EDITORIAL

Multiple anti-Alzheimer disease activities of non-steroidal anti-inflammatory drugs

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Today, several drugs are available for Alzheimer's disease (AD) treatment and have shown suppression of disease progression, but none of them have a dramatic effect. In 1906, exactly 100 years ago, the first AD case was reported by Dr Alois Alzheimer. In 1970s reduction of choline acetyltransferase activity in AD was demonstrated. Choline esterase inhibitors which are clinically used today have been developed based on this observation. In these few decades, basic research strongly suggests that amyloid pathology is more specific and upstream of AD pathology than other pathology including the dysfunction of choline acetyltransferase activity. However, we don't have any drug which directly suppresses amyloid pathology in AD. Anti-amyloid drugs are under intensive development in many institute including mega pharmaceutical companies.

On the other hand, non-steroidal anti-inflammatory drugs (NSAID) have been suggested to protect onset of AD.[1–4] Many epidemiological studies,[1–4] with the exception of some,[1] continuously showed the anti-AD effects by NSAID. Furthermore, it was recently revealed that NSAID can suppress amyloid pathology in *in vitro*[5–8] and *in vivo*[9,10] studies. This unexpected effect of NSAID considerably influences the development of other AD drugs which aim to suppress amyloid pathology. Different kinds of anti-AD mechanism by NSAID have been revealed (Figure 1).[11,12] Ironically, that makes the understanding of NSAID difficult. In the present article, we try to organize these remarkable observations about NSAID and AD.

INFLAMMATION IN THE AD BRAIN

The brain is known as an immunologically-privileged organ. Swelling of the brain which is in a closed space in the skull is potentially lethal. Poor lymphatic tissue, blood–brain barrier results in less inflammation. Nonetheless, chronic inflammation in the AD brain was confirmed. Complement is increased and microglia is activated in AD brain.

Based on these facts, the effects of anti-inflammatory drugs on AD were examined. So far, many epidemiological studies showed anti-AD effects by NSAID. In particular, AD prevention effects were strongly suggested in NSAID users in large cohort studies. Relative AD risk of NSAID users of more than two years was 0.20 (Rotterdam study),[2] 0.51 (Cache County study)[3] and 0.40 (Baltimore longitudinal Study of Aging).[4]

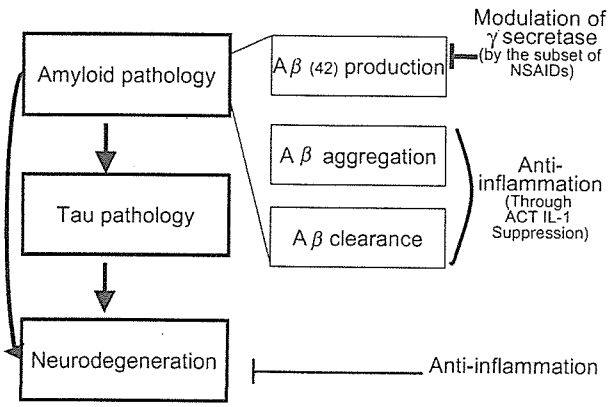

Figure 1 Multiple anti-AD activities. Ibuprofen can suppresses amyloid pathology at pre and post Aβ42 production.

SUPPRESSION OF AMYLOID PATHOLOGY BY NSAID

It has been believed that NSAID prevent neurodegeneration by suppression of inflammation. Using amyloid precursor protein transgenic (APP Tg) mice, an AD model animal, we reported that amyloid pathology is suppressed by NSAID.[9] This surprising observation has been confirmed by at least 11 studies.[11,12] So not only neurodegeneration, but also amyloid pathology which is more upstream and specific AD pathology has begun to be addressed in NSAID studies.

Inflammation includes many different phenomena and molecular pathways. It is not clear yet which kinds of inflammation are related to amyloid pathology. Some kinds of inflammation can accelerate amyloid pathology and/or neurodegeneration but others can clear amyloid pathology (such as activated microglia by Aβ vaccination).[11,12] Recently, we reported that α_1-antichymotrypsin seems to be one of the key molecules which was suppressed by ibuprofen and resulted in the decrease of amyloid pathology.[13]

An unexpected NSAID effect was subsequently revealed. A subset of NSAID can decrease Aβ42 production.[5–8] This does not depend on COX inhibition which is the main anti-inflammatory effect of NSAID.[5] The suppression of Aβ42 seems to be the direct effect of the NSAID[7] and is also independent of the other potential inflammatory target molecules of NSAID (PPARγ, NFκB, etc.).[6,8] Each NSAID has different effect on Aβ42 production. Ibuprofen, sulindac, flurbiprofen etc. can decrease Aβ42 production. Most NSAID do not reduce Aβ42. Some NSAID, especially many COX-2 specific NSAID, even increase Aβ42 production.[5] There is no reliable clinical or epidemiological analysis to examine whether anti-AD effects were observed only in Aβ42-lowering NSAID. Almost no COX-2 specific NSAID were involved in most of epidemiological studies.

γ secretase is the last step for the production of Aβ, the accumulation of which results in amyloid plaque in the AD brain. Therefore, γ secretase is the promising drug target and many inhibitors were developed. These γ secretase inhibitors, however, inhibit many other γ secretase substrate and might result in side-effects.

In contrast, a subset of NSAID is γ secretase modulators, not inhibitors.[5] A subset of NSAID decreases Aβ42 and increases Aβ38.[5] The former powerfully accelerate AD pathology and the latter is much less harmful. Aβ42 lowering NSAID neither inhibit total γ secretase activity nor disturb the production of other γ secretase substrates.[5] It is intriguing that the 30-year-old and cheap ibuprofen has this desirable characters for AD treatment.

One of the problems of Aβ42 lowering NSAID is the requirement of extraordinarily high concentration, around hundred times higher than those of COX inhibition *in vitro*.[5–8] Even with anti-inflammatory doses, the chronic use of NSAID resulted in a significant dropout of AD patients in a clinical study.[14] To reduce the risk of side-effects on vulnerable elderly people, we focused on *R*-enantiomer of profens. Most profens, including ibuprofen, are clinically used as racemate, the mixture of *S* and *R*-enantiomers. *R*-enantiomer of profen poorly inhibits COX. R-flurbiprofen has less side-effects such as gastric ulcers (common side effect of COX inhibitors). *R*-enantiomers can decrease Aβ42 production.[8,10] *R*-flurbiprofen showed suppression of AD progression in phase α clinical studies and moved to phase III study in 2005.

POTENTIAL PROBLEMS AND STUDIES REQUIRED

As mentioned above, NSAID have multiple favorable activities on AD (Figure 1). These anti-AD mechanisms don't conflict each other.

Aβ42 suppression by a subset of NSAID is observed only in extremely high concentration which is quite close to toxic levels and unrealistic to achieve in the human brain.[5–8] New drugs, which have more efficient Aβ42 suppression in low concentrations, are expected.

Many epidemiological studies suggested anti-AD effects by NSAID.[1–4] In an animal AD model, brain amyloid pathology is repeatedly suppressed. Nonetheless, clinical studies have shown little benefit.[14] The NSAID impact on AD might be limited or the selection of drugs and protocol might be poorly chosen. In the case of the *R*-flurbiprofen clinical trial, a subset (higher dose, milder subjects) seemed to be effected. The large prevention trial (Alzheimer's Disease Anti-inflammatory Prevention Trial) was halted in 2004. Well-designed prevention study is still expected, because in epidemiological studies, prevention is supported more than treatment.

To maximize NSAID effects on AD, we should know which NSAID, which stage(s) of AD, what dose and what duration of treatment are most effective. It is not practical to clinically examine all combinations of these conditions. Additional basic research which addresses the anti-AD mechanism of NSAID will make clinical study protocol improve. Different amyloid reductions observed in each APP Tg study might depend on the variable timing of interventions. There is no systematic study to examine the timing and dose of NSAID *in vivo* yet. Most of the AD model animal ibuprofen studies adopt 375ppm/diet (approximately 60 mg/kg body weight)[11,12] which was used in the first report.[9]

Additional clinical and basic studies are expected to establish treatment and/or prevention of AD by NSAID which has plenty of favorable anti-AD characters.

