decreased the secretion of aggregable Aβ42 *in vitro* and *in vivo* without affecting the total Aβ levels (Weggen et al. 2001; Takahashi et al. 2003). Importantly, this class of compounds also affected the cleavage sites by γ-secretase in Notch without apparent inhibition of Notch signaling, suggesting that these compounds modulate only the scissile bond position within TMD of the substrate (Okochi et al. 2006). Thus, it may be possible to avoid the envisaged adverse effects of γ-secretase inhibition by developing Notch-sparing γ-secretase inhibitors and γ-secretase modulators.

Elucidation of the mechanism by which these γ-secretase inhibitors and modulators regulate γ-secretase activity awaits further investigation. Recently, however, chemical biological approach is emerging for the understanding of the mode of action of inhibitors. In fact, the affinity-based labeling and isolation experiments using transition-state inhibitors revealed that PS complex represents the long-sought γ-secretase (Li et al. 2000b; Esler et al. 2000). We recently developed the novel γ-secretase inhibitor-based chemical probes that carry photoaffinity (i.e., benzophenone) and biotin moieties for crosslinking and purification, respectively (Morohashi et al. 2006; Fuwa et al. 2007). These photoprobes enable us to directly identify the molecular target of the small compounds by irradiation experiment.

Using this technique, we unequivocally identified that dipeptidic inhibitors such as DAPT, compound E and DBZ targeted directly to PS fragments, the catalytic subunit of γ-secretase. Importantly, DAPT, the γ-secretase specific inhibitor, bound to PS CTF, while compound E and DBZ that have cross-inhibitory potency against other family protease (i.e., signal peptide peptidase) targeted to PS NTF. These results provide molecular information of the specificity of γ-secretase inhibitors. Identification of the molecular target of the sulfonamide-based Notch-sparing γ-secretase inhibitor is currently underway (Fuwa et al. 2006). Moreover, structural analyses using fluorescence resonance energy transfer and SCAM identified that γ-secretase modulators caused conformational changes in PS polypeptides (Lleo et al. 2004; Isoo et al. 2007), suggesting that the structural modulation of the γ-secretase is the target molecular mechanism for therapeutic intervention. Further attempts to define the molecular mechanism of the compounds and to screen its derivatives specifically relevant to APP cleavage will facilitate not only development of novel therapeutic drug for AD, but also understanding of the unusual intramembrane proteolytic activity.

Conclusion

During the past two decades, the molecular details for the pathogenesis of AD have become clear enough to enable us to develop therapeutic strategies. Genetic and biochemical studies strongly implicate Aβ as a key molecule in the etiology of AD, suggesting that interfering with the production of Aβ could be a promising therapeutic strategy. γ-Secretase is an unusual enzyme that cleaves scissile bond within TMD and one of the plausible molecular targets for AD treatment. Moreover, discovery of several γ-secretase inhibitors and application for biological studies dramatically enhanced our understanding of the enzymatic characteristics of γ-secretase. Selective inhibition of APP cleavage remains to be a critical issue because γ-secretase activity is important for other signaling events. Nonetheless, certain compounds that specifically reduce APP cleavage are recently emerging. Further extensive efforts in both academic and pharmaceutical laboratories may raise high hopes for the promising therapeutics for AD.

Acknowledgments I am grateful to Dr. Takeshi Iwatsubo (The University of Tokyo) and all colleagues who collaborated in the studies cited in this review.

References

Barten DM, Guss VL, Corsa JA, Loo A, Hansel SB, Zheng M, Munoz B, Srinivasan K, Wang B, Robertson BJ, Polson CT, Wang J, Roberts SB, Hendrick JP, Anderson JJ, Loy JK, Denton R, Verdoorn TA, Smith DW, Felsenstein KM (2005) Dynamics of β-amyloid reductions in brain, cerebrospinal fluid, and plasma of β-amyloid precursor protein transgenic mice treated with a γ-secretase inhibitor. J Pharmacol Exp Ther 312:635–643

Borchelt DR, Thinakaran G, Eckman CB, Lee MK, Davenport F, Ratovitsky T, Prada CM, Kim G, Seekins S, Yager D, Slunt HH, Wang R, Seeger M, Levey AI, Gandy SE, Copeland NG, Jenkins NA, Price DL, Younkin SG, Sisodia SS (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ1-42/1-40 ratio *in vitro* and *in vivo*. Neuron 17:1005–1013

Chen F, Hasegawa H, Schmitt-Ulms G, Kawarai T, Bohm C, Katayama T, Gu Y, Sanjo N, Glista M, Rogaeva E, Wakutani Y, Pardossi-Piquard R, Ruan X, Tandon A, Checler F, Marambaud P, Hansen K, Westaway D, St George-Hyslop P, Fraser P (2006) TMP21 is a presenilin complex component that modulates γ-secretase but not ε-secretase activity. Nature 440:1208–1212

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature 391:387–390

Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C (2003) Reconstitution of γ-secretase activity. Nat Cell Biol 5:486–488

Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, Tsai JY, Rahmati T, Xia W, Selkoe DJ, Wolfe MS (2000) Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. Nat Cell Biol 2:428–434

Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, Apfeld J, Nicoll M, Maxwell M, Hai B, Ellis MC, Parks AL, Xu W, Li J, Gurney M, Myers RL, Himes CS, Hiebsch R, Ruble C, Nye JS, Curtis D (2002) *aph-1* and *pen-2* are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. Dev Cell 3:85–97

Fuwa H, Hiromoto K, Takahashi Y, Yokoshima S, Kan T, Fukuyama T, Iwatsubo T, Tomita T, Natsugari H (2006) Synthesis of biotinylated photoaffinity probes based on arylsulfonamide γ-secretase inhibitors. Bioorg Med Chem Lett 16:4184–4189

Fuwa H, Takahashi Y, Konno Y, Watanabe N, Miyashita H, Sasaki M, Natsugari H, Kan T, Fukuyama T, Tomita T, Iwatsubo T (2007) Divergent synthesis of multifunctional molecular probes to elucidate the enzyme specificity of dipeptidic γ-secretase inhibitors. ACS Chem Biol 2:408–418

Goutte C, Tsunozaki M, Hale VA, Priess JR (2002) APH-1 is a multipass membrane protein essential for the Notch signaling pathway in *Caenorhabditis elegans* embryos. Proc Natl Acad Sci USA 99:775–779

Hayashi I, Urano Y, Fukuda R, Isoo N, Kodama T, Hamakubo T, Tomita T, Iwatsubo T (2004) Selective reconstitution and recovery of functional γ-secretase complex on budded baculovirus particles. J Biol Chem 279:38040–38046

Herreman A, Serneels L, Annaert W, Collen D, Schoonjans L, De Strooper B (2000) Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells. Nat Cell Biol 2: 461–462

Ilagan MX, Kopan R (2007) SnapShot: notch signaling pathway. Cell 128:1246

Isoo N, Sato C, Miyashita H, Shinohara M, Takasugi N, Morohashi Y, Tsuji S, Tomita T, Iwatsubo T (2007) Aβ42 overproduction associated with structural changes in the catalytic pore of γ-secretase: common effects of Pen-2 N-terminal elongation and fenofibrate. J Biol Chem 282:12388–12396

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of Aβ 42(43) and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ 42(43). Neuron 13:45–53

Kopan R, Ilagan MX (2004) γ-Secretase: proteasome of the membrane? Nat Rev Mol Cell Biol 5:499–504

Lazarov VK, Fraering PC, Ye W, Wolfe MS, Selkoe DJ, Li H (2006) Electron microscopic structure of purified, active γ-secretase reveals an aqueous intramembrane chamber and two pores. Proc Natl Acad Sci USA 103:6889–6894

Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000a) Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state. Proc Natl Acad Sci USA 97:6138–6143

Li YM, Xu M, Lai MT, Huang Q, Castro JL, DiMuzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvelil JG, Register RB, Sardana MK, Shearman MS, Smith AL, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000b) Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. Nature 405:689–694

Lleo A, Berezovska O, Herl L, Raju S, Deng A, Bacskai BJ, Frosch MP, Irizarry M, Hyman BT (2004) Nonsteroidal anti-inflammatory drugs lower Aβ42 and change presenilin 1 conformation. Nat Med 10:1065–1066

Morohashi Y, Kan T, Tominari Y, Fuwa H, Okamura Y, Watanabe N, Sato C, Natsugari H, Fukuyama T, Iwatsubo T, Tomita T (2006) C-terminal fragment of presenilin is the molecular target of a dipeptidic γ-secretase-specific inhibitor DAPT. J Biol Chem 281:14670–14676

Netzer WJ, Dou F, Cai D, Veach D, Jean S, Li Y, Bornmann WG, Clarkson B, Xu H, Greengard P (2003) Gleevec inhibits β-amyloid production but not Notch cleavage. Proc Natl Acad Sci USA 100:12444–12449

Niimura M, Isoo N, Takasugi N, Tsuruoka M, Ui-Tei K, Saigo K, Morohashi Y, Tomita T, Iwatsubo T (2005) Aph-1 contributes to the stabilization and trafficking of the γ-secretase complex through mechanisms involving intermolecular and intramolecular interactions. J Biol Chem 280:12967–12975

Ogura T, Mio K, Hayashi I, Miyashita H, Fukuda R, Kopan R, Kodama T, Hamakubo T, Iwatsubo T, Tomita T, Sato C (2006) Three-dimensional structure of the γ-secretase complex. Biochem Biophys Res Commun 343:525–534

Okochi M, Fukumori A, Jiang J, Itoh N, Kimura R, Steiner H, Haass C, Tagami S, Takeda M (2006) Secretion of the Notch-1 Aβ-like peptide during Notch signaling. J Biol Chem 281:7890–7898

Parks AL, Curtis D (2007) Presenilin diversifies its portfolio. Trends Genet 23:140–150

