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認知症対策総合研究事業

『認知症治療を目的とした変異型オリゴマーアミロイドペプチドを抗原とする神経免疫療法の開発』に関する研究

平成２２年度　総括研究報告書

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平成２３（２０１１）年　３月

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Ⅰ．総括研究報告

厚生労働科学研究費補助金（認知症対策総合研究事業）
（総括･分担）研究報告書

認知症治療を目的とした変異型オリゴマーアミロイドペプチドを抗原とする神経免疫療法の開発に関する研究

研究代表者　　森　啓　大阪市立大学・大学院医学研究科・教授

研究要旨
アルツハイマー病の病因としてＡβオリゴマーが重要になってきている。我々の発見したAPP E693・変異は、Ａβオリゴマー形成促進をする。この特異な特性を利用したＡβオリゴマーモデルを作成し、病変解析を進めた。同モデルは老人斑を形成しないことは言うに及ばず、すでに8カ月齢頃からニューロン内にＡβオリゴマーを蓄積し始めることで記憶障害を示すことを明らかにした。同時に、脳内炎症反応も惹起されることが脳病理とPET画像で確認した。さらに、神経変性疾患の機構としてミコトコンドリア機能障害を観察し、チトクロームC漏出像を発見することができた。以上より、Ａβオリゴマーのさらなる重要性を示唆する証拠を提示できたと考えている。

Ａ．研究目的

2002 年にハーバード大学の Selkoe 博士が、アルツハイマー病をシナプス傷害によって引き起こされる疾患であると提唱した。これまでの老人斑原因説を否定する事も、肯定することもなく、あたらしくシナプス変性という標的をしめしたが、その病因分子としてＡβオリゴマーが議論され始めるようになった。

文献考証学的には、1998 年にノースウェスタン大学の Klein 博士により ADDLs と呼ばれる可溶性のＡβオリゴマーが発見されたのが発端ともいえる。当時は、分子量 17kDa および 27kDa からなる ADDLs として発表された。Ａβ一分子の分子量が約 4.5kDa として３分子あるいは 6 分子からなる会合体と推測できる。さらに 2002 年には、ハーバード大学の Selkoe 博士によって dimer あるいは low-n Ａβオリゴマーが同定されている。2003 年には、Klein 博士が ADDLs には分子量 56kDa の高分子成分があり、脳 ADDLs にも同じ分子量を同定している。同じ分子量をもつＡβオリゴマーは、2006 年にミネソタ大学の Ashe 博士によってＡβ*56 の別称で議論されたことでＡβオリゴマーの意義がほぼ定着したと云える。

アルツハイマー病（AD）の発症・進行過程におけるＡβオリゴマーの病理学的役割を、我々が家族性認知症患者から同定したアミロイド前駆体蛋白質（APP）の新しい変異 E693Δを発現するトランスジェニックマウス（Tg マウス）を用いて調べることである。

まず、このマウスでの老人斑形成やＡβオリゴマーの蓄積、シナプス機能や学習記憶機能の変化を生化学、組織化学、電気生理学、行動試験などの手法を用いて調べ、Ａβオリゴマーモデルマウスとしての妥当性を検証する。次に、Ａβオリゴマーの蓄積によって、老人斑の形成なしに、シナプスの消失やタウの異常リン酸化、グリア細胞の活性化、ニューロンの消失などの AD の病理変化が起こるかどうかを免疫組織化学的に調べる。Ａβオリゴマーモデルマウスとしての妥当性を検証する。次に、Ａβオリゴマーの蓄積によって、老人斑の形成なしに、シナ

プスの消失やタウの異常リン酸化、グリア細胞の活性化、ニューロンの消失などの AD の病理変化が起こるかどうかを組織化学的に、脳 PET 画像的に調べる。

B．研究方法

＜Aβオリゴマーモデルマウスの作成＞

プリオンプロモーター下流に新変異（E693Δ）を組み込んだヒト APPcDNA 遺伝子コンストラクトのベクター部分を排除した後、顕微鏡下で受精卵前核へ導入し、数時間培養してから偽妊娠状態の雌マウスの卵管に移植した。C57BL/6 にて少なくとも、10 代以上戻し交配したものを確立した。導入遺伝子が常にヘテロと成るようにゲノムチェックをした。

＜生化学的解析＞

Aβの単量体および多量体形成はウェスタンブロットにより、・001 抗体、4G8 抗体、6E10 抗体により検出した。

＜免疫組織学的解析＞

マウス脳組織におけるAβは・001 抗体、4G8 抗体、6E10 抗体、GFAP 抗体、Iba1 抗体、リン酸化タウ抗体(PHF1)、NeuN 抗体により検出を試みた。

＜電気生理学的解析＞

シナプス機能を検討するために LTP 測定を実施した。

＜細胞死検出分析＞

ミトコンドリアレポーター分子（JC-1）染色により、生細胞と死細胞を判別することにした。同時に、ミトコンドリア細胞分画により、細胞質へのチトクローム C 漏出を生化学的および組織染色による観察した。

＜脳 PET 解析＞

マウス個体での脳炎症反応を検出するために、末梢系ベンゾディアゼピン受容体に結合する放射能標識リガンドを使用して活性化ミクログリアの脳画像 PET を撮像することにした。