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アルツハイマー病は予防できるか

①予防薬の将来

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アルツハイマー病を根治させる，あるいは完全に予防する薬剤や方法は，現在のところ存在しません。しかし，今までの疫学・遺伝学・生化学的な検討から少しずつ明らかになってきた病態メカニズムから，その予防および治療に関しての戦略の方向性は示唆されています。治療薬と予防薬とは方向性を共有するため重なるところが多いのですが，発症前に服用するという観点から，ここではおもに抗酸化作用，抗炎症作用，アミロイドβタンパク産生抑制作用，凝集抑制作用について解説します。

アルツハイマー病と酸化ストレスおよび炎症反応

アルツハイマー病の神経病理学的特徴は神経原線維変化と老人斑ですが，この老人斑の主要構成成分はアミロイドβタンパクです。アミロイドβのほとんどはアミロイドβ1-40（40個のアミノ酸から構成）からなりますが，凝集能の強いアミロイドβ1-42（42個のアミノ酸から構成）も少量産生されます。

これらのアミロイドβは，1回膜貫通タンパクであるアミロイド前駆体タンパク（amyloid precursor protin；APP）から切断されて生成されます。APPの多くはαセクレターゼで切断されて分泌型APPとして細胞外に存在するのに対し，アミロイドβタンパクはβセクレターゼおよびγセクレターゼによって切断されることによって産出されます。βセクレターゼはすでにクローニングされ，BACE（Beta-site APP-Cleaving Enzyme）-1およびBACE-2が報告されています。γセクレターゼは，最近の研究によりプレセニリンとそれに結合する諸因子との複合体がその役割を担うものと考えられています。

アミロイドβタンパクは，培養された初代培養の神経細胞や神経芽細胞腫に対して毒性をもち，その際の細胞死の機序には，フリーラジカル産生にともなう酸化ストレスの発生，およびそれを介したミクログリアの活性化が関与していると考えられています。活性化したミクログリアは，腫瘍壊死因子（tumor necrotizing factor）やインターロイキンなどの炎症性サイトカインなどの放出を行ない，結果的に神経細胞死を促進しています。神経病理学的にも，老人斑の周囲には活性化したミクログリアや補体およびサイトカインなどが存在し，一般的には炎症性反応とみられる変化が認められます。

酸化ストレスが関与している他の証拠として，アルツハイマー病脳には，酸化タンパク（advanced glycated end productsなど），酸化脂質（4-hydroxynonenalなど），および

酸化核酸（8-hydroxyguanosine など）の蓄積・増加が報告されています。

抗酸化剤

このように，酸化ストレスがアルツハイマー病の病態と密接に関与していると考えられていますので，酸化を抑制する薬剤がアルツハイマー病の予防処理に有益であるようにみえます。たとえば，脂質に対する抗酸化作用を有するビタミンEは産生されたフリーラジカルを消去するのに役だち，モノアミンオキシダーゼ阻害剤であるセレギリンは，カテコールアミンの酸化的代謝を抑制してフリーラジカルの形成を抑制することによって，神経細胞を保護するものと想定されています。

実際，アルツハイマー病患者にセレギリン（10mg/日）と α-トコフェロール（＝ビタミンE，2000IU/日）とを2年間投与し，死亡，ADL の著明な低下，重症痴呆などにいたるまでの期間を記録したところ，プラセボ投与群（平均440日）に比べセレギリン投与群（平均655日）と α-トコフェロール投与群（平均670日）では有意に遅延していました[1]。これより抗酸化剤であるセレギリンと α-トコフェロールはアルツハイマー病の進行を抑制する可能性が示唆されています。

また，ビタミンEとCの栄養補助剤をいっしょに服用している人はアルツハイマー病になりにくいが，ビタミンE単独，ビタミンC単独，その効果はないことはないという報告もあります。相互作用という意味では，ビタミンEの酸化物はビタミンCによって還元され，酸化されたビタミンCによってひきおこされる核酸の酸化をビタミンEは保護するという効果があることから，抗酸化ビタミンを複合的に服用することにはメリットがある可能性があります。

しかし，アルツハイマー病発症前の軽度認知機能障害のグループにビタミンE（2000IU/日）を投与して，3年間追跡調査を行なうといった大規模な前向きコホート研究の結果では，アルツハイマー病発症率に変化はないという報告もされています[2]。よって，ビタミンE単独では発症後の進行を遅らせるのには役だつ可能性がありますが，発症前の予防としては有効性が低いのではないかという議論もあります。

その他の抗酸化剤としてビタミンAやカロチノイドなども，フリーラジカルを消去するという意味では有用ですが，アルツハイマー病発症抑制に関しては知見が少ないです。グルタチオンは細胞内の酸化還元反応を担い，過酸化水素の除去に重要な因子ですが，経口摂取しても血中に移行しにくいといわれています。このグルタチオンを増加させる物質としてN-アセチルシステインや α-リポ酸，およびセレニウムなどが知られていますが，これらもアルツハイマー病発症抑制に関しては知見が少ないです。

抗酸化剤の服用はアルツハイマー病発症予防に有効な戦略の一つですが，さまざまな組み合わせを複合的に用いる必要性が高く，どの抗酸化剤が必須でどの組み合わせがもっとも有効であるかにに関して，さらに研究が必要であると考えられます。

また，イチョウ葉エキスは銀杏の葉を乾燥させ，アルコールなどを用いて有効成分を抽出したものですが，ドイツでは治療薬として承認されています（EGb761）。イチョウ葉エキスには，ケルセチン，ケンフェロール，イムラムネチンというフラボノイドに糖鎖が付いたフラボノイド配糖体，ギンコライド類およびビロバライドなどのテルペノイドという化合物や，プロアントシアニジンなどが含まれています。イチョウ葉エキスの作用機序としては抗酸化作用および血液凝固抑制作用があり，活性酸素を消去し，血小板の凝集を防ぎ，炎症反応を抑える作用が報告されています。EGb761 は，脳血管型およびアルツハイ

マー型，両方の認知症の症状を改善することが臨床試験で報告されています。

イチョウ葉エキスはさまざまな化学物質を含んでいますが，本邦では効果を立証できなかったため，健康食品として販売されています。フラボノイド類には抗酸化作用，抗炎症作用など多様な作用がありますが，その薬理機序を検討して，より有効な予防薬が開発されることが期待されています。

抗炎症剤（NSAID）

非ステロイド系抗炎症剤（non-steroidal anti-inflammatory drug；NSAID）の長期服用者にアルツハイマー病の発症率が低いことが，数多く報告されています。前向きコホート研究からは2年以上服用していると，統計上有意に発症率が下がるという報告があります。

アミロイドβはミクログリアを活性化して炎症様変化を惹起することから，この過程を抑制することは神経細胞死を抑制するものと考えられています。また，炎症反応にともなうアンチキモトリプシンやインターロイキン-1の産生が，アミロイドβの蓄積に密接に関与していることから，炎症反応を抑制させてアミロイドβタンパクの蓄積を抑制することが実験的に知られています。

さらに驚くべきことに，NSAIDの抗炎症効果と独立して，いくつかのNSAID（すべてのNSAIDではない）が直接γセクレターゼの活性を制御して凝集能の強いほうのアミロイドβ1-42の産生を抑制することが報告されました。また，アミロイドβ産生の第1ステップであるAPPからの切り出し酵素であるBACE-1の発現を抑制する作用も報告されました。

このように，NSAIDはアルツハイマー病発症抑制に非常に効果的であることが知られていますが，アミロイドβ1-42の産生を抑制するようなレベルは，臨床的に用いるには高濃度であり，上部消化管潰瘍などさまざまな問題をひきおこし，治療および予防に用いることが困難です。本来NSAIDは，COX-1またはCOX-2という酵素を阻害することによって抗炎症効果を発揮していますが，上記のような副作用は，NSAIDがCOX-1を阻害するためにおこっている現象なのです。

NSAIDの一つであるibuprofenにはS体とR体の鏡像異性体が存在し，S-ibuprofenもR-ibuprofenどちらもアミロイドβ1-42の産生を抑制することができますが，R-ibuprofenにはCOX-1酵素阻害活性が存在せず，このような副作用を心配せずに用いることができます[3]。R-ibuprofen自体は，治療薬として実用化にいまだいたっていませんが，このようなコンセプトのもとに有効な予防薬が開発される可能性が今後，期待されています。

アミロイドβ重合阻害剤

一般的食品であるカレーの主要成分はクルクミンですが，クルクミンは強力な抗炎症作用と抗酸化作用を有しています。クルクミンは酸化ストレスとなるフリーラジカルを強力に消去すると同時に，COX-1，COX-2，NFκBなどを阻害し，炎症を抑制することができます。

クルクミン自体には，いくつかのNSAIDのように，直接γセクレターゼの活性を制御してアミロイドβ1-42の産生を抑制するといった作用は認められませんが，アミロイドβの重合阻害効果があり，アミロイドβが毒性の強いオリゴマーを形成するのを阻害することが報告されています。また，アミロイド病理を再現するアルツハイマーモデルマウスに投与すると，アミロイドの蓄積を明らかに抑制していました[4]。マウスに投与された有効量のクルクミンをヒトに換算すると，食事で摂取できるレベルではないので，もし

予防薬として使用するとなると，サプリメントとして意図的に摂取する必要があります。

またクルクミンと同様に，抗酸化作用とアミロイドβ重合阻害作用を有する物質として，メラトニン，ポリフェノールなどが報告されています。

このように，食品に関連する物質がアミロイドβ重合阻害作用を有する現象は興味深く，まだ実験的レベルではありますが，予防薬への応用が期待されています。

コレステロール合成阻害剤

コレステロールは身体を構成する重要な物質ですが，高コレステロール血症は冠動脈疾患や脳血管性疾患のリスクとして一般的に知られています。コレステロール合成阻害剤であるスタチンは，このような高コレステロール患者に処方されている有用性の確立された薬剤です。