Petit A, Bihel F, Alves da Costa C, Pourquie O, Checler F, Kraus JL (2001) New protease inhibitors prevent γ-secretase-mediated production of Aβ40/42 without affecting Notch cleavage. Nat Cell Biol 3:507–511

Sato C, Morohashi Y, Tomita T, Iwatsubo T (2006) Structure of the catalytic pore of γ-secretase probed by the accessibility of substituted cysteines. J Neurosci 26:12081–12088

Schmidt B, Baumann S, Braun HA, Larbig G (2006) Inhibitors and modulators of β- and γ-secretase. Curr Top Med Chem 6:377–392

Searfoss GH, Jordan WH, Calligaro DO, Galbreath EJ, Schirtzinger LM, Berridge BR, Gao H, Higgins MA, May PC, Ryan TP (2003) Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional γ-secretase inhibitor. J Biol Chem 278:46107–46116

Shah S, Lee SF, Tabuchi K, Hao YH, Yu C, LaPlant Q, Ball H, Dann CE 3rd, Sudhof T, Yu G (2005) Nicastrin functions as a γ-secretase-substrate receptor. Cell 122:435–447

Siemers E, Skinner M, Dean RA, Gonzales C, Satterwhite J, Farlow M, Ness D, May PC (2005) Safety, tolerability, and changes in amyloid β concentrations after administration of a γ-secretase inhibitor in volunteers. Clin Neuropharmacol 28:126–132

Siemers ER, Quinn JF, Kaye J, Farlow MR, Porsteinsson A, Tariot P, Zoulnouni P, Galvin JE, Holtzman DM, Knopman DS, Satterwhite J, Gonzales C, Dean RA, May PC (2006) Effects of a γ-secretase inhibitor in a randomized study of patients with Alzheimer disease. Neurology 66:602–604

Steiner H, Than M, Bode W, Haass C (2006) Pore-forming scissors? A first structural glimpse of γ-secretase. Trends Biochem Sci 31:491–493

Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T, Iwatsubo T (2003) Sulindac sulfide is a noncompetitive γ-secretase inhibitor that preferentially reduces Aβ42 generation. J Biol Chem 278:18664–18670

Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T (2003) The role of presenilin cofactors in the γ-secretase complex. Nature 422:438–441

Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives *in vivo*. Neuron 17:181–190

Tolia A, Chavez-Gutierrez L, De Strooper B (2006) Contribution of presenilin transmembrane domains 6 and 7 to a water-containing cavity in the γ-secretase complex. J Biol Chem 281:27633–27642

Tomita T, Iwatsubo T (2006) γ-Secretase as a therapeutic target for treatment of Alzheimer's disease. Curr Pharm Des 12:661–670

Tomita T, Maruyama K, Saido TC, Kume H, Shinozaki K, Tokuhiro S, Capell A, Walter J, Grunberg J, Haass C, Iwatsubo T, Obata K (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. Proc Natl Acad Sci USA 94:2025–2030

Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity. Nature 414:212–216

Wolfe MS (2006) The γ-Secretase complex: membrane-embedded proteolytic ensemble. Biochemistry 45:7931–7939

Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. Nature 398:513–517

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, Yu H, Yang DS, Holmes E, Milman P, Liang Y, Zhang DM, Xu DH, Sato C, Rogaev E, Smith M, Janus C, Zhang Y, Aebersold R, Farrer LS, Sorbi S, Bruni A, Fraser P, St George-Hyslop P (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. Nature 407:48–54

Zhou S, Zhou H, Walian PJ, Jap BK (2005) CD147 is a regulatory subunit of the γ-secretase complex in Alzheimer's disease amyloid β-peptide production. Proc Natl Acad Sci USA 102:7499–7504

疾患と神経新生

アルツハイマー病

松原悦朗　柳澤勝彦

Clinical Neuroscience 別冊

Vol. 26 No. 8　2008年8月1日発行

中外医学社

アルツハイマー病

松原悦朗　柳澤勝彦

はじめに

アルツハイマー病は，細胞内外への線維性構造物の蓄積を認める，いわゆる“原因蛋白の蓄積病”をその基本病態とする．このアルツハイマー病の病理過程でみられる最も早期の変化は細胞外に認められるβアミロイド(Aβ)を主要構成成分とする斑状の嗜銀性構造物(老人斑)である．アルツハイマー病患者脳においては，この本来可溶型の生理的Aβの産生・分解・クリアランスの代謝機構が何らかの原因で破綻し，不溶性の高まった病的Aβが脳実質に蓄積し，老人斑としての脳アミロイド沈着を形成すると考えられている．しかし最近，老人斑Aβアミロイド線維自体の発症病態への関与は低く，アミロイド線維形成して沈着・蓄積する前の中間分子(Aβオリゴマー)こそがその本態(いわゆるシナプス機能障害を引きおこす病態惹起性神経毒性分子)で，アルツハイマー病の治療標的であることが認識されてきている．こうしたAβオリゴマーの蓄積が引き金となり，二次的に神経細胞内にタウを主要構成成分とする嗜銀性構造物(神経原線維変化)蓄積をきたす神経変性(タウオパチー)・神経細胞死が引きおこされアルツハイマー病の病像が完成すると考えられている(アミロイドカスケード仮説)が，in vivoにおいてその直接的連関を示す証拠は現時点でまだ得られていない．神経変性疾患の代表格であるアルツハイマー病において，その中枢神経系の神経細胞は非分裂細胞であるため，傷害を受けた脳を再生させることは全くの夢物語と考えられ，本特集のトッピックスである神経新生の問題はほとんど蚊帳の外であった．ところが，哺乳類の成体脳における神経新生が海馬[1]や側脳室周囲[2]の限られた部位でおこっていることが確認され，実際に海馬での神経新生は，学習や記憶など成体脳の可塑性に重要な機能を有していること[3,4]，アルツハイマー病のシナプス機能障害の発症分子基盤がAβオリゴマーであるとの知見から，シナプス機能における神経回路の観点から両者の接点が見えてきた．本稿においては，このアミロイドカスケード仮説における神経新生の病態形成への関与や，神経新生を標的とした再生医療の試みにつき，これまで報告されている現況を整理しつつ概説したい．

まつばら えつろう　国立長寿医療センター研究所/アルツハイマー病研究部室長
やなぎさわ かつひこ　国立長寿医療センター研究所副所長

アルツハイマー病患者脳での神経新生

アルツハイマー病の報告後100年以上が経過したが，アルツハイマー病患者脳における神経新生の報告はわずか2報に留まっている．最初の報告は2004年Jinら[5]によってなされた．驚くことにアルツハイマー病患者脳海馬では神経細胞新生の程度が高まっていたのである．しかしながらアルツハイマー病の確固たる病理所見は神経細胞死であり，この神経新生亢進は結果的にこの神経細胞死を置換できるレベルには至っていない訳である．剖検脳の段階では神経細胞死が神経新生を圧倒している可能性，新生神経細胞が成熟し機能的な神経細胞に成り得ない可能性，アルツハイマー病患者脳の微少環境が新生神経細胞にとって毒性を持っている可能性が推測されていた．2008年，ようやくLiら[6]により，アルツハイマー病患者脳において，これらの推測の一つである海馬新生神経細胞の機能的にも成熟した神経細胞への分化が障害されていることが証明された．この結果は，海馬新生神経細胞が，傷害を受けたアルツハイマー病患者脳において，正常脳と同じような機能的な神経回路ネットワークを再構築することが如何に困難であるかを改めて示した例とも考えられると同時に，如何に新生神経細胞がおかれる微少環境が重要かを示している．

アルツハイマー病モデルマウス脳での神経新生

アルツハイマー病のモデルマウスの登場は，アルツハイマー病の病態解析や治療法開発の環境を一変させ，Aβオリゴマーがアルツハイマー病発症の分子基盤であることやAβワクチン療法などの根本治療法の可能性提示など，そのアルツハイマー病への貢献度は計り知れないものがある．ところが，アルツハイマー病モデルマウスにおいてこれまでなされた神経新生の検討では統一見解が得られていないのが現状である．新生神経細胞の増殖・生存・移動・

0289-0585/08/¥500/論文/JCLS

分化の見地からこれまで報告された結果を整理すると，神経新生亢進・低下・著変なしの如くその報告結果は混乱を招いている．アルツハイマー病患者脳での神経新生増加を報告したJinら[7]はモデルマウスにおいても同様の神経新生増加を報告している．逆に，Haugheyら[8]は新生神経細胞増殖抑制とアポトシーシス誘導による神経細胞死増加を，Dongら[9]は老人斑アミロイド出現以前からの新生神経細胞増殖抑制，またDonovanら[10]は老人斑アミロイド出現以後からの新生神経細胞増殖抑制を報告している．著変なしの報告はZhangら[11]によりなされているが，この報告ではAPP変異とPS1変異を共発現させたノックインマウスでは老人斑アミロイド形成に依存した新生神経細胞増殖抑制(神経芽細胞の形成障害)が認められるとしている．またVerretら[12]は比較的選択的な成熟神経細胞死を報告している．最近nestin enhancer制御下LacZ発現マウスとAPP発現マウスの掛け合わせ結果から，老人斑アミロイド形成により神経新生，特に神経移動・分化が亢進されるが，グリオーシスは影響を受けぬことが報告された[13]．以上の如く報告された知見のばらつきは，使用したモデル動物や神経新生評価法の相違に由来していると考えられるが，図らずともアルツハイマー病患者脳の検証からも示唆されたように，アルツハイマー病モデルマウス脳でも新生神経細胞がおかれる微少環境が果たす役割が十分検討されていないことにも由来していると考えられる．

