-progressive MCI (non-AD). With a cut-off of 5.8 fmol/ml, sensitivity for PMCI 100% (7/7), and specificity for NPMCI 100% (6/6)

Nonetheless, our study suggests that there might be a limitation even in the use of the p-tau assay for a clear-cut distinction to be made between AD and certain tauopathies. In fact, the CSF p-tau 199 levels were over the cut-off value in approximately 30% (16/52) of the non-AD tauopathy group (Figure 23.2). Additional studies reported that pathological tau isoforms purified from tauopathy brains were occasionally hyperphosphorylayed at serine 199.[9,10] Therefore, the CSF p-tau 199 testing may be less accurate in distinguishing AD from other types of degenerative dementia or tauopathies. New and/or modified biomarkers would be necessary to differentiate AD from tauopathies in the future.[11,12]

A substantial proportion of subjects with MCI later developed clinical AD.[13] At autopsy, subjects with MCI showed a broad spectrum of morphological brain changes including typical AD pathological characteristics. Therefore, MCI

partly represents a predementia stage of AD. To maximize the benefit of therapeutic strategies, it is important to identify AD at the stage of MCI. Biochemical markers will be required to establish the diagnosis of MCI.[14] This study showed that CSF p-tau 199 levels in the progressive MCI group were significantly elevated compared to those in the non-progressive MCI and the control groups. We found that CSF p-tau 199 increased in an early stage of AD, the so-called MCI state, and confirmed that CSF p-tau 199 may be useful for the diagnosis of MCI as well as AD.

CONCLUSION

Our results suggest that CSF p-tau 199 is useful for an early diagnosis of AD.

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REVIEW ARTICLE

Studies on diagnostic markers for Alzheimer's disease

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Key words: *acetylcholine receptor α7, cerebrospinal fluid, genetic polymorphism, phosphorylated tau protein, touch panel computer.*

Abstract

In recent years, Alzheimer's disease (AD) has increased in incidence in Japan and elsewhere, and the marketing of donepezil hydrochloride (Aricept®) has allowed for the treatment of AD. These circumstances have encouraged the development of and research in markers for the early diagnosis of AD. Currently, the measurement of phosphorylated tau protein in the cerebrospinal fluid is considered to provide the most reliable and useful diagnostic marker for AD. For this purpose, a screening test using a touch panel computer can be recommended. The results of our study also suggest that the analysis of acetylcholine receptor α7 genetic polymorphism may be useful as a marker in the treatment with acetylcholine esterase inhibitors.

INTRODUCTION

In recent years, Alzheimer's disease (AD) has increased in incidence in Japan and elsewhere, and it accounts for about half the dementing diseases.[1,2] The recent marketing of donepezil hydrochloride (Aricept®) has allowed AD to be treated, and researchers have reported its usefulness.[3,4] Thus, the key to the treatment of AD is whether it can be diagnosed early and reliably. Unfortunately, AD is currently diagnosed solely by exclusion, and the development of diagnostic markers that are more easily accessible to everyone is highly desired. Researchers have tried many approaches in the development of diagnostic markers, of which tau protein-related ones have yielded the best results. This paper reports on the studies of tau protein-related markers and other markers.

DIAGNOSTIC MARKERS FOR AD

The Reagan Institute established to eradicate AD specifies that diagnostic biomarkers for AD must have the following characteristics:[5] They must reflect the disease status, be minimally invasive to the patient and have a high diagnostic accuracy in the differentiation between AD and other dementing diseases; that is, have a detection rate (sensitivity) of more than 80% for AD patients and a non-detection rate (specificity) of more than 80% for non-AD patients. We have previously reported that cerebrospinal fluid total tau protein satisfies most of the above requirements fairly well, but it does not have a sensitivity or specificity of over 80%. However, in combination with amyloid β-protein, it achieves a sensitivity and specificity of over 80% (called the AD index or AD unit).[6,7] The use of total tau protein as a biomarker for AD poses particular problems in that meningoencephalitis and Creutzfeldt–Jakob disease are associated with extremely high levels of total tau protein in the cerebrospinal fluid (Fig. 1).[8] To develop a single marker that meets the above requirements, we analyzed phosphorylated tau protein in the cerebrospinal fluid. Since tau protein in the degenerated neurofibrils in the brains of AD patients is hyperphosphorylated, we postulated that the selective measurement of phosphorylated tau protein would yield better results than the measurement of total tau protein. Focusing on phosphorylation at serine 199, our research group developed a sandwich enzyme-linked immunosor-