高コレステロール血症とアルツハイマー病発症に相関があること，およびスタチンの服用者にアルツハイマー病発症が少ないことが，疫学的調査でいくつか報告されています[5]。スタチンによるコレステロール代謝への作用では，アミロイドβの凝集・沈着およびクリアランスに影響を与えること，脳内の微小循環改善作用，神経細胞保護作用，および抗炎症作用などがあることが示唆されており，予防薬として期待されています。

ただし，前向きコホート試験で有意な差がでておらず，現在までに正確な結論にはいたっていません。そこで，効果の確認のためには，さらに大規模な前向きコホート試験による解析が必要となっています。

ホモシステイン代謝

ホモシステイン代謝経路は，心血管疾患と神経変性疾患にとって重要であることが知られています。血漿中のホモシステイン量とアルツハイマー病のリスクは相関しており[6]，ホモシステイン代謝酵素の遺伝子多型もアルツハイマー病の発症リスクに影響を与えています。さらに，前向きコホート研究によっても，血漿中ホモシステイン量が高いとアルツハイマー病を発症しやすいことが報告されています。そのメカニズムとしては，高ホモシステイン血症による脳の微小血管障害，脳血管の内皮機能障害，酸化ストレスの増大などが考えられています。

そしてさらに，高齢者における血漿ホモシ

ステイン量と血漿アミロイドβ1-40の量が正の相関を示すことも報告されています。そのメカニズムとしては，先述のBACE-1の発現がホモシステイン代謝によって影響を受けることが実験的に示されています。

BACE-1の遺伝子発現を制御するプロモーター領域は，ホモシステイン代謝産物であるS-アデノシルメチオニンによってメチル基を供与され，その発現が抑制されます。実験的には，ビタミンB_{12}および葉酸などのホモシステイン代謝を促進させる物質の存在下では，ホモシステインが減少し，BACE-1の発現も抑制されます。

ホモシステイン代謝を促進させる物質としてはビタミンB_6も存在しますが，このようなビタミン類を摂取した人とそうでない人を比較して，前者では血漿中のアミロイドβが低いという報告もあるものの，実際にアルツハイマー病への発症率を低下させるかどうかに関しては明らかではありません。しかし，血漿ホモシステイン量を低く保つことが，理論的にはいろいろな面からアルツハイマー病発症のリスクを軽減させることから，このようなビタミン群の補給あるいはホモシステインの代謝調整薬などが，将来的にアルツハイマー病の予防に有効である可能性は高いと推測されています。

*

アルツハイマー病の発症予防薬に関連して，現在までに知られている病態機序と可能性の高い化合物に関して概説しました。現在のところその基本的な戦略は，抗酸化作用，抗炎症作用，アミロイドβタンパク産生抑制作用，凝集抑制作用などに絞られるものと考えられています。遺伝的検討から発症リスクの高いグループの選別も可能となってきており，近い将来には，高リスクグループに対して定期的な認知機能の検診と，予防薬服用を積極的に推奨することになるものと考えられます。

概説した予防薬となる可能性のある化合物は多岐にわたりますが，どの組み合わせで服用するのが適切であるのかに関しても，また重要な課題になるものと考えられます。認知症患者の総数は2020年ごろには約300万人になると予想されており，それを防ぐためにも，今後，予防薬の知見がさらに深まることが期待されています。

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The *DYRK1A* gene, encoded in chromosome 21 Down syndrome critical region, bridges between β-amyloid production and tau phosphorylation in Alzheimer disease

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We scanned throughout chromosome 21 to assess genetic associations with late-onset Alzheimer disease (AD) using 374 Japanese patients and 375 population-based controls, because trisomy 21 is known to be associated with early deposition of β-amyloid (Aβ) in the brain. Among 417 markers spanning 33 Mb, 22 markers showed associations with either the allele or the genotype frequency ($P < 0.05$). Logistic regression analysis with age, sex and apolipoprotein E (*APOE*)-ε4 dose supported genetic risk of 17 markers, of which eight markers were linked to the *SAMSN1*, *PRSS7*, *NCAM2*, *RUNX1*, *DYRK1A* and *KCNJ6* genes. In logistic regression, the *DYRK1A* (dual-specificity tyrosine-regulated kinase 1A) gene, located in the Down syndrome critical region, showed the highest significance [OR = 2.99 (95% CI: 1.72–5.19), $P = 0.001$], whereas the *RUNX1* gene showed a high odds ratio [OR = 23.3 (95% CI: 2.76–196.5), $P = 0.038$]. *DYRK1A* mRNA level in the hippocampus was significantly elevated in patients with AD when compared with pathological controls ($P < 0.01$). *DYRK1A* mRNA level was upregulated along with an increase in the Aβ-level in the brain of transgenic mice, overproducing Aβ at 9 months of age. In neuroblastoma cells, Aβ induced an increase in the *DYRK1A* transcript, which also led to tau phosphorylation at Thr^{212} under the overexpression of tau. Therefore, the upregulation of *DYRK1A* transcription results from Aβ loading, further leading to tau phosphorylation. Our result indicates that DYRK1A could be a key molecule bridging between β-amyloid production and tau phosphorylation in AD.

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INTRODUCTION

Alzheimer disease (AD) is the major cause of dementia in the elderly and is pathologically characterized by senile plaques with β-amyloid deposition (Aβ) and neurofibrillary tangles harboring hyperphosphorylated tau in the brain. It is well established that familial autosomal-dominant early onset AD is mostly caused by mutations of the amyloid protein precursor (*APP*) and presenilin 1 and 2 (*PS1* and *PS2*) genes (1). In contrast, Down syndrome (DS) is also highlighted as a model condition predisposing to AD, because patients with DS develop early deposition of Aβ in the brain (2). Therefore, it has been speculated that genetic factors related to AD could exist on chromosome 21, independent of the ε4 allele of the apolipoprotein E gene (*APOE*-ε4), a known strong risk for late-onset AD (3,4). Using the candidate approach, it was reported that duplication of the *APP* gene was transmitted in patients with familial autosomal-dominant early onset AD with cerebral angiopathy (5), whereas an association with the *APP* gene, to the best of our knowledge, was not supported in case–control studies (6–8). The *BACE2* gene, encoding β-secretase of APP, was not associated with AD; however, recent studies showed weak associations (9–11). In contrast, with the positional approach, genome scans of late-onset AD showed positive linkage on chromosome 21 (12,13). Although this linkage remains controversial (14–16), a locus strongly influencing age at onset was also found on chromosome 21 (17). To search for genetic factors for late-onset AD on chromosome 21, we scanned throughout this chromosome using patients with Japanese late-onset AD and population-based controls, by a stepwise single nucleotide polymorphism (SNP) scan. We report that the *DYRK1A* gene is a genetic factor related to the progression of AD.

RESULTS

Chromosome 21 scan

An exploratory scan of chromosome 21 was performed in 188 AD and 375 controls, using 417 SNPs at an average interval of <100 kb, including at least one SNP in each coding region. Selected SNP markers were distributed between base positions 14 440 543 and 46 915 057 based on NCBI Build 35, whereas no SNP closer to the centromere was included because of the duplicated region in the chromosome 21 sequence (18). Using a threshold of $P < 0.05$ for allele frequency, we detected 14 SNPs, which is less than the predicted 21 markers. Therefore, to reduce type II error, we also tested genotype frequency in both dominant and recessive models (Fig. 1). Finally, the exploratory scan detected 42 SNPs in total (10.0%), among which 14 SNPs were significant in both allele and genotype frequencies, of which one positive region was identified in the Down syndrome critical region (DSCR) (19–21). The confirmatory scan targeting the selected 42 SNPs indicated that 22 SNPs were still significant for either allele or genotype frequency (Table 1). Among those, 17 SNPs were also significant by logistic regression for the risk genotype with age, sex and APOE-ε4 dose. Genes linked to these SNPs were the

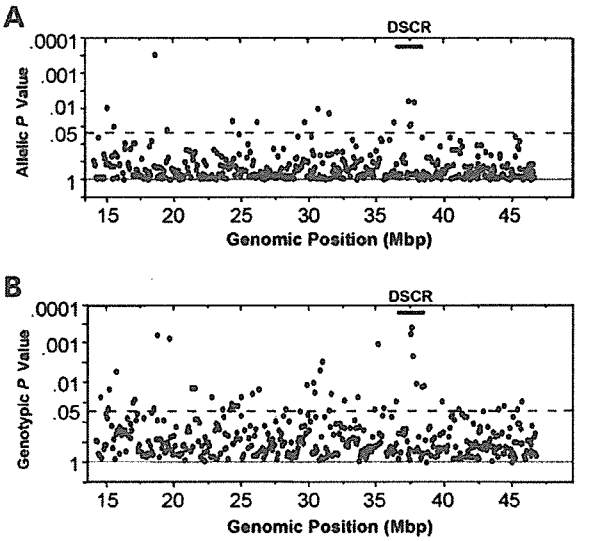

Figure 1. Exploratory scan using 417 markers. (**A**) *P*-values for allele frequency in chi-squared test. (**B**) *P*-values for genotype frequency in better fitting models. Genomic position is based on NCBI Build 35.