（倫理面への配慮）

臨床研究ではない、組み換え動物実験を使用した基礎医学的研究であるために、倫理面での配慮は不必要である。ただし、DNA 組み換え、動物実験についての承認は取得済みである

C．研究結果

1．細胞内オリゴマーの蓄積と脳内病変

モデルマウス脳内では、Aβオリゴマーが 8 カ月齢頃より大脳皮質や海馬のニューロン内に蓄積し、さらにシナプス障害が生じることも示した（図１）[1]。このニューロン内蓄積が、$Aβ_{1-40}$ あるいは $Aβ_{1-42}$ に依るものかについては決着しておらず推測の域が出ない。

細胞内蓄積Aβは、細胞外へ分泌されるAβと、ある意味で逆比例の関係にある。蓄積が増加すると言うことは、分泌量低下になることが示唆されるが、大阪変異をもつ遺伝子発現をする細胞では、Aβオリゴマーが増加するという特徴の他に、細胞内Aβオリゴマーの蓄積と細胞外への分泌低下が観察されている。細胞外への分泌には、細胞内コレステロールが重要である[2]。これには、$Aβ_{1-40}$ あるいは $Aβ_{1-42}$ の化学的な性質が大きく関係することが in vitro の系で示唆された[3]。

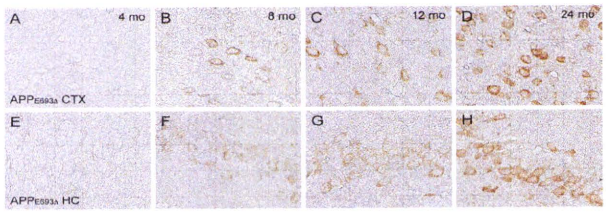

図1.　Aβオリゴマーモデル（APPE693Δ）マウスにおける脳病理組織像の経時変化.
オリゴマー型ＡＤマウスの4mo、8mo、 12mo、24moでのAβオリゴマーをオリゴマー特異抗体（NU-1）にて免疫染色。CTX、HCは大脳皮質、海馬領域を示す.

アストログリア、ミクログリアの経時的増加も観察されたことから、脳内炎症を示唆する免疫反応に関与する生体変化が細胞内Aβオリゴマー蓄積と同期していることが観察された。これは、マウスの生体レベルでの PET を使用した脳画像でも確認することが出来ている[4]。この結果より、脳神経炎症のトリガーは、アミロイド老人斑だけでなく、非線維性Aβオリゴマーも脳炎症の原因と成ることが示された。更なる考慮のために、ヒト型タウ遺伝子を発現するモデルマウスとの交配を計画している。

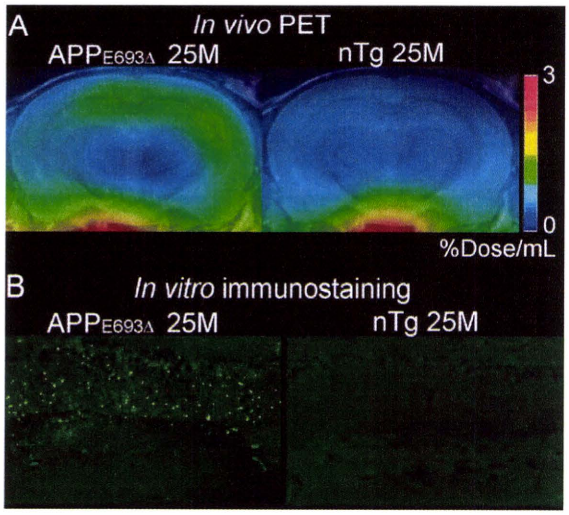

図２．　Aβオリゴマーモデル（APPE693Δ）マウスの脳内炎症変化
A：活性化ミクログリア反応は、18kDaの輸送分子として知られる末梢系ベンゾディアゼピン受容体に結合する放射能標識リガンドを使用して活性化ミクログリアの脳画像PETをしめす．B:リガンド抗体（NP155）による海馬CA1染色。生体脳画像と脳組織標本の一致が確認されていることがわかる．