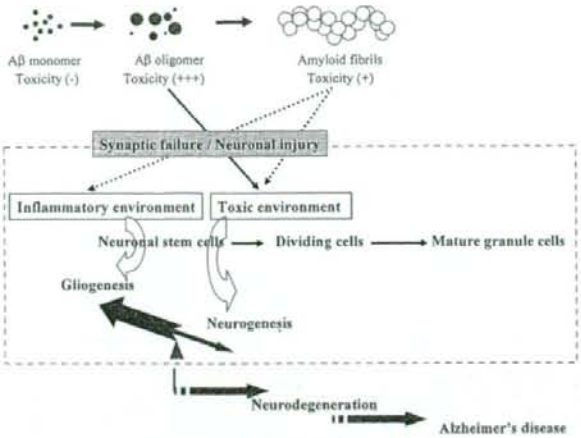
新生神経細胞がおかれる微少環境

では，神経新生を左右するアルツハイマー病の微少環境の特徴は何であろうか．アルツハイマー病患者脳に沈着する典型的老人斑周囲にはミクログリア活性化などの慢性炎症反応が認められ，多くの疫学調査が非ステロイド系抗炎症薬(NSAIDs)の長期服用でアルツハイマー病の発症が減ることを報告している．さらにアルツハイマー病患者脳ではミクログリア活性化に加え，IL6発現増強[14]も報告されている．またこうしたミクログリア活性化による炎症反応環境の存在がアルツハイマー病モデル動物でも示されている[15,16]．興味深いことに慢性炎症やミクログリア活性化が生体脳における海馬神経新生を抑制し，抗炎症薬(イブプロフェン)や抗IL6薬(ミノサイクリン)投与でその回復が図られることがわかっている[17,18]．一方，IL6にはグリオーシス，オリゴデンドロサイトーシスを促進させる作用が報告されている．従って，ミクログリア活性化された慢性炎症反応下では神経新生自体の抑制とアストログリオーシス[19]やオリゴデンドログリオーシス[20]への誘導による間接的な神経細胞新生が抑制される微少環境にあると考えられる．

アルツハイマー病患者脳においては前脳基底部におけるアセチルコリン合成酵素活性が低下しており，この補充目的に現在アセチルコリン分解酵素阻害薬が広く使用されているのは周知の如くである．アルツハイマー病患者脳やモデルマウス脳では成熟新生神経細胞への分化に重要なGABA作動性神経細胞は比較的保たれた環境にあるが，最近，グルタミン酸作動性神経細胞からなる腫大変性神経突起の存在が両脳で明らかとなった．従って，アルツハイマー病患者脳やモデルマウス脳では結果的にGABAやグルタミン酸の入力低下を招き，神経新生にとり抑制的な微少環境が形成されていると推測される[21]．

最近，孤発性アルツハイマー病患者脳においてBACE1活性が上昇していることが明らかとされ，相対的な分泌型APPの減少がアルツハイマー病の病態形成に重要である可能性が推測されている．前述したIL6と同様にこの分泌型APP自体に神経幹細胞からアストロサイトへの分化促進作用があることがin vivoとin vitroにて確認されており[22]，アルツハイマー病患者脳では，その相対的活性低下は神経新生にとり保護的な環境に貢献していると考えられる．

アルツハイマー病患者脳の発症基盤を担う本質的な分子変化はAβやAβオリゴマーの蓄積である．前述したアルツハイマー病モデルマウスやアルツハイマー病患者脳では統一的な見解は得られていないが，Aβ自体の直接的な神経新生への効果を検証したin vitro研究においてHaugheyら[23]は，① 脳室内投与したAβが成体マウス脳室下層の神経新生を障害すること，② Aβ自体が培養神経前駆細胞増殖・分化抑制とアポトーシス誘導による神経細胞死効果を持つことを報告した．逆にLópez-Toledanoら[24]はAβ，特にAβオリゴマー[25]が神経前駆細胞へ作用し神経細胞への分化を促進し神経細胞数を増加させることを報告した．Calafioreら[26]も脳室内投与したAβの成体マウス脳室下層の神経前駆細胞の神経細胞への分化促進効果を報告している．相反する結果でいずれが正しいのかの結論は得られていないが，Aβによる神経細胞分化誘導は成熟神経細胞まで至らぬ，仮に至ったとしてもその成熟神経細胞で神経毒性を示し死滅させてしまう，もしくはその神経回路に組み込みを障害するなど負の側面が大きいと推測される．いずれにしても，細胞死が優位な状況にあるアルツハイマー病では神経前駆細胞にとり有害な微少環境にある，もしくは成熟神経細胞にとり有害な微少環境が優位に立っている



神経新生とアルツハイマー病の病態

と考えられ，この微少環境の改善は神経新生の側面から極めて重要な問題と考えられる．

アルツハイマー病における再生医療の可能性

Magavi ら[27]は神経変性疾患における脳内在性の神経前駆細胞をその場で操作し，神経細胞への置換治療の可能性を報告している．Koketsu ら[28]は Magavi らが報告した障害後の特殊な条件下では大脳皮質でも神経細胞再生がおこるが，健常脳では大脳皮質における神経細胞新生は極めて稀であることを明らかとした．Nakatomi ら[29]は，一過性の脳虚血後認められる死滅海馬錐体細胞の，脳室内への神経成長因子投与による内在性神経幹細胞からの神経新生による海馬錐体細胞補充と，その機能回復が確認されている．以上の知見を総合し神経新生とアルツハイマー病における病態を整理すると，一過性でなく，慢性持続型の，どちらかというと神経新生にとり不利益な微少環境でのアルツハイマー病における神経新生の側面が浮き彫りとなる(図)．現時点においてはアルツハイマー病における再生医療への道程はまだまだ長いようである．

文　献

1) Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat : age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 ; 16 : 2027-33.
2) Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron. 1993 ; 11 : 173-89.
3) Wiskott L, Rasch MJ, Kempermann G. A functional hypothesis for adult hippocampal neurogenesis : avoidance of catastrophic interface in the dentate gyrus. Hippocampus. 2006 ; 16 : 329-43.
4) Lledo PM, Alonso M, Grubb MS. Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci. 2006 ; 7 : 179-93.
5) Jin K, Peel AL, Mao XO, et al. Increased hippocampal neurogenesis in Alzheimer's disease. Proc Natl Acad Sci USA. 2004 ; 101 : 343-7.
6) Li B, Yamamori H, Tatebayashi Y, et al. Failure of neuronal maturation in Alzheimer disease dentate gyrus. J Neuropathol Exp Neurol. 2008 ; 67 : 78-84.
7) Jin K, Galvan V, Xie L, et al. Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPsw, Ind) mice. Proc Natl Acad Sci USA. 2004 ; 101 : 13363-7.
8) Haughey NJ, Nath A, Chan SL, et al. Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. J Neurochem. 2002 ; 83 : 1509-24.
9) Dong H, Goico B, Martin M, et al. Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. Neuroscience. 2004 ; 127 : 601-9.
10) Donovan MH, Yazdani U, Norris RD, et al. Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. J Comp Neurol. 2006 ; 495 : 70-83.
11) Zhang C, McNeil E, Dressler L, et al. Long-lasting impairment in hippocampal neurogenesis associated with amyloid deposition in a knock-in mouse model of familial Alzheimer's disease. Exp Neurol. 2007 ; 204 : 77-87.
12) Verret L, Jankowsky JL, Xu GM, et al. Alzheimer's-type amyloidosis in transgenic mice impairs survival of newborn neurons derived from adult hippocampal neurogenesis. J Neurosci. 2007 ; 27 : 6771-80.
13) Gan Li, Qiao S, Lan W, et al. Neurogenic responses to amyloid-beta plaques in the brain of Alzheimer's disease-like transgenic (pPDGF-APPsw, Ind) mice. Neurobiol Dis. 2008 ; 29 : 71-80.
14) Lukiw WJ. Gene expression profiling in fetal, aged, and Alzheimer hippocampus : a continuum of stress-related signaling. Neurochem Res. 2004 ; 29 : 1287-97.
15) Benzing WC, Wujek JR, Ward EK, et al. Evidence for glial-mediated inflammation in aged APP (SW) transgenic mice. Neurobiol Aging. 1999 ; 20 : 581-9.
16) Matsuoka Y, Picciano M, Malester B, et al. Inflammatory responses to amyloidosis in a transgenic mouse model of Alzheimer's disease. Am J Pathol. 2001 ; 158 : 1345-54.
17) Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. Science. 2003 ; 302 : 1760-5.
18) Ekdahl CT, Claasen JH, Bonde S, et al. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci USA. 2003 ; 100 : 13622-37.
19) Van Wagoner NJ, Benveniste EN. Interleukin-6 expression and regulation in astrocytes. J Neuroimmunol. 1999 ; 100 : 124-39.
20) Valerio A, Ferrario M, Dreano M, et al. Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells. Mol Cell Neurosci. 2002 ; 21 : 602-15.
21) Bell KFS, Ducatenzeiler A, Riberio-da-Silva A, et al. The amyloid pathology progresses in a neurotransmitter-specific manner. Neurobiol Aging. 2006 ; 27 : 1644-57.
22) Kwak Y-D, Brannen T, Qu T, et al. Amyloid precursor protein regulates differentiation of human neural stem cells. Stem Cells Dev. 2006 ; 15 : 381-9.
23) Haughey NJ, Liu D, Nath A, et al. Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal precursor cells in culture, by amyloid beta-peptide : implications for the pathogenesis of Alzheimer's disease. Neuromolecular Med. 2002 ; 1 : 125-35.
24) López-Toledano MA, Shelanski ML. Neurogenic effect of beta-amyloid peptide in the development of neural stem cells. J Neurosci. 2004 ; 24 : 5439-44.
25) López-Toledano MA, Shelanski ML. Increased neurogenesis in young transgenic mice overexpressing human APP (Sw, Ind). J Alzheimers Dis. 2007 ; 12 : 229-40.
26) Calafiore M, Battalglia G, Zappalà A, et al. Progenitor cells from the adult mouse brain acquire a neuronal phenotype in response to β-amyloid. Neurobiol Aging. 2006 ; 27 : 606-13.
27) Magavi SS, Leavitt BR, Macklis JD. Induction of neurogenesis in the neocortex of adult mice. Nature. 2000 ; 405 : 951-5.
28) Koketsu D, Mikami A, Miyamoto Y, et al. Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. J Neurosci. 2003 ; 23 : 937-42.
29) Nakatomi H, Kuriu T, Okabe S, et al. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell. 2002 ; 110 : 429-44.