SAMSN1, *PRSS7*, *NCAM2*, *RUNX1*, *DYRK1A* and *KCNJ6* genes and those linked to unknown open reading frames were C21ORF 63, 55 and 5. In logistic regression, the *DYRK1A* gene, located in the middle of the DSCR, showed the highest significance [OR = 2.99 (95% CI: 1.72–5.19), $P = 0.001$], whereas the *RUNX1* gene showed a very high odds ratio [OR = 23.3 (95% CI: 2.76–196.5), $P = 0.038$].

Haplotype analysis of *DYRK1A*

SNPs located in the *DYRK1A* gene region were genotyped to determine the haplotype associated with AD. Linkage disequilibrium was identified in the control group from 30 kb upstream of exon 1 to intron 9, but not in exon 13 genotyped by rs1803439 which was not in Hardy–Weinberg equilibrium, and the AD group showed similar results (Fig. 2). Haplotype analysis indicated that three haplotypes had significantly different frequencies between AD and controls, whereas the permutation test supported significant differences in two haplotypes. Considering the haplotype frequencies, rs8126696 alleles could represent the risk haplotype (Table 2). We also sequenced all coding regions of the *DYRK1A* gene in six patients and three controls homozygous for the risk allele, but no sequence alteration was found.

DYRK1A mRNA in hippocampus of AD

DYRK1A mRNA in the hippocampus was measured by quantitative polymerase chain reaction (PCR) to examine the relation with the occurrence of AD and with the genotype of rs8126696. *DYRK1A* mRNA level in the patients was significantly different ($P < 0.01$), being ~7-fold greater than that in pathological controls (Fig. 3A). In contrast, patients homozygous for the risk rs8126696-c allele showed a tendency for a decrease in *DYRK1A* mRNA level compared with the others,

Table 1. Genes linked to markers associated with AD on chromosome 21

Marker	Association[a] (*P*)		Logistic regression[b]		Gene
	Allele	Genotype[c]	Odds (95% CI)	*P*-value	
rs723856	0.019	0.012 (aa)	1.53 (1.08–2.18)	0.0181	*SAMSN1*
rs2268437	0.008	0.008 (aa)	2.09 (1.24–3.55)	0.0059	*PRSS7*
rs2212624[d]	0.058	0.003 (gg)	1.66 (1.17–2.35)	0.0046	*NCAM2*
rs2833844	0.033	0.030 (cc)	1.74 (1.11–2.73)	0.0166	C21 orf 63
rs28360609[d]	0.128	0.017 (aa)	3.43 (1.31–8.95)	0.0119	C21 orf 55
rs4816501	0.224	0.004 (tt)	23.3 (2.76–196.5)	0.0038	*RUNX1*
rs1023367	0.054	0.036 (cc, ct)	1.40 (0.96–2.05)	0.0839	C21 orf 5
rs2835740	0.035	0.001 (cc)	2.99 (1.72–5.19)	0.0001	*DYRK1A*
rs2835908	0.024	0.056 (cc)	1.55 (0.99–2.43)	0.0546	*KCNJ6*

[a]One-sided *P*-value in chi-squared test.
[b]Logistic regression of risk genotype with age, sex and APOE-ε4 dose under no interaction.
[c]Risk genotypes in a better fitting model are shown in parentheses.
[d]AD group showed deviation from the Hardy–Weinberg equilibrium.

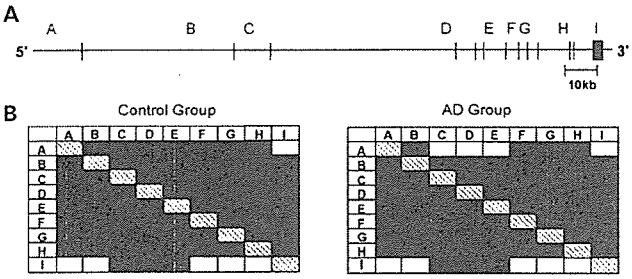

Figure 2. Linkage disequilibrium in *DYRK1A* gene region. (**A**) Genomic structure of the *DYRK1A* gene is shown. Horizontal bar indicates exons, and letters indicate SNPs, such as rs28360609 (A), rs2251085 (B), rs2835740 (C), rs10470178 (D), rs11701810 (E), rs1024294 (F), rs2835773 (G), rs2835774 (H) and rs1803439 (I). (**B**) r^2 (upper right) and $|D'|$ values (lower left) were judged significant at less than 0.5 and 0.9, respectively, and significant values are shown by dark boxes.

Table 2. Haplotype case–control study for *DYRK1A* gene

Haplotype[a]	Frequency			*P*-value	
	Overall	AD	Control	Chi-squared	Permutation
2-1-2-1-1-1-2-1	0.500	0.467	0.532	0.0147	0.013
1-2-1-2-2-2-1-2	0.312	0.337	0.287	0.0395	0.051
1-2-2-1-1-2-1-2	0.065	0.064	0.067	0.8369	0.844
1-1-2-1-1-1-2-1	0.065	0.080	0.050	0.0216	0.017
2-1-2-1-1-1-2-1	0.031	0.025	0.038	0.1582	0.171
1-2-1-2-2-2-1-2	0.016	0.018	0.015	0.6984	0.745
1-2-2-2-2-2-1-2	0.011	0.010	0.013	0.6268	0.632

[a]Haplotypes were constructed with markers composed of rs8126696 (allele 1 = c, allele 2 = t)–rs2251085 (c/g)–rs2835740 (c/t)–rs10470178 (a/g)–rs11701810 (a/c)–rs1024294 (c/t)–rs2835773 (a/g)–rs2835774 (a/t). Chi-squared for the overall haplotypes (df = 6) was significant by the EM algorithm ($P = 0.040$) as well as by the permutation method ($P = 0.038$).

but this was not significant (Fig. 3B). Thus, the increased expression of *DYRK1A* mRNA is possibly a consequence of AD.

DYRK1A mRNA and Aβ in transgenic mouse brain

We examined whether Aβ loading is related to *DYRK1A* mRNA level in the brain in PS1^{I213T}KI and Tg-PS1/APP mice. Aβ1–40 level in PS1^{I213T}KI mice was low, but Aβ1–40 was almost undetectable, whereas both Aβ1–40 and Aβ1–42 were elevated in Tg-PS1/APP mice (Fig. 4A and B), suggesting that Tg-PS1/APP mice have an Aβ burden in their brain. Quantitative PCR showed that the *DYRK1A* mRNA level was significantly increased in Tg-PS1/APP mice when compared with that in PS1^{I213T}KI mice ($P < 0.05$) by 1.2-fold (Fig. 4C). Thus, the expression of *DYRK1A* mRNA increased along with Aβ loading in the mouse brain.

DYRK1A mRNA, Aβ and tau phosphorylation in cell models

We examined whether Aβ, a major component of senile plaques in the AD brain, induces expression of *DYRK1A* mRNA in cultured neuroblastoma cells. SH-SY5Y cells were incubated with Aβ, and then total RNA was extracted and quantified (Fig. 5). *DYRK1A* mRNA level was significantly increased by 1.6-fold ($P < 0.05$) with 0.5 μM Aβ1–42 and by 1.3-fold ($P < 0.01$) with 25 μM Aβ25–35, compared with the level in non-treated cells, but was not changed with control 25 μM Aβ35-25. Thus, Aβ loading resulted in an increase in the *DYRK1A* transcription. In an *in vitro* experiment, DYRK1A protein not only phosphorylates itself, but also has a large repertoire of phosphorylation (22). Therefore, we examined whether *DYRK1A* overexpression induces phosphorylation of tau at the cellular level. An immunoblot of HEK293T cells transiently transfected with the *MAPT* expression vector showed a detectable amount of tau along with those phosphorylated at Thr^{212} (Fig. 6A). Tau phosphorylated at Thr^{212} was increased by co-transfection of the *DYRK1A* expression vector, compared with that of mock vector, whereas tau level was similar (Fig. 6A). Densitometric quantification supported the induction of phosphorylation by 1.5-fold ($P < 0.01$) (Fig. 6B). Thus, the increase in the *DYRK1A* transcription under overexpression of tau induced tau phosphorylation at Thr^{212}.