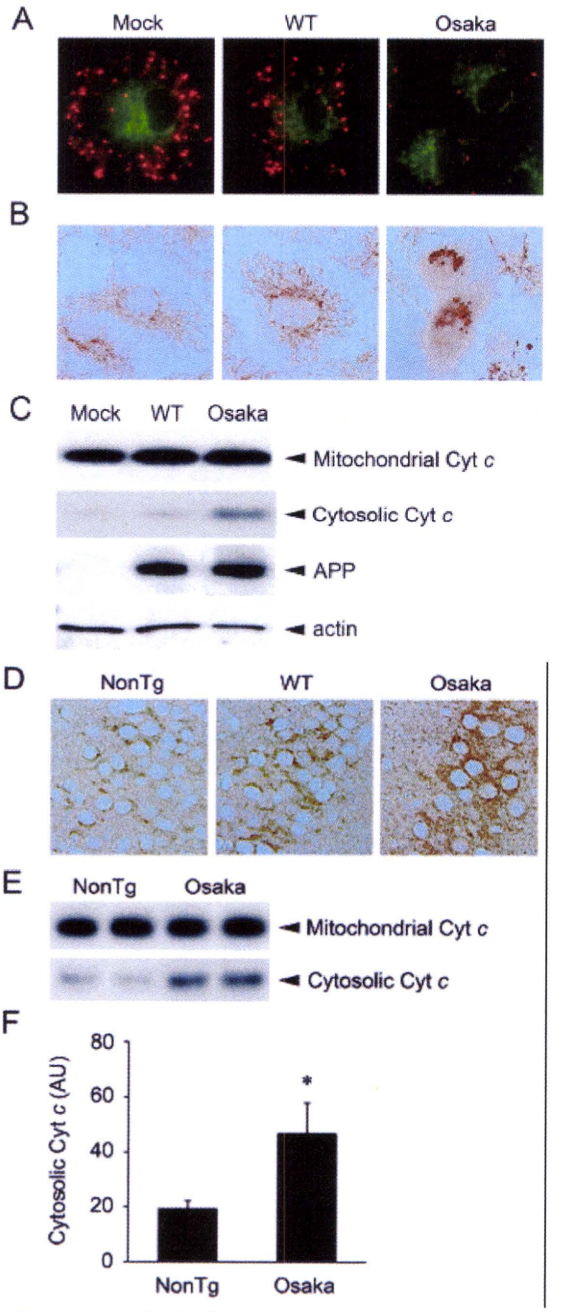

図３．　Aβオリゴマーによるミトコンドリア機能への影響
A：ミトコンドリアの COS 細胞内分布．ミトコンドリアレポーター分子（JC-1）染色したもの。B：同細胞の DAB 染色像．C:チトクロームC染色が細胞質にあることを、細胞分画した後にウエスタンブロットにより検出．D-F：Aβオリゴマーモデル（APPE693Δ）マウス脳におけるミトコンドリア観察。D:脳組織の免疫染色像、E：ミトコンドリアを脳組織から細胞分画したウエスタンブロット像、F：E を定量．

2．細胞死分子機構

小胞体でのAβオリゴマー蓄積は、小胞体ストレスを介して細胞死を誘導する事が知られているが、細胞死におけるミトコンドリアの関与は老化における酸化ストレスの議論を考慮した場合重要なファクターとなる。そこで、Aβオリゴマーによるミトコンドリア機能への影響について検討した。

ミトコンドリア内膜成分として電子伝達系作用を担っている機能性チトクロームC（赤色）がAPPE693Δ発現をした細胞では機能低下が示唆されている。実際、ミトコンドリア画分から細胞質画分への漏出があることを確認をしている（図3．B，C）。これらの培養細胞を用いた結果はAβオリゴマーモデル（APPE693Δ）マウス脳内においても検証された。以上を総合して、Aβオリゴマーの細胞内発現と蓄積によって、ミトコンドリアの形態はチューブ状からドット状もしくは凝集体へと変化するだけでなく、ミトコンドリア内膜のチトクロームC分子の細胞質への漏出が生じることが明らかとなった[3]。

D．考察

Aβオリゴマーの蓄積は、ニューロン内に観察されたが、細胞外濃度の交信については決着していない。これには、脳PETリガンドの開発あるいは、髄液等における水溶液中の可溶性Aβオリゴマー定量測定計の開発が必要である。この木のためにも、標準物質と成るAβオリゴマーの再構成は、必須の課題であることがわかる。

細胞外のAβオリゴマーについての議論を深めるまでもなく、細胞内Aβオリゴマーの存在と異常蓄積は、特筆すべき脳変化である。その根拠は、モデルマウスに特異的に出現したこと、加齢に伴う蓄積であること、さらには蓄積時期と行動異常あるいは学習障害の時期が一致していることが指摘される。これらをさらに補強するために、電気生理学的な長期増強（Long-term potentiation: LTP）の低下が確認されていることは強調しておかなければならない。現在の処、モデルマウス脳から抽出するAβオリゴマーは、dimerあるいはtrimerといったlow-nAβオリゴマーであるが、高分子量域にあるADDLsやAβ*56様Aβ・オリゴマーを追試できていない。おそらくは、実験条件による相違であるか、発表されていないノウハウがある可能性を否定できない。Ashe自身も、恒常的に高分子Aβオリゴマーを再現していない点でも明らかなように、高分子オリゴマーの安定性を担保するファクターについては、未だ解明されていないことは特筆しておきたい。

本研究により、ADの発症・進行過程におけるAβオリゴマーの役割を明らかにすることができた。また同時に、本研究により、Aβオリゴマーの病理学的意義やADの定義を考える上で重要な情報になると考えられる。

E．結論

AD発症原因分子として注目されているAβオリゴマーに特化した実験的モデルマウスを作成した。本モデルは、診断および治療薬開発の標的モデルマウスとして今後の研究に有用である。

F．健康危険情報

該当する内容はない。

G．研究発表

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H. 知的財産権の出願・登録状況 (予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

III. 研究成果の刊行物・別刷

Hirosaki Med. J. 60(Suppl.) : S105—S110, 2010

OLIGOMERIC Aβ IS THE SOLE CULPRIT MOLECULE TO CAUSE ALZHEIMER'S DISEASE?