BRAIN RESEARCH 1219 (2008) 169–179

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Plasma antibodies to Aβ40 and Aβ42 in patients with Alzheimer's disease and normal controls

Wuhua Xu[a,b], Takeshi Kawarabayashi[c], Etsuro Matsubara[d], Kentaro Deguchi[a], Tetsuro Murakami[a], Yasuo Harigaya[e], Masaki Ikeda[f], Masakuni Amari[g], Ryozo Kuwano[h], Koji Abe[a], Mikio Shoji[c,*]

[a]Department of Neurology, Neuroscience, Biophysiological Science, Okayama University Graduate School of Medicine, Dentistry and Pharmacy, 2-5-1 Shikatacho, Okayama 700-8558, Japan
[b]Department of Neurology, Guangzhou Red Cross Hospital, Fourth Affiliated Hospital of Jinan University Medical College, Guangzhou 510220, China
[c]Department of Neurology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan
[d]Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan
[e]Department of Neurology, Maebashi Red Cross Hospital, 3-21-36, Asahimachi, Maebashi, Gunma 371-0014, Japan
[f]Department of Neurology, Gunma University School of Medicine, 3-39-22 Showamachi, Maebashi, Gunma 371-8511, Japan
[g]Department of Neurology, Geriatrics Research Hospital, 3-26-8 Otomomachi, Maebashi, Gunma 371-0847, Japan
[h]Department of Bioinformatics, Genome Science Branch, Center for Bioresource-based Researches, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata 951-8122, Japan

ARTICLE INFO

Article history:
Accepted 15 February 2008
Available online 4 March 2008

Keywords:
Amyloid
Aβ
Alzheimer's disease
Antibodies
Plasma

ABSTRACT

Antibodies to amyloid β protein (Aβ) are present naturally or after Aβ vaccine therapy in human plasma. To clarify their clinical role, we examined plasma samples from 113 patients with Alzheimer's disease (AD) and 205 normal controls using the tissue amyloid plaque immunoreactivity (TAPIR) assay. A high positive rate of TAPIR was revealed in AD (45.1%) and age-matched controls (41.2%), however, no significance was observed. No significant difference was observed in the MMS score or disease duration between TAPIR-positive and negative samples. TAPIR-positive plasma reacted with the Aβ40 monomer and dimer, and the Aβ42 monomer weakly, but not with the Aβ42 dimer. TAPIR was even detected in samples from young normal subjects and young Tg2576 transgenic mice. Although the Aβ40 level and Aβ40/42 ratio increased, and Aβ42 was significantly decreased in plasma from AD groups when compared to controls, no significant correlations were revealed between plasma Aβ levels and TAPIR grading. Thus an immune response to Aβ40 and immune tolerance to Aβ42 occurred naturally in humans without a close relationship to the Aβ burden in the brain. Clarification of the mechanism of the immune response to Aβ42 is necessary for realization of an immunotherapy for AD.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

AD brains are invariably characterized by two pathological features: initial Aβ amyloidosis characterized by extracellular deposition of Aβ42 (43) and Aβ40, and subsequent tauopathy characterized by intracellular accumulation of neurofibrillary tangles consisting of abnormally phosphorylated tau. Since familial AD-linked mutations of amyloid β protein precursor

* Corresponding author. Fax: +81 172 39 5143.
E-mail address: mshoji@cc.hirosaki-u.ac.jp (M. Shoji).

0006-8993/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2008.02.060

(βAPP), presenilin-1 (PS-1), and presenilin-2 (PS-2) increase the extracellular concentration of Aβ42 (43) and protofibril Aβ, these peptides are likely to be initiating factors in the pathogenesis of all types of AD (Hardy and Selkoe, 2002, Selkoe, 2002). Transgenic mouse models reproducing substantial brain Aβ amyloid support these hypotheses, and the appearance of neurofibrillary tangles (NFT) enhanced by Aβ amyloid in Tg2576 x tau P301L double transgenic mice further indicates that Aβ amyloidosis is the most important target for curing AD (Lewis et al., 2001).

Recent studies suggested that Aβ immunotherapy is the most promising among the many candidate therapies for AD. Schenk and others showed that an Aβ42 peptide vaccine clearly reduced the Aβ amyloid burden in transgenic model mice (Schenk et al., 1999; Weiner et al., 2000; Janus et al., 2000; Morgan et al., 2000). Passive immunization using anti-Aβ antibodies was also shown to be effective for reduction of the Aβ amyloid burden (Bard et al., 2000). These findings suggest peripheral antibodies to Aβ may serve a protective role against AD. A detectable increase in antibodies to Aβ42 was observed in about 25% of patients who received AN1792 in a Phase I study (Orgogozo et al., 2003; Nicoll et al., 2003). Analysis of serum samples by ELISA indicated that 15 of 18 patients experiencing meningoencephalitis in a Phase II study had antibodies against Aβ42. CSF antibodies to Aβ42 were present in 6 of 8 patients tested after the onset of encephalitis. However, titers of antibodies to Aβ42 were not correlated with the occurrence or severity of symptoms or relapses (Orgogozo et al., 2003). An autoantibody to Aβ40 was first detected in human B cell lines from AD patients (Gaskin et al., 1993). Naturally occurring antibodies to synthetic Aβ40 were confirmed by ELISA in the CSF and plasma of non-immunized humans and titers were significantly higher in healthy controls than in patients with AD (Du et al., 2001). Titers of anti-Aβ42 peptide antibodies were lower in AD patients compared with healthy individuals (Weksler et al., 2002), or elevated in AD patients and elder transgenic mice (Nath et al., 2003). Naturally occurring anti-Aβ42 antibodies were detected at very low levels by ELISA in over 50% of elderly individuals and at modest levels in 5% of them. Neither the presence nor the amount of naturally occurring anti-Aβ42 antibodies correlated with the presence, or age of AD onset, or the plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001). Normal levels of antibodies to Aβ42 and Aβ40 were present in both AD and control groups, even in a young population (Baril et al., 2004). Thus, the previous reports suggested complex relationships for naturally occurring antibodies to Aβ.

In the Zurich cohort of a Phase II study, patients who generated antibodies to β-amyloid plaques in the plasma as determined by tissue amyloid plaque immunoreactivity (TAPIR) assay showed significantly slower rates of decline for cognitive functions and daily living activities suggesting that antibodies against β-amyloid plaques may be protective against AD (Hock et al., 2002, 2003; Gilman et al., 2005; Bombois

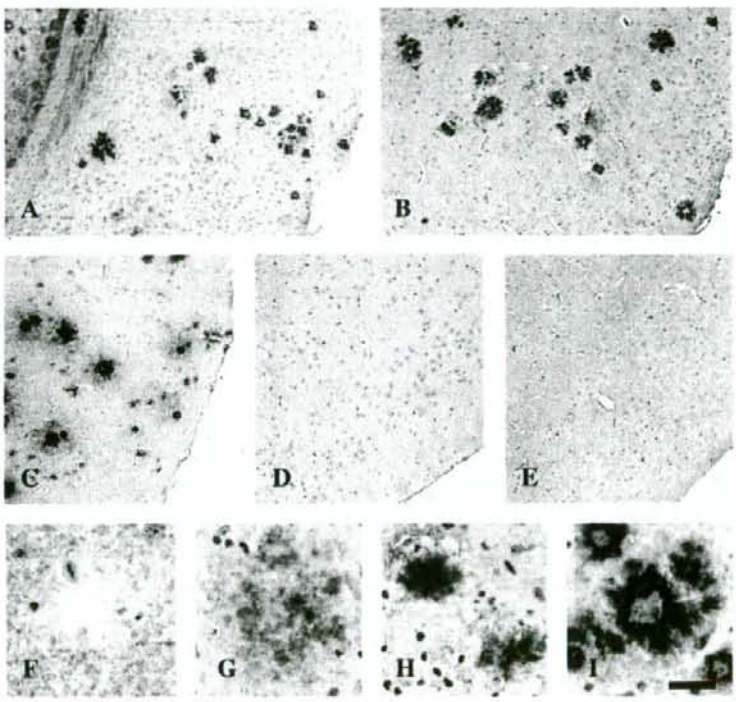


Fig. 1 – TAPIR using plasma and Tg2576 mouse brains. Many senile plaques in Tg2576 brains were labeled prominently in A (AD group, TAPIR grading ++) and B (aNC group, TAPIR grading ++). C: control Aβ immunostaining with Ab9204; no senile plaques were labeled in D (AD group, TAPIR grading –) and E (aNC group TAPIR grading –). F to I are examples of TAPIR grade. F: TAPIR –; G:±; H: +; I: ++. Scale bar=6.25 μm in A–E and 25 μm in F–I.

et al., 2007). Here, we examined 113 AD cases and 155 age-matched normal controls by TAPIR assay in order to clarify the positive rates, antibody characters, correlations with clinical symptoms, and clinical roles of naturally occurring antibodies against β-amyloid plaques. Modification of plasma Aβ40 and Aβ42 concentrations by antibodies to Aβ was also studied based on age- or AD-dependent alterations of plasma Aβ levels.