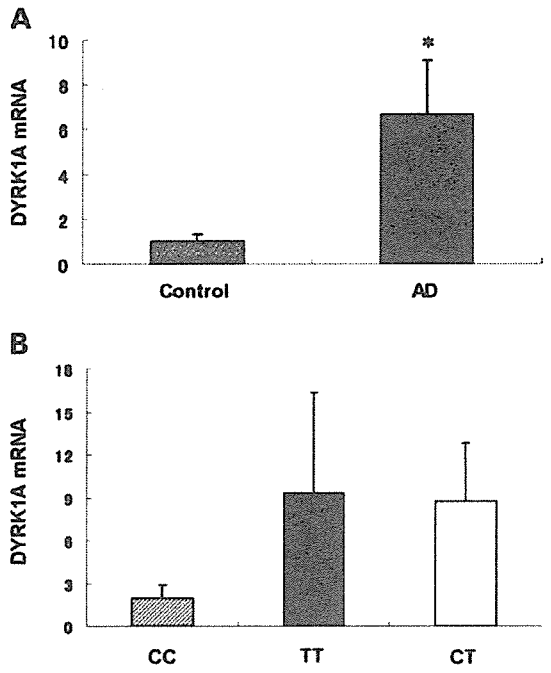

Figure 3. Expression of *DYRK1A* mRNA in human hippocampus. (**A**) Quantitative real-time PCR of *DYRK1A* mRNA in AD ($n = 22$) and controls ($n = 12$). (**B**) *DYRK1A* mRNA level in AD brain divided by rs28360609 genotypes, where CC is the risk genotype. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean ± SEM. $^{*}P < 0.01$ by Mann–Whitney's *U*-test.

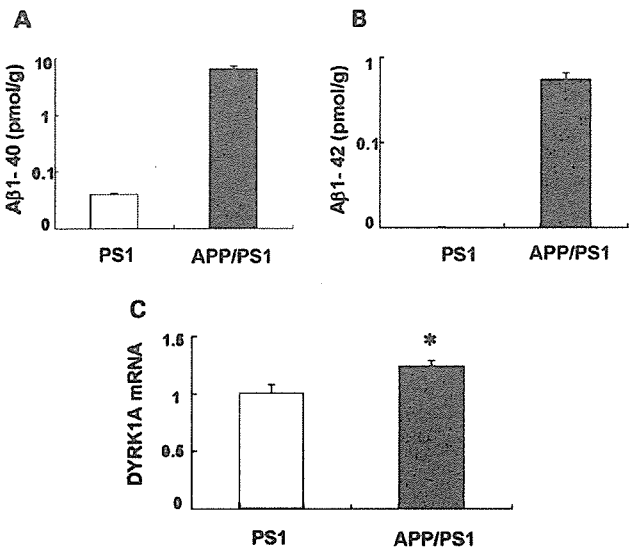

Figure 4. Aβ-level and expression of *DYRK1A* mRNA in transgenic mouse brain. Heterozygous PS1^{I213T}KI (PS1, $n = 6$) and Tg-APP/PS1 (APP/PS1, $n = 6$) mice were sacrificed at 9 months of age. Aβ-level was measured by ELISA. *DYRK1A* mRNA level was measured by quantitative real-time PCR. (**A**) Aβ1–40 level, (**B**) Aβ1–42 level and (**C**) amount of *DYRK1A* mRNA. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean ± SEM. $^{*}P < 0.01$ by Student's *t*-test.

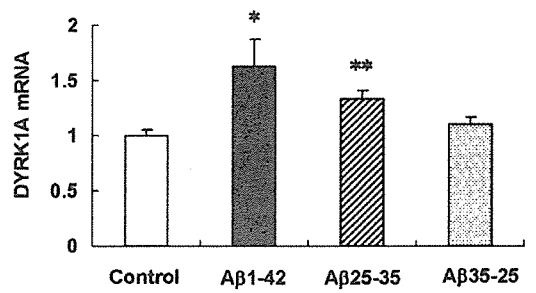

Figure 5. Aβ-induced expression of *DYRK1A* mRNA in SH-SY5Y cells. SH-SY5Y cells were incubated with Aβ1–42, Aβ25–35 and Aβ35–25. *DYRK1A* mRNA level was measured by quantitative real-time PCR. Values were normalized to those in untreated cells. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean ± SEM of four independent measurements. $^{**}P < 0.01$ and $^{*}P < 0.05$ by Student's *t*-test compared with control.

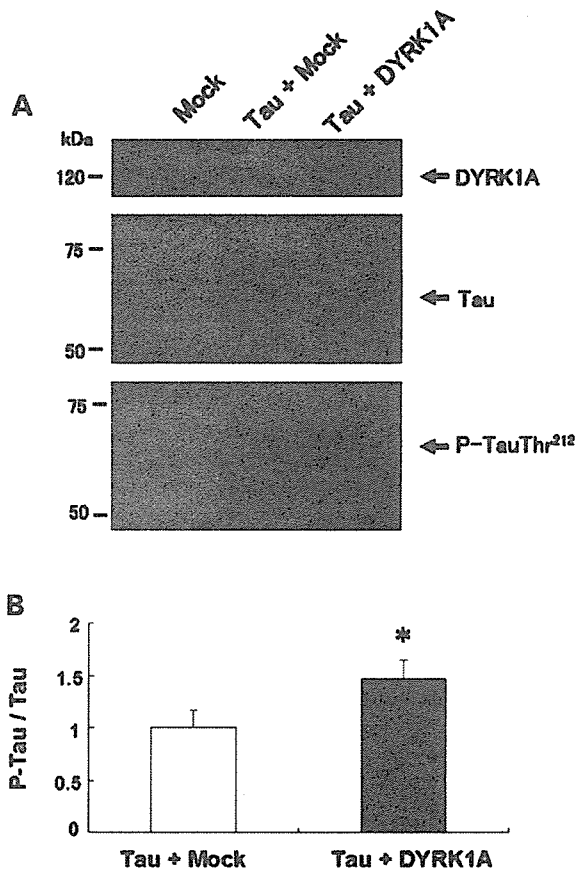

Figure 6. Tau hyperphosphorylation in DYRK1A-overexpressing cells. (**A**) HEK293T cells were transfected with either the *MAPT* expression vector (Tau) or both the *MAPT* and *DYRK1A* expression vectors (Tau + DYRK1A). After 24 h incubation, lysates were immunoprecipitated with anti-FLAG M2 agarose and then subjected to immunoblotting with anti-DYRK1A (DYRK1A), anti-tau (Tau) or anti-phosphotau (P-TauThr^{212}). (**B**) P-TauThr^{212}/Tau ratio was measured as integrated optical density values. Data are shown as mean ± SEM of four independent measures. $^{*}P < 0.01$ by Student's *t*-test.

Discussion

Genome scanning using case–control studies, based on linkage disequilibrium, is a strategy to identify genetic factors of polygenetic diseases. In general, many susceptibility genes have been reported, but it remains difficult to replicate the results in different studies. This could possibly be caused by selection bias in patients as well as in controls, because hospital-based control subjects often suffer from another disease, leading to an additional background of that disease. Therefore, we used population-based controls to match the phenotypic background. From the exploratory and confirmatory scans, we identified 22 candidate SNPs associated with late-onset AD on chromosome 21. Although we showed their risk effects in logistic regression with age, sex and *APOE*-ε4 dose, a known major risk for AD (3), these candidates need to be confirmed, because *P*-values were inconclusive when considering the comparison of multiple loci.

We found associations of AD with markers linked to six known genes, but not with reported candidates, the *APP* and *BACE2* genes. The *SAMSN1* gene encodes a member of putative adaptors and scaffold proteins containing SH3 and sterile alpha motif domains, expressed mainly in immune tissues and hematopoietic cells and also at lower levels in the heart, brain, placenta and lung (23). The *DYRK1A* gene, located in the DSCR, is a candidate gene responsible for learning and memory impairment in patients with DS (24,25). The *PRSS7* gene encodes enteropeptidase (EC 3.4.21.9), an intestinal enzyme initiating activation of pancreatic proteolytic proenzymes such as trypsin, chymotrypsin and carboxypeptidase A, which are highly expressed in the intestines and at a low level in the brain of rat (26), but is downregulated in amniotic fluid cells in patients with DS (27). The neural cell adhesion molecule 2 (*NCAM2*) gene is expressed in fetal and adult brains (28), sharing many features with immunoglobulins and mediating adhesion among neurons and between neurons and muscle (29) and having a potential regulatory role in the formation of selective axonal projections of olfactory sensory neurons in mice (30). The *RUNX1* gene, also called *AML1*, encodes runt-related transcription factor 1, which is required for active repression in CD4-negative/CD8-negative thymocytes, and a defective *RUNX1* gene causes a familial platelet disorder with predisposition to acute myelogenous leukemia (31). The mouse *RUNX1* homolog is expressed in selected populations of post-mitotic neurons of the embryonic central and peripheral nervous systems (32). The *KCNJ6* gene, located in the DSCR, encodes a G protein-coupled inwardly rectifying potassium channel and is expressed in the brain and pancreatic beta cells (33,34). A *kcnj6* mutation was found in the weaver mouse characterized by ataxia with reduced size of the cerebellum because of depletion of granule cell neurons (35).