Hiroshi Mori[1)], Takami Tomiyama[1)], Kennichi Ishibashi[1)], Kiyouhisa Ohnishi[1)], Rie Teraoka[1)], Akiko Fukushima[1)], Hiroshi Takuma[1)], Hiroyuki Shimada[1)], Suzuka Ataka[1)], Tomohiro Umeda[1)], Erika Kitajima[1)], Yuki Fujita[1)], Yuki Yamashita[1)], Keiichi Yamamoto[1)], Takami Miki[1)], Shogo Matsuyama[2)], Hiroyuki Iso[3)], Tetsu Nagata[4)], Tomoyuki Nishizaki[4)], Yasuhiro Wada[5),6)], Eito Yoshioka[5),6)] and Yasuyoshi Watanabe[5),6)]

Abstract Alzheimer's disease (AD) is the major and common disease usually for aged people to show progressive neurodegenerative disorder with the dementia. Amyloid-beta (also β-protein and referred here to as Aβ) is a well-established seminal peptide in AD that is produced from the amyloid precursor protein (APP) by consecutive digestions with β-secretase of BACE and gamma-secretase of the presenilin complex. Abnormal cerebral accumulation of Aβ such as insoluble fibrils in senile plaques and cerebral amyloid angiopathy (CAA) are observed as a neuropathological hallmark of AD. In contrast to such insoluble fibrillary Aβ, a soluble oligomeric complex is discussed as ADDLs, Aβ oligomer, low-n oligomer Aβ, Aβ*56 or so. Despite their different names, it is proposed as the current hypothesis that oligomeric Aβ is the direct molecule to cause synaptic toxicity and cognitive dysfunction in the early stages of AD. We identified a novel APP mutation (E693delta; referred to as the Osaka mutation) in a pedigree with probable AD resulting in a variant Aβ lacking glutamate at position 22. Based on theoretical prediction and *in vitro* studies on synthetic mutant Aβ peptides, the mutated Aβ peptide showed a unique aggregation property of enhanced oligomerization but no fibrillization. This was further confirmed by PiB-PET analysis on the proband patient. Collectively together, we conclude that the Osaka mutation is the first human evidence for the hypothesis that oligomeric Aβ is involved in AD.

Hirosaki Med. J. **60, Supplement** : S105—S110, 2010

Key words: Alzheimer's disease; amyloid genetics; oligomeric Aβ

Amyloid beta (Aβ) oligomers are suggested to cause synaptic dysfunction in the early stages of Alzheimer's disease (AD)[1-6]. However, their precise contribution to the AD pathology is unknown: It is not evident whether oligomer formation of Aβ is sufficient for the progression of the disease in the absence of fibril formation. We had a chance to study a patient with a Japanese familial AD who was supposed to have Aβ oligomers without fibrils.

The proband was a 62-year-old woman with a history of suspected familial AD. She noticed memory disturbance at the age of 56. She had no history or symptoms of other neurological disorders. Her Hachinski's ischemic and Mini Mental State Examination (MMSE) scores were normal. MRI and PET showed no cortical atrophy or abnormal metabolism, while SPECT demonstrated bilateral mild hypoperfusion in the temporal lobes. Electroencephalogram showed bilateral intermittent generalized slow theta activity. Thus, she was diagnosed as having mild cognitive impairment at that time. At the age of 59, she showed ideomotor apraxia, and her MMSE score was 22/30 points. According to the Diagnostic and Statistical Manual of

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Mental Disorders (DSM-IIIR) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke, AD and Related Disorders Association (NINCDS-ADRDA), she was diagnosed as having AD. At the age of 62, her MMSE score dropped to 5 and she exhibited cerebellar ataxia. The axial T1 weighted MRI images showed only mild parietal lobe atrophy. Genetic analysis was examined after an appropriate consultation in which they gave their informed consent to participate in this study. This study was approved by the institutional ethics committee of Osaka City University Graduate School of Medicine. Exons 16 and 17 of APP and all exons of presenilins 1 and 2 were amplified from the patient's genomic DNA by PCR. The DNA sequence of each product was analyzed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Since this patient was found to have a mutation in APP exon 17 but not in the presenilin 1 or 2 exons, only APP exon 17 was examined for other family members. ApoE genotyping was performed by detection of the restriction site polymorphism, as described previously[7].

From her and her family members, we identified a novel mutation (hereafter referred to as the Osaka mutation) in APP exon 17 but not in presenilin 1 or 2 exons. This mutation is the deletion of codon 693 encoding glutamate (E693delta) at position 22 in the Aβ sequence. The patient had a homozygous deletion while her unaffected older and younger sisters showed only heterozygous deletions. ApoE genotype appeared not to be associated with this familial case. This Osaka mutation is the first deletion-type mutation in APP. The same homozygous deletion was recently found in another pedigree with AD and the heterozygous deletion in two other pedigrees, one of which was with mild cognitive impairment and the other was normal. These findings strongly suggest that the Osaka mutation is a cause of AD. In addition, this mutation might represent the first recessive one linked to familial AD, though any conclusion cannot be drawn from the little information. To identify Aβ species produced from the mutant APP, we examined the molecular mass of Aβ secreted from HEK293 cells transfected with the APP construct. The resultant Aβ was found to start and end at normal positions but lack a glutamate at position 22. The secretion of the mutant Aβ1-42 and Aβ1-40 were both reduced to about 60% of wild-type Aβ but the ratio Aβ1-42/ Aβ1-40 was unaffected. This lowered Aβ secretion may explain why the present mutation appears to recessive. This issue remains to be further investigated.