2. Results

2.1. *High positive TAPIR rate but no difference between AD and aNC groups*

Some plasma samples from AD and aNC groups strongly labeled nearly all amyloid plaque cores (Fig. 1A, B and I; grading ++) as did Ab9201 (Fig. 1C). Other plasma samples from both groups showed negative (Fig. 1D, E, F, grading –), weak (Fig. 1G, grading ±), or positive (Fig. 1H, grading +) labeling. The TAPIR staining was detected by anti-IgG second antibody, but not with anti-IgM or IgA antibodies (not shown), thus TAPIR-positive antibody was shown to be IgG antibody. The specificity of TAPIR-positive antibody was examined by immunoprecipitation of Aβ as described in 2.3. In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were grading +, and 7 (6.2%) were ++. Fifty one of 113 AD patients were ++ and +, suggesting frequent appearance (45.1%) of naturally occurring antibodies to amyloid plaque cores. In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++. Sixty-four cases of 155 aNC group (41.3%) were TAPIR ++ or +. No significant differences were detected by Mann–Whitney's *U* tests in the positive rates of naturally occurring antibodies to amyloid plaque cores among groups ($p=0.77$), or comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +) and negative aNC (± and –) group ($p=0.54$) (Table 1).

2.2. *TAPIR was not correlated with clinical symptoms*

There were no significant differences in gender or mean age in both AD and aNC groups (Table 1). No significant differences were observed in MMS scores and disease duration among the TAPIR –, ±, +, ++ subgroups of AD samples (Table 1 and Fig. 2A, B). There were also no significant differences in the progressive decline of MMS scores among these AD subgroups (Fig. 2C). The presence of naturally occurring antibodies to Aβ as detected by TAPIR may therefore not improve prognosis of AD.

2.3. *TAPIR-positive plasma recognized Aβ40 and FAβ, but Aβ42 very weakly*

As indicated in Fig. 3, freshly prepared Aβ40 and Aβ42 were composed of monomers and dimers. However, formic acid extractable Aβ (FAβ) exhibited polymerization as shown by the higher molecular mass of its oligomers (Fig. 3, left panel). Immunoprecipitation with TAPIR ++/+ plasma obtained from the AD and aNC groups retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected (Fig. 3 right panels). These findings suggest that TAPIR-positive plasma reacts with Aβ, but its reactivity to Aβ42 is very weak.

2.4. *Antibodies to Aβ appeared before Aβ amyloid deposits in the brain*

In order to clarify when these antibodies against Aβ appear, we additionally examined the remaining 50 plasma samples from subjects younger than 43 yeas old in the tNC group. Surprisingly, TAPIR revealed that antibodies to Aβ appeared in a 2 year-old child and also in some young subjects (TAPIR +; Fig. 4A, B and C). TAPIR-positive rates were 57% by 10 years old ($n=7$; 4 TAPIR +), 64% by 20 years old ($n=11$; 6 TAPIR +), 20% by 30 years old ($n=10$; 2 TAPIR +) and 10% by 40 years old ($n=10$; 1 TAPIR +). To confirm further this early appearance of antibodies to Aβ, immunoprecipitation was performed. Essentially identical finding to those seen in the AD and aNC groups were revealed (Fig. 4 D–F). Aβ40 and FAβ monomers and dimers were strongly immunoprecipitated (arrows). However, immunoprecipitation of the Aβ42 monomer was also weak and the Aβ42 dimer was absent in TAPIR-positive plasma from younger controls.

This was also the case in plasma obtained from Tg2576 model mice. Plasma from younger and older Tg2576 mice labeled

Table 1 – Summary of tissue amyloid plaque immunoreactivity (TAPIR) in AD and control groups

	Grade	Cases	rate (%)	Gender (M/F)	Mean age (SD), yr	Mean MMSE (SD)	Mean duration (SD), mo
AD ($n=113$)	–	42	37.2	11/31	75.4 (7.2)	14.7 (7.2)	50.9 (34.4)
	±	20	17.8	5/15	75.0 (8.0)	15.4 (7.3)	39.5 (27.4)
	+	44	38.9	14/30	74.5 (8.2)	14.9 (6.3)	37.5 (24.7)
	++	7	6.2	3/4	77.3 (5.3)	14.7 (6.2)	47.7 (19.7)
aNC ($n=155$)	–	54	34.8	21/33	74.7 (9.5)	29.9 (0.3)	–
	±	37	23.9	16/21	76.0 (8.7)	29.6 (0.5)	–
	+	44	28.4	19/25	77.9 (8.0)	29.7 (0.4)	–
	++	20	12.9	3/17	74.2 (11.8)	29.9 (0.3)	–

In the AD group, 42 cases (37.2%) were TAPIR –, 20 (17.8%) were ±, 44 (38.9%) were +, and 7 (6.2%) were ++; 51 of 113 AD patients were ++ and +, suggesting high rate of TAPIR (45.1%). In the aNC group, 54 cases (34.8%) were TAPIR –, 37 (23.9%) were ±, 44 (28.4%) were +, and 20 (12.9%) were ++; 64 of 155 aNC controls (41.3%) were TAPIR-positive. No significant differences were found in the positive TAPIR rate within each group ($p=0.77$), or in comparisons between the positive AD group (++ and +), negative AD group (± and –), positive aNC group (++ and +), and negative aNC (± and –) group ($p=0.54$). There were no significant differences in gender, mean age, mean MMS score or mean disease duration according to TAPIR grade in both AD and aNC groups. yr: years old; mo: months.

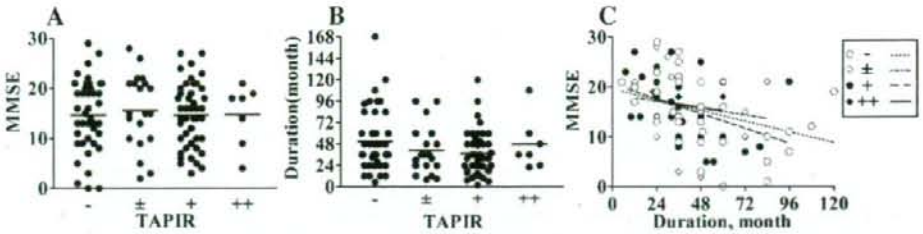


Fig. 2 – TAPIR was not correlated with clinical symptoms. There were no significant differences in MMS scores (A), disease duration (B) or decline of the clinical course of AD according to TAPIR grade. No significant difference in the decline of MMS scores according to duration was shown among AD subgroups (C). Y=−0.09X+19.54, r^2=0.19, p=0.01 in TAPIR − (○); Y=−0.06X+18.50, r^2=18.50, p=0.52 in TAPIR ± (◊); Y=0.12X+20.59, r^2=0.17, p=0.02 in TAPIR + (•); Y=−0.06X+18.63, r^2=0.04, p=0.72 in TAPIR ++ (•).

amyloid cores in AD brains (Fig. 4G–I). The appearance rate was 1/3 at 4 months old (1 TAPIR +), 3/3 at 8 months old (1 TAPIR ++ and 2 TAPIR +), 1/1 at 16 months old (1 TAPIR ++) and 1/1 at 23 months old mice (1 TAPIR +).

Finally, we summarized age-dependent TAPIR-positive rates (TAPIR grading + and ++) in 10 year increments in both AD and tNC groups (Fig. 4J). TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after 50. No differences were observed between AD and tNC samples from 50 to 91 years old. Thus, the appearance of antibodies to Aβ preceded Aβ amyloid deposition in human and model mouse brains.

2.5. *Levels of plasma Aβ40 and Aβ42 were age-dependently regulated in the tNC group*

To examine the effect of antibodies to Aβ on plasma Aβ concentrations, we measured levels of Aβ40 and Aβ42 in 318 plasma samples by specific ELISA. In the tNC group, plasma Aβ40 levels increased after 40 years of age (Fig. 5A; $p<0.0001$). On the contrary, plasma Aβ42 levels increased between the teens and twenties, then gradually declined with age (Fig. 5B; $p=0.0158$). The Aβ ratio (Aβ40/Aβ42) was stable until ~30 years old and then gradually increased (Fig. 5C; $p<0.0001$).

2.6. *Plasma Aβ ratio is increased in AD*

Significantly increased levels of plasma Aβ40 were observed in the AD group (112±39.51 pmol/L) compared to the aNC group (95.38±32.30; $p<0.0002$; Fig. 5D). Aβ42 levels were significantly decreased in the AD group (10.29±13.80 pmol/L) compared to the aNC group (12.13±12.29; $p<0.0001$; Fig. 5E). Based on these changes, the Aβ ratio (Aβ40/Aβ42) was more strongly increased in the AD group (14.42±10.00) than in the aNC group (8.34±3.83; $p<0.0001$; Fig. 5F). ROC analysis of the Aβ ratio indicated that the significant cut off value was 9.0, which provided high sensitivity (78.8%) and low specificity (30.3%) for clinical diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and the specificity was 96%. When AD was divided into 3 subgroups according to clinical stage, increasing Aβ40 levels and Aβ ratio, as well as decreasing Aβ42 levels progressed from the early stage to the advanced stage (Fig. 5G–I). Thus, the plasma Aβ ratio can be used as a specific biomarker for AD although the sensitivity and specificity are lower than those of CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002).

2.7. *TAPIR did not modify Aβ concentration*

Finally, we examined whether antibodies to Aβ could affect levels of plasma Aβ40 and Aβ42. There were no significant differences in the concentrations of plasma Aβ40 or Aβ42, or in the Aβ ratio among AD and aNC classified by TAPIR score (Fig. 6A–C).