DYRK1A is a mammalian ortholog of the *Drosophila minibrain* gene, which is essential for normal post-embryonic neurogenesis (36). In rodents, *DYRK1A* mRNA is expressed ubiquitously in various tissues during development and is also strongly expressed in the adult brain and heart (20, 37–39). In humans, *DYRK1A* mRNA is expressed especially in the brain, and immunoreactive DYRK1A is found in the cerebral cortex, hippocampus and cerebellum and is overexpressed in the DS brain in a dose-dependent manner (40,41). Transgenic mice overexpressing full-length *DYRK1A* mRNA exhibit neurodevelopmental delay, motor abnormalities and cognitive deficit, suggesting a causative role of the *DYRK1A* gene in mental retardation and motor anomalies of DS (24,25). It was noted that all adults with DS over the age of 40 years develop sufficient neuropathology for a diagnosis of AD (42). The identification of the *DYRK1A* gene as a genetic factor strongly supports that the *DYRK1A* gene is involved in the development of AD.

We demonstrated an increase in the *DYRK1A* mRNA level in post-mortem brains, coinciding with the recent report of DYRK1A immunoreactivity in the neocortex and hippocampus in AD (41). The risk genotype of the *DYRK1A* gene showed a tendency for a decrease in the *DYRK1A* mRNA level, but our observation needs to be carefully considered because the result might be caused by the reduction of neuronal cells in the AD brain. However, no studies have yet examined the relationship between the *DYRK1A* gene and Aβ. Genetic and pathological evidence strongly supports the amyloid cascade hypothesis that Aβ42, a proteolytic derivative of the APP protein, has an early and pivotal role in all cases of AD. It is thought that Aβ42 forms aggregates that initiate the pathogenic cascade, leading ultimately to neural loss and dementia (43). We demonstrated that Aβ, especially Aβ42, results in an increase of *DYRK1A* transcription in human neuroblastoma cells and is also observed in transgenic mouse models. Therefore, the increase in *DYRK1A* transcription is a common feature of AD and DS and could relate to the cognitive impairment in patients with AD.

The DYRK1A enzyme has dual substrate specificity: autophosphorylation for self-activation takes place on the Tyr^{321} residue in the active loop of the catalytic domain (44) and target protein phosphorylation occurs on serine/threonine residues in several proteins, including STAT3, FHKR, Gli-1, eIF2Bε, tau, dynamin, glycogen synthase, 14-3-3, CREB, cyclin L2, Arip4, Hip-1 and PAHX-AP1, indicating that DYRK1A may participate in many biological pathways (22). We showed that overexpression of the *DYRK1A* gene phosphorylates tau at Thr^{212} in HEK293T cells overproducing tau, suggesting that tau phosphorylation at Thr^{212} by DYRK1A could be a downstream consequence of Aβ overproduction. It was shown in an *in vitro* experiment that DYRK1A phosphorylates tau at Thr^{212}, which primes tau for phosphorylation by GSK3-β at Ser^{208}, leading to the formation of paired helical filaments composed of highly phosphorylated tau, a component of neurofibrillary tangles (41). However, transgenic mice overexpressing DYRK1A did not show this phosphorylation, and this phosphorylation is highly susceptible to dephosphorylation by protein phosphatase-1, which is expressed in the frontal lobes of the brain, indicating that tau phosphorylation at Thr^{212} could be prohibited *in vivo* (45,46). On the contrary, it was noted that peptides of tau phosphorylated at Thr^{212} completely block Aβ binding, and DYRK1A mediated phosphorylation of Huntingtin-interacting protein 1 (Hip-1) in response to βFGF, resulting in the blockade of Hip-1-mediated neuronal cell death as well as the enhancement of neurite outgrowth (47,48). Therefore, tau phosphorylation at Thr^{212} could be a protective response against neuronal cell death. Although overexpression of DYRK1A could be a common phenomenon

between AD and DS, neuropathological studies might elucidate how the pathway from overexpression of DYRK1A to phosphorylation of tau is related to the severity of Alzheimer pathology.

Our study provides evidence that the *DYRK1A* gene is a genetic factor for AD, whose expression is increased by Aβ loading in neuroblastoma cells and transgenic mice, resulting in hyperphosphorylation of tau at Thr^{212} under overexpression of tau. The *DYRK1A* gene could be responsible for learning and memory deterioration in DS (24,25), and a DYRK1A inhibitor has been proposed as a novel drug to address learning and memory deficit in DS (49). Our findings suggest that *DYRK1A* upregulation is a key phenomenon as a consequence of Aβ loading in AD, connecting the condition to DS, and we propose a possible relation between the *DYRK1A* gene and memory impairment in AD.

MATERIALS AND METHODS

Sample-set characteristics

Patients with late-onset AD were diagnosed as having definite or probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (50). Non-demented control subjects, tested by a questionnaire including the date, orientation and past history, were obtained from population-based elderly subjects. Written informed consent to participate in this study was obtained, and then peripheral blood was drawn and subjected to DNA extraction. The number of patients for the scan was 374 (70.6% female), composed of 73 with definite and 301 with probable AD; mean ± SD age at onset was 73.0 ± 8.0, range 60–94 years and age at blood drawing was 78.2 ± 8.3, range 60–98 years. Controls were composed of 375 individuals (54.7% female); age at assessment was 75.5 + 4.85, range 66–92 years. Brain hippocampal tissue was also obtained from the post-mortem brains of 22 patients with AD (age: 82.8 ± 8.5 years, 63.6% female) and 12 pathological controls (age: 89.0 ± 7.0 years, age at onset: 72.9 ± 7.2 years and 58.0% female). DNA was extracted from peripheral blood nuclear cells by phenol–chloroform method or using a QIAamp DNA Blood Kit (Qiagen, Tokyo, Japan). The procedure to obtain the specimens was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine, Ehime University and the Ethical Committee of Fukushimura Hospital.

Genotyping and sequencing

An exploratory scan was performed in 188 patients (67.0% female) (age at onset: 75.0 ± 7.2 and range 60–92 years) and 375 controls matched for age. A confirmatory scan was performed in 374 patients including 175 who underwent exploratory scan, and the data were compared with the genotype data of controls in the exploratory scan. The whole genomic DNA was amplified by degenerate oligonucleotide-primed-PCR and used in the confirmatory scan, because of the small amount of DNA (51). The accuracy of genotyping in the confirmatory scan was monitored by comparison with data obtained in the exploratory scan. The selected markers were 417 SNPs distributed in chromosome 21, spanning a region of 33 Mb, which was sequenced and reported by the Chromosome 21 Mapping and Sequencing Consortium (18). Mean interval of the markers in NCBI Build 35 was 78.1 kb, and their range was 7.7–240.0 kb, and 15 intervals were over 100 kb where no coding region was predicted on the basis of the SNP information in using SNPbrowser Software Version 3.5 on NCBI Build 35, available from http://www.appliedbiosystems.com/. Genotyping was performed by a quantitative genotyping method using the TaqMan SNP Genotyping System (Applied Biosystems, Foster City, CA, USA). DNA obtained from six patients and three controls homozygous for the risk genotype of the *DYRK1A* gene was subjected to direct sequencing of its exons, using the primers listed in Table 3.

Table 3. Primer sequences for *DYRK1A* gene

	Primer sequences (5′–3′)		
Exon	Forward	Reverse	Product size (bp)
1	gtttttcttcacacagtg	cccccactaactgct	207
1	gtttttcttcacacagtg	cccccactaactgct	207
2	atgtcaaatgatacaaaca	ttttcccaatccataatc	394
3	gcaggttacagaagaggga	agggtaaatagtcacact	258
4	ctcaaatgtcaactgtag	aacaacaagattcactaag	359
5	ttgaatagaaatagatggc	tgtccaacagaaataaaca	445
6	taactgaactctgcgtttg	atacctacactgtcctacc	471
7	gaagttaatcaatggaac	tattcaaactgacctcac	413
8	ctgtatgctggatgtct	aacacactgatttcaagt	372
9	attatgtgagtgtttacg	gtaactgctccccac	481
10	ttaaccagacttcattgt	gtcattctaaaggcacct	433
11	tgaatgtatttgggatttgtgt	actgtgactgggatgtgg	1063
11	tatttgggattttgtg		(For sequencing)
11	ctgctcctcttgg		(For sequencing)
11	caagattctatggagg		(For sequencing)
11	cgtctactccaatcc		(For sequencing)

Quantitative real-time PCR

Total RNA was isolated from frozen brains using the acid guanidine–phenol–chloroform RNA extraction method provided as ISOGEN (Nippon Gene, Toyama, Japan), and purified using an RNAeasy Mini kit (Qiagen). RNA samples with an A_{260}/A_{280} absorption ratio over 1.9 were subjected to cDNA synthesis using a High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was carried out in an ABI PRISM 7900HT (Applied Biosystems), and primers/probe sets for the *DYRK1A* and *GAPDH* genes of human and mouse were purchased from TaqMan Gene Expression Assay Products (Applied Biosystems). All quantitative PCR reactions were duplicated, and the ratio of the amount of *DYRK1A* cDNA to that of *GAPDH* internal control cDNA at a threshold in the mid-log phase of amplification was used to compare the amount of *DYRK1A* mRNA.