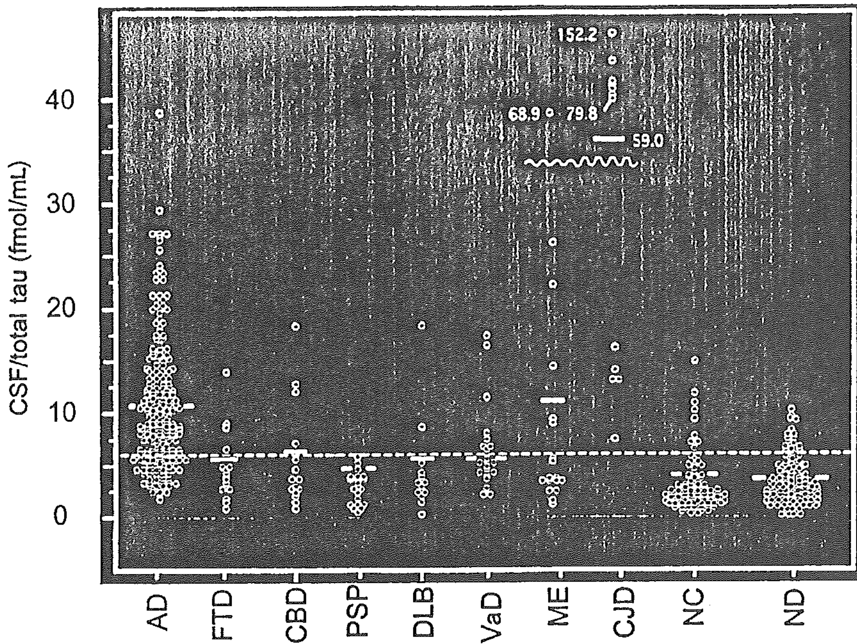

Figure 1 Quantification of total tau in cerebrospinal fluid (CSF). Total tau in CSF was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) employing anti-tau antibodies. AD, Alzheimer's disease; CBD, corticobasal degeneration; CJD, Creutzfeldt-Jakob disease; DLB, dementia with Lewy body; FTD, frontotemporal dementia; ME, meningoencephalitis; NC, normal controls; ND, non-dementia; PSP, progressive supranuclear palsy; VaD, vascular dementia.

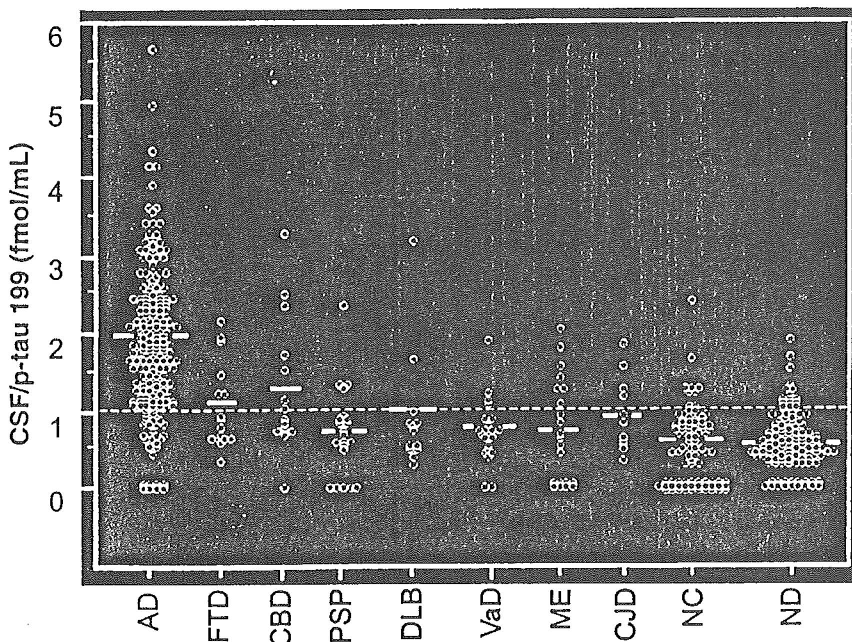

Figure 2 Quantification of phosphorylated tau at Ser 199 in cerebrospinal fluid (CSF). Phosphorylated tau in CSF was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) employing antiphosphorylated tau antibodies. AD, Alzheimer's disease; CBD, corticobasal degeneration; CJD, Creutzfeldt-Jakob disease; DLB, dementia with Lewy body; FTD, frontotemporal dementia; ME, meningoencephalitis; NC, normal controls; ND, non-dementia; PSP, progressive supranuclear palsy; VaD, vascular dementia.

bent assay (ELISA) to quantify N-terminal fragments of phosphorylated tau protein,[9] which gave better results than total tau protein measurement. In particular, phosphorylated tau protein levels are low in patients with meningoencephalitis and Creutzfeldt–Jakob disease, which are associated with high total tau protein levels (Fig. 2). Thus, the receiver operating characteristic (ROC) analysis also showed improved results, with a sensitivity and specificity of over 80% (Table 1).[10] In addition to our method of quantifying tau protein phosphorylated at serine 199, methods of quantifying tau protein phosphorylated at threonine 181 and threonine 231 have been reported. All these methods have yielded good results, making them the

most reliable diagnostic markers. To further increase the diagnostic accuracy of biomarkers, attempts have been made to increase the ability to differentiate AD from tauopathies typified by corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP),[11,12] such as the method of separately determining the levels of tau isoforms in the cerebrospinal fluid. As tau protein occurs mainly as the 4-repeat isoform in AD, and as the 3-repeat isoform in CBD and PSP, its differentiation is theoretically possible.