To examined their aggregation property, the mutant Aβ1-40deltaE and Aβ1-42deltaE peptides, which lack a glutamate at position 22, were synthesized (American Peptide Company, Sunnyvale, CA). Molecular weight and amino acid composition of the peptides were confirmed by electro spray mass spectral analysis and amino acid analysis, respectively. The purity of the Aβ1-40deltaE and Aβ1-42deltaE peptides, which was determined by reverse-phase HPLC, was 95.0% and 91.0%, respectively. Control wild-type Aβ1-40 and Aβ1-42 peptides were obtained from American Peptide Company and Peptide Institute (Osaka, Japan). In the thioflavin T fluorescence assay, wild-type peptides showed a quick increase of fibril aggregation, whereas the mutant peptides exhibited little or no increase. In Western blotting, synthetic Aβ peptides were initially dissolved to 0.1 mM in the alpha-helix promoting solvent hexafluoroisopropanol (HFIP) (Sigma) and the solvent was evaporated under vacuum using a Savant Speed Vac system (GMI, Ramsey, MN). The dried peptides were resuspended to 1 mM in 0.1% NH_4OH and dispensed, in quadruplicate, into tubes containing PBS to make a peptide concentration of 100

microM. The peptide solutions were incubated at 37° C for 7 days; aliquots were taken every 24 hr to monitor peptide aggregation by ThT fluorescence assay[8]. For Western blotting, the aliquots were diluted 10-fold in SDS sample buffer and boiled for 5 min. After a further 200-fold dilution in SDS sample buffer, 4 micro-litre (equivalent to 0.2 pmol Aβ peptide) of sample was separated by SDS-PAGE on a 12% NuPage Bis-Tris gel (Invitrogen, Tokyo, Japan) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Tokyo, Japan). The membranes were boiled in PBS for 10 min to enhance signals and blocked overnight with 3% BSA/1% skin milk/0.1% Tween 20/150 mM NaCl/50 mM Tris-HCl, pH 7.6. Aβ peptides were probed with 6E10 or beta001 followed by horseradish peroxidase (HRP) -labeled anti- mouse or anti-rabbit antibody (Bio-Rad Laboratories, Tokyo, Japan), respectively. Wild-type peptides showed a rapidly decrease of monomers, reflecting their aggregation into fibrils, but those of the mutant peptides only gradually decreased. However, the mutant peptides showed massive formation of SDS-stable oligomers (dimers, trimers and tetramers) immediately after solubilization.

The peptides in aliquots were adsorbed onto 200-mesh Formvar-coated copper grids and negative-stained with 2% uranyl acetate. The specimens were viewed using a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan), showing that wild-type Aβ1-42 peptide formed abundant fibrils during 7-day incubation, whereas virtually no fibrillization was observed in the mutant peptide. Thus, the mutant peptides were shown to rapidly form stable oligomers but not to transform into fibrils.

The unique aggregation property of the mutant Aβ was suggestive of no amyloid formation in the patient's brain. To assess this possibility, we performed PET amyloid imaging of the patient's brain with [^{11}C]- Pittsburgh compound-B (PiB) using a PET scanner Eminence-B (Shimadzu Corp., Kyoto, Japan) which was composed of 352 detector blocks, each with a 6 x 8 array of 3.5 x 6.25 x 30 mm^3 bismuth germinate oxyorthosilicate crystals, arranged as 32 crystal rings with 208 mm axial field of view. Transmission scans were performed before PiB administration for 5 min in singles mode with ^{137}Cs point source to obtain attenuation correction data. Emission data were acquired over 60 min (29 frames: 6×30 s, 12×60 s, 5×180 s, 6×300 s). Images were reconstructed with segmented attenuation correction, using Fourier rebinning followed by two-dimensional filtered back-projection applying Ramp filter cutoff at Nyquist frequency. Three-dimensional Gaussian filter with a kernel full-width of a half maximum of 5 mm was applied to the images as a post filter. All subjects had an intravenous bolus injection of 150-300 MBq PiB with a high specific activity (average 20-30 GBq/micro mol). PiB retention data were given as standard uptake values, as described previously[9]. Slight but significant PiB retention signals were observed in temporal, parietal and occipital lobes and cerebellum but not in frontal lobe, which was apparently different

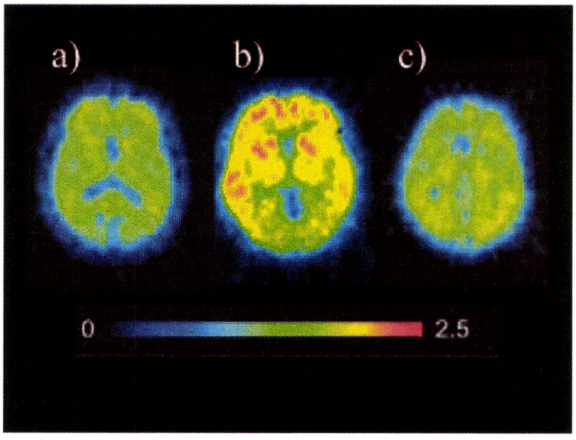

Figure 1 PiB-PET analysis

Amyloid imaging of the patient's brain with [^{11}C]PiB. PiB standardized uptake value images summed over 40 to 60 minutes are shown. (a) aged control (81-yr, female); (b) sporadic AD (71-yr, female); (c), the present case (62-year-old, female).