Transgenic mice

The $PS1^{I213T}$KI mouse, with a 'knocked-in' human *PS1* I213T mutation in the mouse presenilin 1 gene (52,53), was bred with Tg2576 mice expressing the human *APP* gene harboring

the K670N/M671L Swedish mutation (Taconic) (54). PS1^{I213T}KI and double transgenic (Tg-APP/PS1) mice were maintained on the B6 background. Six heterozygous Tg-APP/PS1 and six PS1^{I213T}KI mice were sacrificed at age 9 months under anesthesia, and their brains were dissected and stored at −80°C until use. All animal procedures were reviewed by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. Every effort was made to minimize the number of animals used and their suffering.

Cell culture

Human neuroblastoma (SH-SY5Y) cells were grown in F12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), and human embryonic kidney (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS. Amyloid peptides (Sigma-Aldrich, St Louis, MO, USA) were dissolved in phosphate-buffered saline, followed by incubation at 37°C for 72 h. SH-SY5Y cells were incubated for 20 h with Aβ at 0.5 μM for Aβ1–42 and at 25 μM for Aβ25–35 and Aβ35–25. Total RNA was isolated from harvested cells using an RNAeasy Mini kit, and then synthesized cDNA was subjected to quantitative PCR. The human long isoform of *MAPT* cDNA, obtained from Dr Goedert (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK), was cloned in pcDNA3.1 (Invitrogen), and the FLAG epitope-tagged *DYRK1A* expression vector was cloned in pEGFPC2 (55,56). These vectors were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen), followed by their expression for 24 h, and the cells were harvested and subjected to biochemical experiments.

Biochemical experiments

In transgenic mice, the hemisphere of each brain was homogenized in Tris-buffered saline (TBS) composed of 137 mM NaCl and 20 mM Tris, pH 7.6, containing 1% Triton X-100 with Complete™ protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA), followed by ultracentrifugation, and the supernatant was subjected to measurement of Aβ1–40 and Aβ1–42 levels using a sandwich ELISA kit (Biosource International, Camarillo, CA, USA). In cell experiments, cells were lysed in lysis buffer composed of 150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). After centrifugation at 10 000*g* for 15 min at 4°C, protein extracts were obtained as the supernatant and quantified using BCA Protein Assay (Pierce). For immunoprecipitation, 300 μg of protein lysate was incubated with 20 μl anti-FLAG M2 agarose (Sigma-Aldrich) with gentle rotation at 4°C overnight, and after centrifugation, the precipitate was dissolved in SDS sample buffer, electrophoresed in 8% SDS–PAGE and blotted onto nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). After blocking with 5% milk in TBS buffer composed of 0.1% Tween-20, 140 mM NaCl and 10 mM Tris–HCl, pH 7.6, the membranes were incubated overnight at 4°C with primary antibodies, such as polyclonal antibody to phosphotau (P-TauThr^{212}) (Biosource International) diluted to 1:500 or polyclonal antibodies to DYRK1A (Abcam, Cambridge, MA, USA) at 1:200 or to tau (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500. The membranes were washed and then incubated with peroxidase-conjugated secondary antibodies against mouse, rabbit or sheep immunoglobulin (Promega, Madison, WI, USA), followed by washing and developing with an ECL Plus Western Blotting Detection System (GE Healthcare Bio-Sciences). The immunoreactive bands on films were digitized with an HP 2355 and subjected to densitometric quantification using Image J version 1.36 (National Institute of Health).

Statistical analysis

To reduce type II errors, the exploratory and confirmatory scans were assessed for associations by one-sided chi-squared test for both allele and genotype frequencies in dominant and recessive models, where each α-level was 0.05. For markers showing significant associations in the confirmatory scan, the Hardy–Weinberg equilibrium was tested. The risk genotypes in the better fitting model were given a value of 1 and the other genotypes 0, and then logistic regression was performed along with age, sex and the *APOE*-ε4 dose under no interaction, using StatView software (SAS Institute, Cary, NC, USA). Linkage disequilibrium in the *DYRK1A* gene was also assessed by $|D'|$ and r^2 values; those less than 0.9 and 0.5, respectively, were judged significant (57). Case–control haplotype analysis was performed with the EM algorithm (58) and with the permutation test at 1000 iterations (59), using SNPAlyze software (DYNACOM, Japan). Normally distributed variables were compared by Student's *t*-test; otherwise non-parametric Mann–Whitney's *U*-test was applied. A *P*-value less than 0.05 was considered significant.

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ORIGINAL ARTICLE

Inhibition of endocytosis activates alternative degradation pathway of βAPP in cultured cells

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Abstract

Background: Alzheimer's disease associated βAPP is sequentially endoproteolyzed by α/β-secretase and γ-cleavage. In the process, extracellular shedding by α-secretase (ADAM 9/10/17) or β-secretase (BACE 1/2) at position L^{17} or D^{1} (Aβ numbering) are prerequisites for the generation of P3 or Aβ, respectively. In addition, several alternative extracellular cleavage sites in βAPP have been reported at position I^{-6}, V^{-3}, R^{5}, E^{11}, F^{20}, and A^{21}. Among these sites, position R^{5} is considered to be cleaved by α-secretase-like activity, whereas position E^{11}, F^{20} and A^{21} are cleaved by β-secretase. Therefore, extracellular shedding of βAPP is thought to be mediated exclusively by α/β-secretase activities. However, so far the characteristics of cleavages at position V^{-3} and I^{-6} are not well understood. The aim of this study is to characterize these two cleavages of βAPP.

Methods: We analyzed the conditioned media of βAPP wt or sw expressing cells with or without pharmacological agents.

Results: Here, we show that the cleavage at position I^{-6} of βAPP has characteristics distinct from that of α/β-secretase, while the cleavage at V^{-3} seems to be mediated by β-secretase. Although inhibition of endocytosis enhances the cleavages at both V^{-3} and I^{-6}, PMA, an α-secretase stimulator, treatment enhances neither of these cleavages. Interestingly, a β-secretase inhibitor, z-VLL-CHO, suppressed V^{-3} but not I^{-6} cleavage. The pathological βAPP Swedish mutant adjacent to the cleavage sites shows similar effects.

Conclusions: Our data demonstrate that neither α nor β-secretase undergoes extracellular shedding at I^{-6} of βAPP. Therefore, our data may indicate a novel alternative βAPP degradation pathway which is up-regulated upon low level of endocytosis.

Key words: *Alzheimer's disease, Amyloid-β peptide, α-secretase, β-secretase, βAPP, endocytosis, presenilin.*

INTRODUCTION

Amyloid-β peptide (Aβ) is a constituent of senile plaque, which is a pathological hallmark of Alzheimer's disease (AD).[1] βAPP is endoproteolyzed by α/β-secretase and γ-cleavage, sequentially. α- or β-secretase cleavage leads to the secretion of N-terminal ectodomain of βAPP and the retention of βAPP CTF-α or -β, respectively (shedding). These CTF-stubs of βAPP are cleaved at the center of the transmembrane domain by presenilin (PS) dependent γ-secretase in several sites.[1]

ADAM 9/10/17, α-secretases, cleave the extracellular domain of βAPP at position L^{17} (Aβ numbering) on the plasma membrane.[2–6] On the other hand, BACE1/2, β-secretases, cleave βAPP at D^{1} mainly on Trans-Golgi-Network and/or endosome.[7–11] However, additional cleavage sites of βAPP are reportedly identified at I^{-6}, V^{-3}, R^{5}, E^{11}, F^{20} and A^{21}. α-secretase- and β-secretase-like activities cleave at R^{5},[12] and at E^{11}, F^{20} and A^{21}, respectively,[7,13,14] (Fig. 1). In the literature, extracellular shedding of βAPP is thought to be mediated exclusively by either α- or β-secretase.

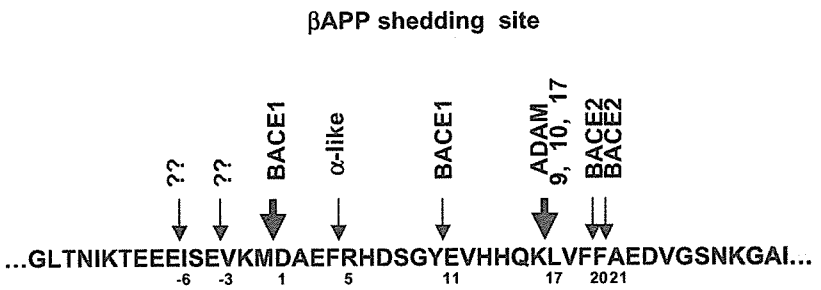

Figure 1 Schematic presentation of known βAPP extracellular shedding site. The gray box denotes the transmembrane domain of βAPP. The secretase activity involving V^{-3} and I^{-6} is unknown (see also the Introduction).