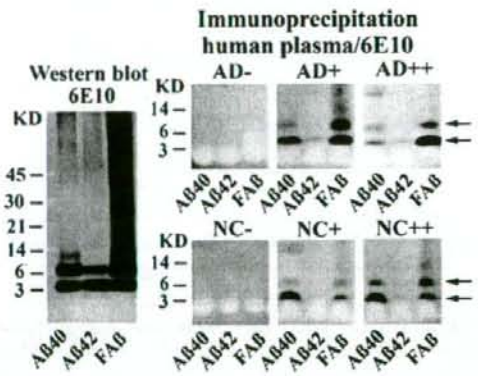


Fig. 3 – TAPIR-positive plasma immunoprecipitated Aβ40 and amyloid Aβ, but Aβ42 very weakly. On direct western blotting of synthetic Aβ40, Aβ42, and FAβ from the AD brain, antibody 6E10 detected monomers and dimers of Aβ40, Aβ42 and brain amyloid Aβ with smear aggregates (left panel). Immunoprecipitations of Aβ40, Aβ42, and FAβ using TAPIR −, +, and ++ plasma from the AD group (right upper panel, AD) or the aNC group (right lower panel, NC) were labeled by antibody 6E10, showing that monomers (arrow) and dimers (arrow) of Aβ40 were recognized by TAPIR-positive plasma (grading + and ++) in addition to Aβ42 monomers, and brain Aβ amyloid monomers and dimers with smear aggregates, which showed weak signals.

3. Discussion

In our study, a high positive rate of TAPIR was found in both AD (45.1%) and aNC (41.2%) groups, but no significant difference was found between these groups. Essentially the same findings were observed even in strongly positive (++) subgroups of AD (6.2%) and aNC (12.9%). Non-parametric analysis revealed that neither MMSE score nor disease duration correlated with TAPIR grade, indicating that the physiological impact of naturally occurring anti-Aβ antibodies is below clinical significance. This is consistent with previous reports describing frequent presence of low levels of antibodies to Aβ40 or Aβ42 peptides as detected by ELISA in plasma and CSF. A large scale study by Hyman et al. showed by ELISA that there were low and modest levels of anti-Aβ42 peptide antibodies in 52.3% and 4.7% of 365 plasma samples from AD and age-matched controls, respectively (Hyman et al., 2001). Neither the presence nor the amounts of anti-Aβ antibodies correlated with the likelihood of developing dementia or plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001; Orgogozo et al., 2003; Moir et al., 2005; Li et al., 2007). Our study indicated that TAPIR-

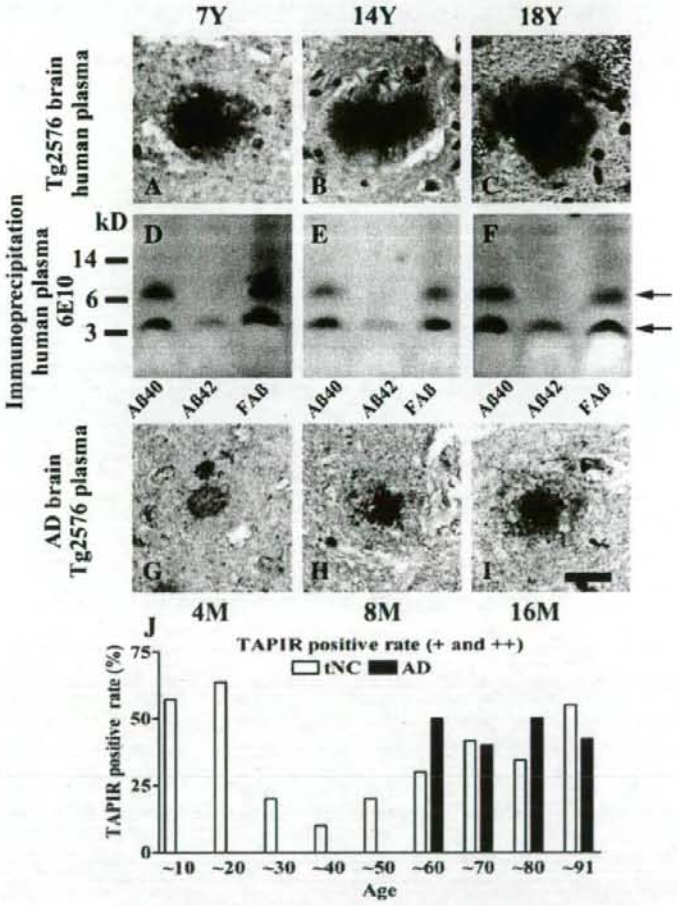


Fig. 4 – Antibodies to Aβ appeared before Aβ amyloid deposits in the brain. TAPIR was positive in 7 years old (TAPIR +; A, 7Y), 14 years old (TAPIR +; B, 14Y), and 18 years old young persons (TAPIR +, C, 18Y). TAPIR-positive plasma strongly immunoprecipitated monomers and dimers (arrow) of Aβ40 and FAβ, and weakly immunoprecipitated monomers of Aβ42 and Aβ amyloid (D, E and F; corresponding plasma of upper panels; D and A 7Y, E and B 14Y and F and C 18Y). Plasma from younger and older Tg2576 mice also labeled amyloid cores in AD brains (G: 4 months old TG; H: 8 months old Tg and I: 16 months old Tg). Bar scale = 15 μm. J: TAPIR-positive rates in the tNC group according to age. Columns show the TAPIR-positive rate (TAPIR grading + and ++) for 10 year increases in the AD (black columns) and tNC (white columns) groups. TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after age 50. No differences were observed between AD and tNC groups in samples from subjects 50 to 91 years old.

positive antibodies to Aβ amyloid plaques also occur naturally and frequently in human plasma and that their titers are not sufficient to prevent development of dementia. High titer of antibodies are necessary to improve the Aβ burden as shown in AD patients treated with an Aβ vaccine (Hock et al., 2002) or an anti-Aβ antibody infusion therapy (Dodel et al., 2002).

TAPIR is a new method to detect anti-Aβ antibodies (Hock et al., 2002, 2003). The fact that cognitive impairment was improved in patients who generated anti-Aβ antibodies after Aβ vaccination leads us to hypothesize that TAPIR-positive anti-Aβ antibodies are substantially different from naturally occurring anti-Aβ peptides antibodies in their specificity for Aβ40 and Aβ42 species or conformational epitopes of Aβ oligomers (Mirra et al., 1991; Kayed et al., 2003). Antibodies labeling Aβ amyloid plaques were more effective for the clearance of the Aβ burden of transgenic mice in passive immunization experiments (Bard et al., 2000). Direct action of the anti-Aβ antibody through the blood–brain barrier without T-cell proliferation as well as microglial clearance via the Fc or non-Fc portion of the antibodies mediated disruption of the plaque structure (Bard et al., 2000; Bacskai et al., 2002; Lombardo et al., 2003). Binding of an IgG2a antibody to the special N-terminal region of Aβ correlated with a clearance response (Bard et al., 2003). Injected antibodies may bind and sequestrate blood Aβ completely and disturb the balance between CSF Aβ and blood Aβ, leading to increased clearance from the brain into the blood (DeMattos et al., 2001; DeMattos et al., 2002). Clearance of diffusible Aβ oligomers that impair cognitive function was considered to be another target for passive immunization (Kayed et al., 2003). Recently a 56-kDa soluble amyloid-β assembly termed Aβ*56 has been shown to disrupt memory (Lesné et al., 2006), and Aβ oligomers have been shown to be increased in CSF from AD patients (Georganopoulou et al., 2005).

These reports all support the hypothesis that naturally occurring TAPIR-positive antibodies to Aβ recognize special Aβ species. Our immunoprecipitation study suggested that

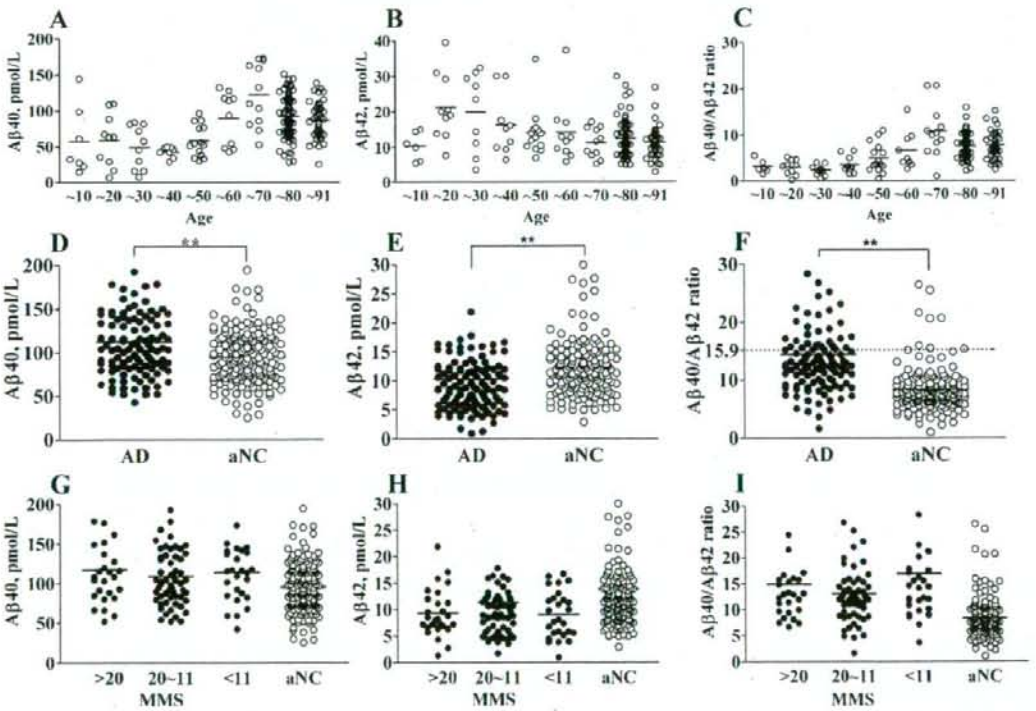


Fig. 5 – Age-dependent regulation of plasma Aβ levels in controls, and their alteration in AD. Plasma Aβ40 and Aβ42 levels showed different age-dependent alterations in the tNC group. Aβ40 levels increased from age 50 and decreased from age 70 (A). Aβ42 levels were high in the teens and twenties, then gradually decreased with age (B). Based on these different changes, the Aβ ratio (Aβ40/Aβ42) progressively increased from age 40 (C). Significantly increased levels of Aβ40 (D: $p=0.0002$) and increased Aβ ratio (F: $p<0.0001$) as well as decreased levels of Aβ42 (E: $p<0.0001$) were shown between the AD and aNC groups. When the mean +2SD of the Aβ ratio in the aNC group was used as a diagnostic marker for AD, the cut off value 15.9 (dot line) provided 24% sensitivity and 96% specificity (F). Constant alterations of plasma Aβ levels in AD were recognized at the early (MMS score >20), moderate (MMS score 20–11), and advanced stages (MMS score <11) (G–I). A, D, G: Aβ40; B, E, H: Aβ42; C, F, I: Aβ ratio. Bars show mean levels.