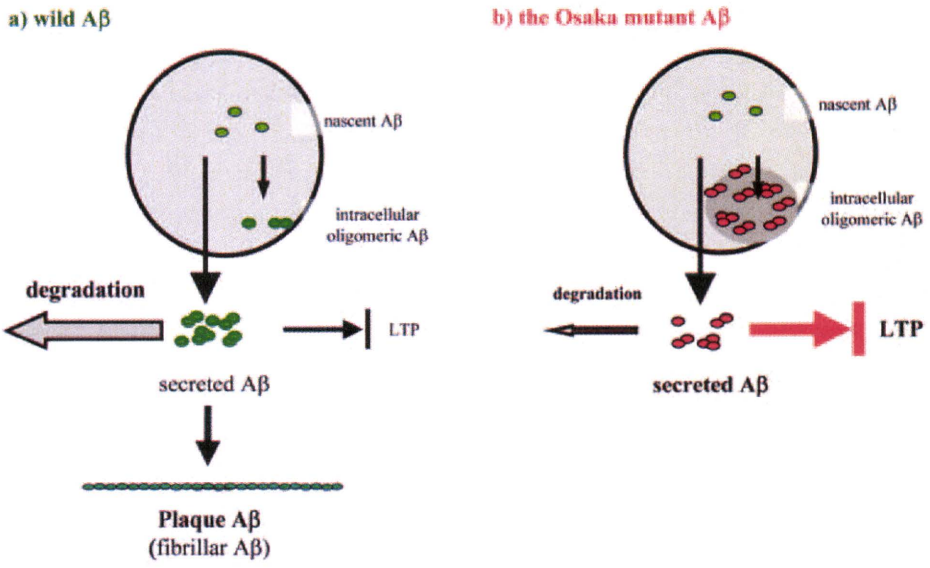

Figure 2 Scheme of Aβ pathway

Aβ was produced from APP by the consecutive digestions with β-secretase and γ-secretase. (a) Wild Aβ was produced from APP and its majority was secreted out in media. Some of secreted Aβ was in an oligomeric form that causes the synaptic LTP function. (b) The mutant Aβ was enhanced to form an oligomeric state due to the lack of the 22nd glutamate residue resulted in less secretion and in intracellular accumulation. The mutant Aβ was less vulnerable to proteases such as IDE or neprilysin and showed stronger inhibiting activity on LTP than wild Aβ.

from most cases of AD. Thus, the absence of fibril formation of the mutant Aβ was observed both *in vitro* and *in vivo*. It has been proposed that the formation of a beta-turn at positions 22 and 23 in Aβ molecules plays a crucial role in peptide aggregation[10]. The Osaka mutation at position 22 may cause disruption of the secondary structure of the peptide necessary for its formation into fibrils. The lack of a polar amino acid (glutamate) should lead to increased hydrophobicity of the peptide, which may result in accelerated assembly of the peptides into oligomers.

The recent findings of Aβ oligomer-induced synaptic dysfunction[3,4] led us to examine effects of the mutant Aβ on synaptic plasticity in comparison with wild-type Aβ. Synthetic Aβ1-42 peptides were injected into rat cerebral ventricle and hippocampal LTP was examined *in vivo*. As shown previously[3], wild-type peptide caused a significant inhibition of LTP ($p = 0.0497$ vs. PBS). Notably, the mutant peptide showed a much stronger inhibition than wild-type peptide ($p < 0.0001$ vs. PBS; $p < 0.0001$ vs. wild-type). The observed result appears to reflect the ability of the mutant peptide to form oligomers.

Beside excellular Abeta, several reports have suggested that synaptic dysfunction and neurodegeneration are associated with intraneuronal Aβ[11-17]. We also examined their occurrence in cells transfected with wild and mutated APP cDNA.

Unlike other APP mutations[18-22], the Osaka mutation neither increased total Aβ or Aβ1-42 production nor promoted Aβ aggregation into fibrils but markedly enhanced Aβ oligomerization. Our results suggest that this novel mutation causes AD by enhancing Aβ oligomerization. Furthermore, it is suggested that Aβ fibrillization is not a definite requirement to induce AD, rather its oligomerization may be a crucial event in the pathogenesis of the disease. Alternatively, the present case may represent not a typical but variant type of AD in which the enhanced oligomerization of Aβ enables

to induce the disease without Aβ fibrillization. Finally, as a model to represent Aβ oligomer-related neuropathology, our $APP_{E693delta}$-Tg mice have an advantage in that they control for the influence of Aβ fibrils. Therefore, they will be useful not only for investigating the pathogenic roles of Aβ oligomers but for evaluating therapeutic strategies for AD targeting Aβ oligomers. This will be hoped to break through a current problem on Aβ oligomers. Probably our main goal is to vividly show and specify "the Aβ oligomer" as the culprit of the disease.