In 1995 Haass *et al.* reported cleavage at I^{-6} and V^{-3} using cultured K293 cells treated with bafilomycin, but they could not detect an increase in cleavages at I^{-6} and V^{-3} with Swedish (sw) mutant βAPP expressing cells.[15] They speculated that those cleavages at I^{-3} and V^{-6} sites might be due to a novel protease, other than β-secretase, which might be less active in βAPP sw expressing K293 cells. However, it remains to be elucidated whether I^{-6} and V^{-3} positions are cleaved by α/β-secretase.

We show that cleavages at both I^{-6} and V^{-3} of βAPP occur near the plasma membrane. Interestingly, characteristics of I^{-6} cleavage are distinct from those of α- or β-secretase, while that of V^{-3} is similar to β-secretase.

MATERIALS AND METHODS

Cell culture and cDNA construction

Human embryonic kidney 293 (K293) cells stably expressing wild-type (wt) βAPP, wt PS1/βAPP sw or PS1 L166P/βAPP sw, were cultured as described elsewhere.[16–18] HeLa cells expressing a dominant-negative mutant Dynamin1 K44A under the control of a tetracycline transactivator were kindly provided by Drs Sandra L. Schmid (Scripps Institute, La Jolla, CA, USA).[19] These cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL G418, 200 ng/mL puromycin and 1 μg/mL tetracycline. βAPP sw were stably transfected with the Dynamin1 K44A expressing HeLa cells by using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the supplier's instructions. The HeLa cells were then cultured without tetracycline for 72 h to induce expression of Dyn-1 K44A. Cells were treated with 100 nM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO, USA) with or without 20 ng/mL PMA (Sigma-Aldrich) a α-secretase stimulant, and with or without 10 μM z-VLL-CHO (Calbiochem, San Diego, CA, USA), a β-secretase inhibitor, for 12 h prior to medium collection.

Combined immunoprecipitation and semiquantitative MALDI-TOF MS

IP-MS analysis was carried out as previously described.[20] 4G8 (Senetec PLC, Napa, CA, USA), an antibody against residue 17–40 of Aβ, was used for immunoprecipitation. The height of the MS peaks and the size of molecular masses were calibrated with angiotensin (Sigma-Aldrich) and a bovine insulin β-chain (Sigma-Aldrich). For semiquantitative analysis, the peak heights of Aβ in the MS spectra were measured and the peak heights relative to the peak height of 1 pmol of bovine insulin beta-chain (internal control) were calculated. These relative peak heights were used to calculate the relative levels of Aβ contained in conditioned medium. Subsequently, the amounts of the conditioned media were adjusted to contain the same levels of Aβ using a standard curve for Aβ, and subjected again to IP-MS analysis. After confirming that the Aβ peak has the same height as the peak of the internal control, peak heights of each sample were measured and the peak heights were calculated relative to the internal control.

RESULTS

Bafilomycin treatment increases cleavages at I^{-6} and V^{-3} but decreases cleavage at D^{1} of βAPP

Bafilomycin treatment of βAPP wt expressing cells increases N-terminally elongated Aβ whose amino (N)-termini are V^{-3} and I^{-6} (Fig. 1).[15] To understand the cleavages at V^{-3} and I^{-6}, we first examined the conditioned media of wt βAPP expressing K293 cells using IP-MS with antibody 4G8 to demonstrate that the fragment produced cleaved at V^{-3} and I^{-6}. Three major peaks consisting of 4330, 4638 and 4985 Da were

observed in the conditioned media of βAPP wt cells (Fig. 2A left panel). These peaks matched the molecular masses of Aβ and N-terminally elongated Aβ species, of which N-termini started with V^{-3} (4638) and I^{-6} (4985) (Table 1). We refer to them as Aβ (D^{1}-V^{40}), Aβ (V^{-3}-V^{40}) and Aβ (I^{-6}-V^{40}), respectively. The peak heights of Aβ (V^{-3}-V^{40}) and Aβ (I^{-6}-V^{40}) were increased using bafilomycin treatment of wt βAPP expressing cells (Fig. 2A right panel). Concomitantly, the peak height of Aβ (D^{1}-V^{40}) decreased (Fig. 2A right panel).

Next we examined whether cleavages at V^{-3} and I^{-6} increase upon bafilomycin treatment using βAPP sw expressing cells. βAPP sw expressing cells mainly secreted Aβ (D^{1}-V^{40}) in the non-treated condition (Fig. 2B upper panels). When treated with bafilomycin, the peak heights of Aβ (V^{-3}-V^{40}) and Aβ (I^{-6}-V^{40}) increased, while the peak height of Aβ (D^{1}-V^{40}) decreased compared with the untreated condition (Fig. 2B middle panels). These data indicate that in both βAPP wt expressing cells and βAPP sw expressing cells, bafilomycin treatment increased the secretion of Aβ (V^{-3}-V^{40}) and Aβ (I^{-6}-V^{40}) and decreased secretion of Aβ (D^{1}-V^{40}) in a similar manner.

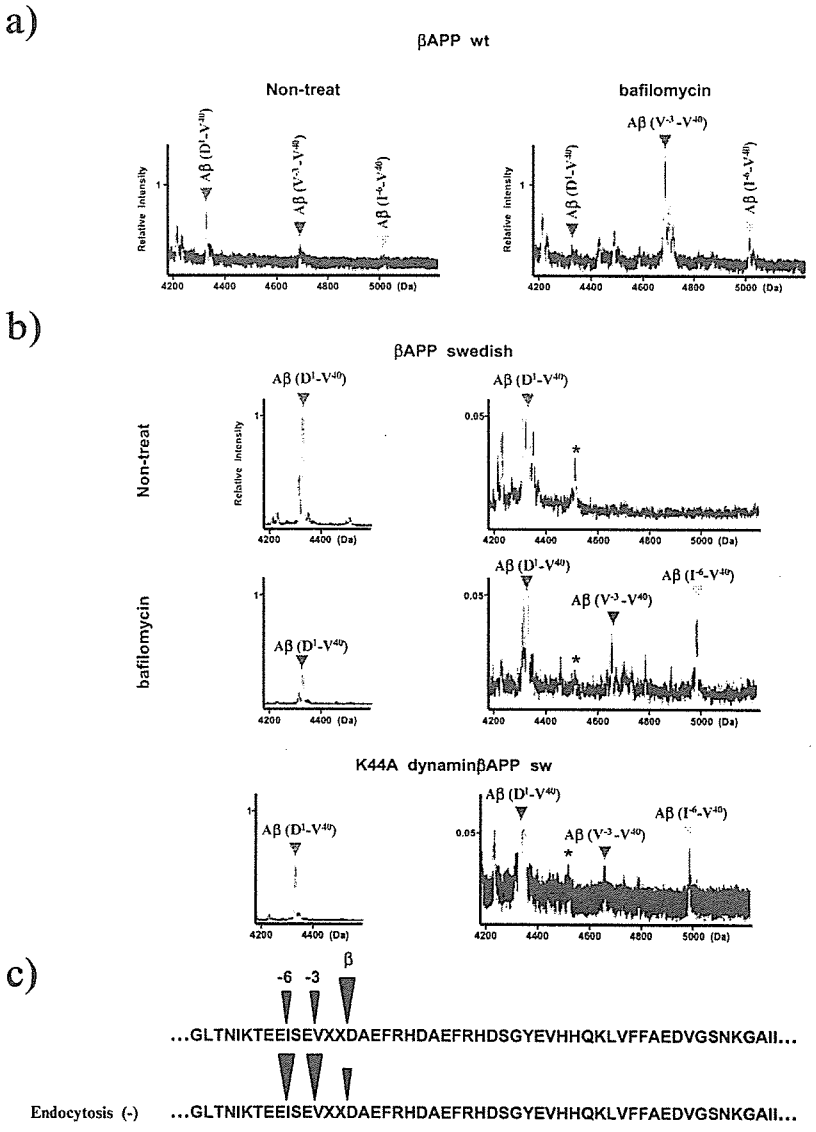

Figure 2 Effect of endocytosis inhibition on Aβ and N-terminally elongated Aβ secretion. βAPP wt or sw expressing K293 or dynamin K44A/βAPP sw expressing HeLa cells were cultured with and without bafilomycin. Conditioned media were analyzed by IP-MS. (A) Mass spectra of Aβ recovered from non-treated (left panels) or bafilomycin treated (right panels) conditioned media of βAPP wt expressing K293 cells. (B) Mass spectra derived from non-treated (upper panels) or bafilomycin treated (middle panels) βAPP sw expressing K293 cells. Mass spectra derived from non-treated dynamin K44A and βAPP sw expressing HeLa cells (lower panels). Asterisks indicate the peaks of Aβ (D^{1}-A^{42}). Molecular masses of each peak are shown in Tables 1 and 2. (C) Schematic presentation of the effect of endocytosis inhibition on the D^{1}, V^{-3} and I^{-6} cleavage. XX denotes KM (wt) or NL (Sw).