SCREENING TEST FOR AD

As described above, the measurement of phosphorylated tau in the cerebrospinal fluid is currently the most reliable marker, but it is not easy to perform a cerebrospinal fluid examination. Therefore, a simple screening test is necessary. One approach is to develop a test that can be performed on blood or urine. Regrettably, no useful markers have been developed so far. Although our group has been measuring blood tau and Aβ levels, we have not achieved satisfactory results. Thus, as another approach, we developed a simple screening method for AD using a touch panel computer.[13] We used questions assessing temporal orientation, delayed recognition and space perception (choosing cubes and triangular prisms), which are sensitive test items. The computer program was developed using Microsoft Visual Basic 6.0, and was made to operate on a PC running a Windows operating system. Hardware with an audio output was used to provide audio information as well as visual information. As elderly individuals are not accustomed to using a mouse, a touch panel was adopted. Incorrect answers were given a mark of 0, and results were graded on a scale of 0 to 15. Tests on 49 AD patients and 30 control subjects showed that almost all the subjects in the control group obtained full marks (with one or two incorrect answers, if any), whereas subjects in the AD group failed to give the correct answer to three or more questions (Fig. 3). Thus, at a cut-off value of 12, the ROC analysis indicated that the screening test has a very high accuracy with a sensitivity of 96% and a specificity of 97%. Because this test can be easily performed anywhere, and is non-invasive, highly sensitive, and highly specific, it is extremely useful.

Table 1 Receiver operating characteristic (ROC) analysis of total tau and phosphorylated tau assays

	Cut-off level	Sensitivity	Specificity
AD vs NC + ND			
Total tau	4.8 fmol/mL	82.7%	82.0%
p-tau 199	0.96	87.3	87.4
AD vs others			
Total tau	6.0 fmol/mL	77.1%	77.6%
p-tau 199	1.05	85.2	85.0

ROC analysis also showed an improved sensitivity and specificity of over 80% in the case of phosphorylated tau in the cerebrospinal fluid (CSF) assay. AD, Alzheimer's disease; NC, normal controls; ND, non-demented controls.

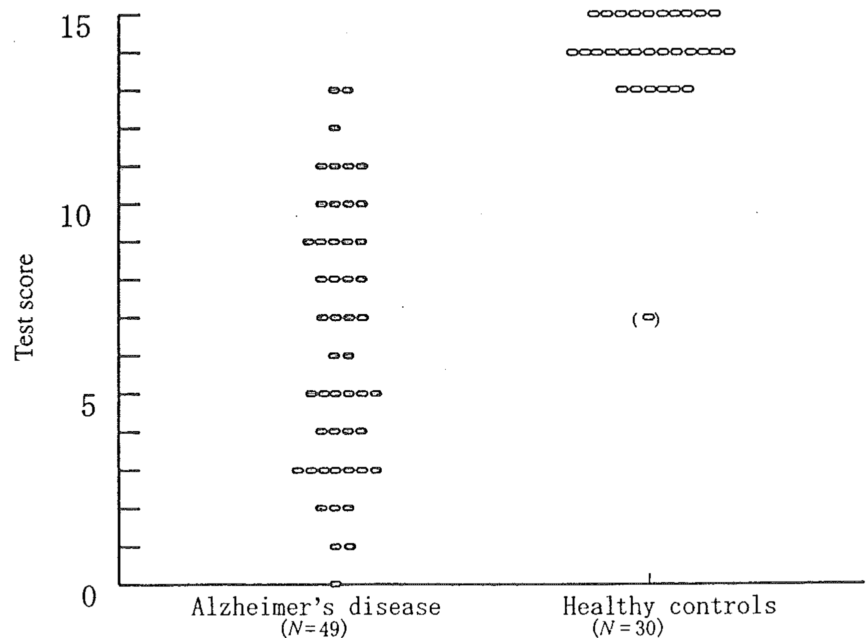

Figure 3 Results of the simple screening test for dementia using a touch panel computer. A simple screening method for Alzheimer's disease (AD) using a touch panel computer was developed. Tests on 49 AD patients and 30 control subjects showed that almost all subjects in the control group obtained full marks.