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Regulation of Cholesterol Efflux by Amyloid β Secretion

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Amyloid β (Aβ) is a key molecule in the pathogenesis of Alzheimer's disease, but its physiological function remains unclear. Aβ is produced from amyloid precursor protein (APP) by β- and γ-secretases, which is enhanced by high levels of cellular cholesterol, so cholesterol is a risk factor for Alzheimer's disease. This linkage led us to hypothesize that Aβ is produced to regulate cellular cholesterol levels in response to high-cholesterol stimulation. Here we show that Aβ production caused a reduction of cellular cholesterol levels in transfected HEK293 cells and neuronal IMR-32 and Neuro2a cells, which was accompanied by an increase in efflux of cholesterol from cells. Fractionation of the culture media by ultracentrifugation and subsequent immunoelectron microscopic observation revealed that Aβ assembled high-density lipoprotein-like particles with cellular cholesterol during its secretion. This assembly was mediated by the ATP-binding cassette transporter A1. APP transgenic and knockout mice exhibited lower and higher levels of cellular cholesterol in their brains, suggesting that Aβ-mediated regulation of cellular cholesterol is physiological. Furthermore, we found that, when injected into mouse cerebral ventricle, reconstituted lipoproteins with Aβ were excreted into the peripheral tissues more efficiently than those without Aβ. This result suggests that Aβ mediates cholesterol transport from the brain to the circulation. We propose, based on these findings, a novel, apolipoprotein-like function for Aβ that is involved in maintenance of cellular and cerebral cholesterol homeostasis.

Key words: Alzheimer's disease; risk factor; apolipoprotein; HDL; ABCA1

Cerebral accumulation of amyloid β (Aβ) is a hallmark of Alzheimer's disease (AD). Aβ is generated from amyloid precursor protein (APP) by β- and γ-secretases at the cellular membranous compartments and secreted into the extracellular space. Several factors have been shown to affect Aβ production and thereby influence the incidence of AD. For example, high levels of plasma cholesterol are known to be a risk factor for AD (Solomon and Kivipelto, 2009; Stefani and Liguri, 2009). Cholesterol loading of cells causes increased Aβ generation via activation of both β- and γ-secretases (Frears et al., 1999; Xiong et al., 2008), whereas cholesterol depletion results in reduced Aβ production (Simons et al., 1998; Frears et al., 1999; Grimm et al., 2008). In transgenic mouse models of AD, diet-induced hypercholesterolemia increased Aβ levels in the brain and thus accelerated Aβ deposition (Refolo et al., 2000; Shie et al., 2002), whereas treatment with statin, an inhibitor of the cholesterol biosynthesis enzyme hydroxymethylglutaryl-CoA (HMG-CoA) reductase, attenuated amyloid pathology (Petanceska et al., 2002). Although the relationship between cholesterol and AD is still somewhat controversial, the above-mentioned observations indicate that cerebral Aβ accumulation is evidently influenced by the levels of cholesterol. Nevertheless, neither the physiological function of Aβ nor the biological significance of cholesterol-induced Aβ production is well understood.

A recent study has demonstrated that Aβ (particularly Aβ40) reduces cholesterol de novo synthesis by inhibiting HMG-CoA reductase activity (Grimm et al., 2005). This negative feedback effect of Aβ on cellular cholesterol may account for why high levels of cellular cholesterol increase Aβ production. On the other hand, it has been reported that Aβ can bind to lipids and exists on lipoproteins in the cerebrospinal fluid together with apoE and/or ApoA-I (Koudinov et al., 1996). Some investigators have thus speculated that Aβ may influence cholesterol transport in the brain (Yao and Papadopoulos, 2002; Kontush, 2004). In addition to its existence on brain lipoproteins, Aβ, as well as apoE, has been shown to bind to lipoprotein receptors and to be transported from the brain to the circulation across the blood–brain barrier (Deane et al., 2008). These

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observations led us to hypothesize that Aβ is produced to regulate cellular and cerebral cholesterol levels in an apolipoprotein-like manner.

To test this hypothesis, we examined the effects of Aβ on cellular cholesterol levels and on cholesterol transport in vitro and in vivo. We found that Aβ enhanced cholesterol efflux from cells by assembling lipoprotein-like particles during its secretion, which was mediated by the ATP-binding cassette transporter A1 (ABCA1). This effect of Aβ was confirmed in vivo by measuring cellular cholesterol levels in APP transgenic and knockout mouse brains. Furthermore, we demonstrated that Aβ promoted cholesterol transport from the brain to the peripheral tissues in mice. Our findings suggest a novel, apolipoprotein-like function for Aβ that is involved in maintenance of cellular and cerebral cholesterol homeostasis.