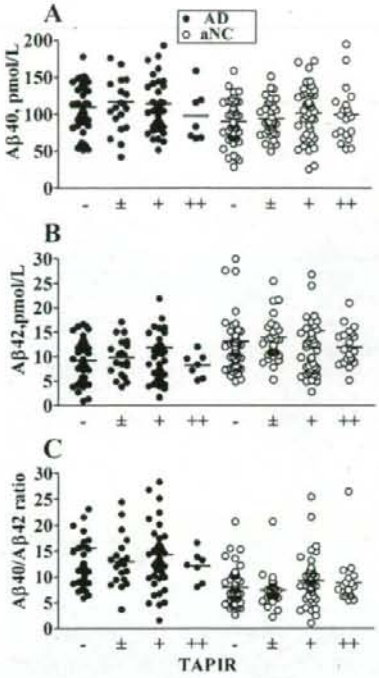


Fig. 6 – TAPIR did not modify Aβ concentration. No significant differences were found in Aβ40 and Aβ42 concentrations as well as Aβ ratios among all TAPIR grades (–, ±, + and ++) in AD (•) and aNC (○) group (A, B and C).

TAPIR ++/+ plasma obtained from AD and aNC subjects retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected under our testing conditions. The absence of anti-Aβ42 dimer antibodies and the relatively low levels of anti-Aβ42 monomers were characteristic of naturally occurring antibodies to Aβ. These findings are considered to be another reason why naturally occurring antibodies to Aβ are not sufficient for prevention of development of dementia.

Our TAPIR assay also showed that anti-Aβ antibodies were naturally present throughout the entire human life span. It is relevant to note that naturally occurring anti-Aβ antibodies were unequivocally detected in young human subjects as well as young Tg2576 mice. In relative terms, the positive rates of anti-Aβ antibodies were highest in young individuals, lowest in those middle-aged and higher in the elderly. The presence of anti-Aβ antibodies in young human subjects was characterized by the subsequent immunoprecipitation study. Anti-Aβ antibodies retrieved Aβ40 monomers and dimers as well as high molecular mass oligomers in FAβ fractions, but they retrieved fewer Aβ42 dimers. To our knowledge, this is the first report showing the relatively selective presence of anti-Aβ40 antibodies, and reduced amounts of anti-Aβ42 antibodies in young individuals. We also found that this was the case in normal elderly as well as AD patients, suggesting that the immune response to Aβ was unchanged in the two groups tested. Impaired spontaneous production of anti-Aβ42 antibodies also took place in elderly human subjects as well as AD patients. It is unknown why these antibodies to Aβ appeared more frequently in the young and the elderly populations and how specific immune tolerance for Aβ42 monomers and oligomers could be present. However, it should be noted that naturally occurring antibodies to Aβ appear in young human subjects and young Tg2576 mice, which do not develop an Aβ burden in their brain. The appearance of naturally occurring antibodies to Aβ is not correlated with the Aβ burden in the brain.

The exact mechanism underlying spontaneous anti-Aβ antibody production remains unknown. Although increased Aβ42 levels have been detected in transgenic animal models (Kawarabayashi et al., 2001), immune hyporesponsiveness to Aβ42 was also shown (Monsonego et al., 2001). Increased T-cell reactivity to Aβ42 was shown to increase in elderly individuals and patients with AD (Monsonego et al., 2003). However, the previous findings and our results could not show increased titers of anti-Aβ42 antibodies in these groups. Thus, hyopoimmunue responses to Aβ42, especially to the Aβ42 oligomer, actually occurred in AD and healthy populations. Since Aβ42 is highly pathogenic and neurotoxic, Aβ42 may be sequestered and spontaneous immune responses to Aβ may be suppressed in human populations. For effective immunotherapy as shown in transgenic mice studies and Aβ vaccine trials (Orgogozo et al., 2003; Hock et al., 2003), it is necessary to further generate antibodies to Aβ42 oligomers as well as monomers and monitor their titers. Furthermore, in order to prevent unexpected adverse reaction as seen in the Phase II trials of AN1792, detection of these spontaneous antibodies to Aβ will be necessary before treatment.

Recent studies have shown that plasma concentrations of Aβ40 and Aβ42 are possible biomarkers (Ertekin-Taner et al., 2000; Fukumoto et al., 2003; Mayeux et al., 1999, 2003; van Oijen et al., 2006; Graff-Radford et al., 2007) and can be used to monitor the effects of special treatments for AD (Dodel et al., 2002; DeMattos et al., 2001, 2002). After administration of an antibody to Aβ, the rapid increase in plasma Aβ was highly correlated with the amyloid burden in the brain (DeMattos et al., 2002), suggesting the possibility that naturally occurring anti-Aβ antibodies may cause increases the plasma Aβ concentration. In order to clarify this effect, we first analyzed age-dependent levels of plasma Aβ40 and Aβ42, and then examined alterations of Aβ40 and Aβ42 levels according to the presence or absence of AD and antibodies to Aβ. In the tNC group, plasma Aβ40 levels increased from age 40. Plasma Aβ42 levels increased between age 10 and 20, then gradually declined with age. The Aβ ratio (Aβ40/Aβ42) was stable until about 30 years and then gradually increased. These natural time courses were identical to those of CSF Aβ40 levels, but completely different from those of CSF Aβ42 levels. CSF levels of Aβ40 and Aβ42 showed U-shaped age-dependent curves, suggesting their correlation with brain development and decline (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). The correlation was prominent between the appearance of naturally occurring anti-Aβ antibodies and increased Aβ40 levels in

the CSF and plasma. Increased opportunities for immunological exposure to Aβ40 monomers and oligomers in immature or declining brains in young and elderly indivisuals may be sources for the naturally occurring immune response to Aβ40.

Based on these natural time courses of plasma Aβ concentrations, a comparison between AD and aNC groups was performed that provided intriguing findings. Significantly increased levels of plasma Aβ40, increased Aβ ratio and decreased levels of Aβ42 were revealed in the AD group when compared to the aNC group. Since a clear separation was obtained in the Aβ ratio between the AD and aNC groups, we evaluated the value of the Aβ ratio as a diagnostic or monitor maker of AD. ROC analysis indicated high sensitivity (78.8%) and low specificity (30.3%) for diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and specificity was 96%. When AD was divided into 3 groups according to clinical stage, the Aβ ratio increased progressively from the early stage to the advanced stages of AD. These findings show that plasma Aβ ratio can be used as an easy, non-invasive, and useful biomarker for diagnosis and monitoring of clinical symptoms of AD, although the sensitivity and specificity are lower than those in CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). However, naturally occurring antibodies to Aβ did not affect plasma Aβ40 or Aβ42 levels, or the Aβ ratio. There was a possibility that our ELISA system could not detect increased levels of Aβ40 and Aβ42 oligomers. However, all results taken together, suggest that the titer and specificity of naturally occurring anti-Aβ antibodies were not sufficient to elevate plasma Aβ concentrations and increase Aβ clearance from the brain to the peripheral blood with subsequent improvement of clinical symptoms. Higher titers of antibodies to Aβ42 oligomers will likely be necessary to facilitate Aβ clearance from brain amyloid to peripheral blood for AD treatment.

4. Experimental procedures

4.1. Patients and normal controls

After informed consent was given, blood samples were collected into 0.1% EDTA from a total of 318 subjects including 113 patients with AD (AD group) and 205 normal controls (total normal control group: tNC group). As age-matched controls (aNC group), 115 samples from subjects over 43 years old were selected from the tNC group. The basic findings for the respective groups are summarized in Table 2. The clinical diagnosis of AD was based on NINCDS–ADRDA criteria (McKhann et al., 1984). Appropriate diagnostic studies including magnetic resonance imaging and single photon emission computed tomography were used to exclude other disorders of dementia. The clinical severity of AD was evaluated using the Mini-Mental State Examination (MMS) (Folstein et al., 1975). AD patients were divided into 3 subgroups according to clinical stages: early stage MMS score >20, moderate stage MMS score 10–20, advanced stage MMS score <10. Controls were judged to be normal based on their MMS score (>28 points) and follow-up with neurological evaluation. After separation of plasma from blood cells, plasma was stored frozen at −80 °C until use.

Table 2 – Summary of the study subjects

	No. of subjects	Gender (M/F)	Mean age (range), yr	Mean MMS Score (SD)	Mean duration (SD), mo
AD	113	32/81	75 (55–89)	14.9 (6.7)	44 (28)
tNC	205	84/121	64 (1–91)	29.8 (0.3)	–
aNC	155	59/96	76 (43–91)	29.7 (0.4)	–
Total	318	116/202	68 (1–91)		

AD: Alzheimer's disease patients; tNC: total normal controls; aNC: age-matched controls over 43 years old selected from the tNC group; M/F: male and female; yr: years old; MMS: Mini-Mental State Examination; SD: standard deviation; Duration: duration from onset, mo months.