MATERIALS AND METHODS

APP_{695}, C99, C83, C59, and C50 cDNA Constructs

APP_{695}, C99, C83, C59, and C50 cDNA constructs were prepared by using a pCI mammalian expression vector (Promega, Madison, WI), as described previously (Nishitsuji et al., 2009).

Aβ ELISA

Aβ concentrations in culture media were determined by ELISA using human amyloid β (1–40) (N) and (1–42) (N) kits (IBL, Takasaki, Japan).

Cellular Cholesterol

Human embryonic kidney (HEK293) cells were transfected with the above-mentioned constructs using the Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Two days after transfection, cells were loaded with cholesterol by incubation at 37°C for 1 hr with 1 mM cholesterol/methyl-β-cyclodextrin (MβCD) complex (Sigma, St. Louis, MO) in serum-free DMEM. After being washed with PBS, cells were harvested and subjected to protein assay and Western blotting with a polyclonal antibody to the C-terminal region of APP (C40; Suga et al., 2004) to confirm expression of the constructs. Cellular cholesterol was extracted from cell pellets by pipetting and vortexing in chloroform/methanol (2:1). Five hundred microliters of chloroform/methanol was used for cells from each well in six-well culture plates. After a brief centrifugation, 300 μl of the chloroform fraction was transferred into a new tube, and the solvent was evaporated under a vacuum. The dried lipid was resuspended in 20 μl of 10% Triton X-100/isopropanol, and 1 μl of the sample was subjected to cholesterol assay using a Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, Inc., Mountain View, CA). The obtained cholesterol levels were normalized to the protein levels. In some experiments, transfected HEK293 cells were treated overnight with 100 μM α-secretase inhibitor TAPI-I (Peptides International, Louisville, KY), 10 μM β-secretase inhibitor II (Calbiochem, EMD Chemicals, Madison, WI), or 1 μM γ-secretase inhibitor L-685,458 (Peptide Institute, Minoh, Japan) before cholesterol loading. In another set of experiments, untransfected HEK293 cells were loaded with cholesterol for 1 hr, then incubated for a further 1 hr with synthetic Aβ1–40 peptide (Peptide Institute) at 10 ng/ml. This Aβ concentration was similar to that in 2-day conditioned media of APP_{SW}-expressing cells. Alternatively, untransfected HEK293 cells were loaded with cholesterol for 1 hr using cholesterol/MβCD complex solubilized in 2-day conditioned media of APP_{SW}-expressing cells.

Human neuroblastoma IMR-32 and mouse neuroblastoma Neuro2a cells were also treated overnight with β- or γ-secretase inhibitor and then loaded with cholesterol. In ABCA1 knockdown experiments, IMR-32 cells were transfected with the Silencer Select Predesigned siRNA to ABCA1 (s846; Ambion, Applied Biosystems, Foster City, CA) at a concentration of 5 nM using the siPORT NeoFX reagent (Ambion). 2 days after transfection, cholesterol loading was performed as described above. Expression levels of ABCA1 were determined by Western blotting with a monoclonal antibody to ABCA1 (AB.H10; Applied Biological Materials, Richmond, Britich Columbia, Canada). In some experiments, IMR-32 cells were loaded with cholesterol for 1 hr in the presence of a monoclonal antibody to the N-terminus (82E1; IBL) or C-terminus (6A for Aβ40 and 11C for Aβ42; Lippa et al., 1999) of Aβ at 10 ng/ml. This antibody concentration was approximately fourfold higher in molar ratio than the concentration of Aβ (about 50 pg/ml) secreted from IMR-32 cells for 1 hr in response to cholesterol loading. Neither 82E1 nor 6A/11C binds to APP holoprotein. As a control, a mouse monoclonal antibody to FLAG (M2; Eastman Kodak Company, New Haven, CT) was used.

Cellular Cholesterol in Mouse Brains

Tg2576 mice (Hsiao et al., 1996) were purchased from Taconic Farms (Hudson, NY) and mated with wild-type C57/BL6 mice to obtain heterozygotes (+/–) and nontransgenic littermates (–/–). Homozygous APP knockout mice (Zheng et al., 1995) were cross-bred with the APP_{WT} transgenic mice (Matsuyama et al., 2007) to establish human APP (+/–), mouse APP (–/–) mice. As controls for APP knockout mice, wild-type C57/BL6 mice were used. Brains were removed from 6- or 12-month-old mice and incubated in 2.5 ml of 0.25% trypsin-EDTA solution (Sigma) containing 15 kU/ml of DNase (Washington Biochemical, Lakewood, NJ) at 37°C for 10 min. Enzyme reactions were stopped with 50% horse serum. The brain tissues were washed with PBS, and cells in the tissues were dissociated by pipetting several times. After brief centrifugation, the resulting cell pellets were subjected to cholesterol and protein assays. The obtained cholesterol levels were normalized to protein levels.

Immunoprecipitation

Nontransfected IMR-32 and APP_{SW}-transfected HEL293 cells were homogenized by sonication in 500 μl of 1% Triton X-100/Tris-buffered saline (100 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing protease inhibitor cocktail P8340 (Sigma). Cell lysates were immunoprecipitated with an antibody to ABCA1 (AB.H10), or APP (C40), and Protein G Sepharose (Pharmacia, Piscataway, NJ) at 4°C overnight. As a