4.2. Tissue amyloid plaque immunoreactivity (TAPIR)

Five micrometers serial paraffin sections of brains from Tg2576 mice (16–18 months old) or Alzheimer's patients were used. Sections were immersed in 0.5% periodic acid for blocking intrinsic peroxidase and treated with 99% formic acid for 3 min to increase Aβ staining. Sections were then immersed with blocking solution with 5% normal serum in 50 mM phosphate-buffered saline (PBS) containing 0.05% Tween20 and 4% Block Ace (Snow Brand Milk Products, Saporo, Japan) for 1 h; goat serum was used to stain human plasma, and horse serum was used to stain mouse plasma. Sections were incubated at 4 °C overnight with human or mouse plasma diluted with blocking solution (1:100). Sections were then incubated with biotinyzed second antibody (anti-human goat antibody or anti-mouse horse antibody), and horseradish peroxidase-conjugated avidin–biotin complex of Vectastain Elite ABC kit (Vector, Burlingame, CA). Immunoreactivity was visualized by incubation with 0.03% 3, 3′-diaminobenzidine, and 0.02% H_2O_2. Tissue sections were counterstained with hematoxylin. Immunostaining with Ab9204 (Saido et al., 1995) (1:1000, antibody to a synthetic Aβ peptide starting from the amino-terminus of normal L-aspartate) or without the primary antibody were used as positive and negative controls, respectively.

4.3. Grading of TAPIR

TAPIR findings were classified into 4 levels: negative −, no senile plaque core (Fig. 1F); weakly positive ±, senile plaque cores were stained weakly and less than 5 cores were stained in each brain section on a slide (Fig. 1G); positive +, ≥5 senile plaque cores were stained clearly in at least one brain section per slide (Fig. 1H); strongly positive ++, most senile plaque cores were strongly labeled when compared to Ab9204 immunostaining (Fig. 1I). Immunostaining findings of diffuse plaques, amyloid angiopathy, positive neurons, degenerative neurites and glial cells were excluded from this grading.

4.4. Purification of amyloid Aβ (FAβ)

An autopsy brain fulfilling the CERAD criteria for definite AD (Mirra et al., 1991) was selected. About 2 g of gray matter of the AD brain was homogenized with 4 volumes of TBS (10 mM Tris, 150 mM NaCl, pH 8) with protease inhibitors (1 μg/ml

Leupeptin, 1 μg/ml TLCK, 0.1 μg/ml Pepstain A, 1 mM phenylmethysulfonyl fluoride and 1 mM EDTA), and centrifuged at 100,000 ×g for 1 h. The resulting pellet was extracted with 10 ml of 10% sodium dodecyl sulfate (SDS) in TBS and then with 1 ml of 99% formic acid (FA). The final supernatant was lyophilized, dissolved with 20 μl of 99% dimethylsulfoxide (DMSO), and stored at −80 °C until use (formic acid soluble amyloid Aβ fraction: FAβ) (Harigaya et al., 1995; Matsubara et al., 1999).

4.5. *Immunoprecipation*

20 μl of protein G agarose (Roche diagnostic GmbH, Germany) was washed 3 times with 1 ml RIPA buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% cholic acid and 150 mM NaCl, pH 8.0). Prewashed protein G agarose was mixed with 600 ng synthetic Aβ40, 600 ng synthetic Aβ42 (Sigma, Mo) or 300 ng FAβ in 1 ml of RIPA buffer and incubated at room temperature for 30 min. After centrifugation, the resulting supernatant was mixed again with 20 μl of prewashed protein G agarose and 10 μl of plasma, incubated at room temperature for 3 h, and then centrifuged. The pellet was boiled with 1× NuPage LDS sample buffer containing 0.1 M dithiothreitol for 10 min at 70 °C and separated on a 4 to 12% NuPage Bis–Tris gel (Invitrogen, CA). After electro-transfer, the blot membrane was blocked with 10% skim milk (Snow Brand Milk Products, Saporo, Japan) in TBS with 0.05% Tween 20 (TBST), and incubated with monoclonal 6E10 (specific to Aβ1-16, 1:1000, Signet Lab. Inc. MA) at 4 °C overnight. After washing and incubation with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:2000, Amersham Biosci, Buckinghamshire, UK) at RT for 2 h, the signal was developed by SuperSignal west Dura extended duration substrate (Pierce Biotechology, CA), and quantified by a luminoimage analyzer (LAS 1000-mini, Fuji film, Japan).

4.6. *Quantification of plasma Aβ40 and Aβ42 concentrations by ELISA*

Sandwich ELISA was used to specifically quantify whole plasma Aβ, as previously described (Matsubara et al., 1999). Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aβ11-28, specific Aβ11-16) and sequentially incubated with 100 μl of samples followed by horseradish-peroxidase-conjugated BA27 (anti-Aβ1-40, specific Aβ40) or BC05 (anti-Aβ35-43, specific Aβ42 and Aβ43) (Kawarabayashi et al., 2001). Synthetic Aβ40 (peptide content: 79.95%, Sigma, MO) and Aβ42 (peptide content:76.58%, Sigma, MO) were used for standard Aβ. The sensitivity was 40 fmol/ml in the Aβ40 assay and 10 fmol/ml in the Aβ42 assay. Both intra-assay coefficients of variation were less than 10% (Matsubara et al., 1999).

4.7. *Statistical analysis*

Comparisons among the groups using Student's *t*-test, one-way analysis of variance or a non-parametric test with post hoc tests, a receiver-operating characteristic (ROC) curve analysis to determine the cut off value, Mann–Whitney *U* test for appearance rates, and 1st order regression analysis of the relationship between MMS score and AD duration were all performed using SPSS 11.0 (SSPS Inc., IL) and GraphPad Prism, Version 4 (GraphPad Software, San Diego, CA).

Acknowledgments

We thank Yasuko Nogami and Hisae Narihiro for technical assistance, and Dr. TC Saido for kind gift of Ab9204. All animal experiments were performed according to guidelines established in the "Guide for the Care and Use of Laboratory Animals". This study was performed with permission of the ethical committee of Okayama University. Supported by a Grant-in-Aid from the Grants-in-Aid for Primary Amyloidosis Research Committee (M.S.), of the Ministry of Health, Labor and Welfare of Japan and by Grants-in-Aid for Scientific Research (B) (M.S.: 19390233, K.A.: 18390257), (C) (M.I.: 19590980, T.K.: 19590976, A.S.: 18500276, E.M.: 18590968), from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

Bacskai, B.J., Kajdasz, S.T., McLellan, M.E., Games, D., Seubert, P., Schenk, D., Hyman, B.T., 2002. Non-Fc-mediated mechanisms are involved in clearance of amyloid-β in vivo by immunotherapy. J. Neurosci. 22, 7873–7878.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T, Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D., Yednock, T., 2000. Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat. Med. 6, 916–919.

Bard, F., Barbour, R., Cannon, C., Carretto, R., Fox, M., Games, D., Guido, T., Hoenow, K., Hu, K., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, C., Lee, M., Motter, R., Nguyen, M., Reed, A., Schenk, D., Tang, P., Vasquez, N., Seubert, P., Yednock, T., 2003. Epitope and isotype specificities of antibodies to β-amyloid peptide for protection against Alzheimer's disease-like neuropathology. Proc. Natl. Acad. Sci. U. S. A. 100, 2023–2038.

Baril, L., Nicolas, L., Croisile, B., Crozier, P., Hessler, C., Sassolas, A., McCormick, J.B., Trannoy, E., 2004. Immune response to Aβ-peptides in peripheral blood from patients with Alzheimer's disease and control subjects. Neurosci. Lett. 355, 226–230.

Bombois, S., Maurage, C.A., Gompel, M., Deramecourt, V., Mackowiak-Cordoliani, M.A., Black, R.S., Lavielle, R., Delacourte, A., Pasquier, F., 2007. Absence of β-amyloid deposits after immunization in Alzheimer disease with Lewy body dementia. Arch. Neurol. 64, 583–587.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Dodart, J.C., Paul, S.M., Holtzman, D.M., 2001. Peripheral anti-Aβ antibody alters CNS and plasma Aβ clearance and decreases brain Aβ burden in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 98, 8850–8855.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M., Holtzman, D.M., 2002. Brain to plasma amyloid-β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. Science 295, 2264–2267.

Dodel, R., Hampel, H., Depboylu, C., Lin, S., Gao, F., Schock, S., Jackel, S., Wei, X., Buerger, K., Hoft, C., Hemmer, B., Moller, H.J., Farlow, M., Oertel, W.H., Sommer, N., Du, Y., 2002. Human antibodies against amyloid β peptide: a potential treatment for Alzheimer's disease. Ann. Neurol. 52, 253–256.

Du, Y., Dodel, R., Hampel, H., Buerger, K., Lin, S., Eastwood, B., Bales, K., Gao, F., Moeller, H.J., Oertel, W., Farlow, M., Paul, S.,

2001. Reduced levels of amyloid β-peptide antibody in Alzheimer disease. Neurology 57, 801–805.
Ertekin-Taner, N., Graff-Radford, N., Younkin, L.H., Eckman, C., Baker, M., Adamson, J., Ronald, J., Blangero, J., Hutton, M., Younkin, S.G., 2000. Linkage of plasma Aβ42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees. Science 290, 2303–2304.
Folstein, M.F., Folstein, S.E., McHugh, P.R., 1975. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J. Psychiatry Res. 12, 189–198.
Fukumoto, H., Tennis, M., Locascio, J.J., Hyman, B.T., Growdon, J.H., Irizarry, M.C., 2003. Age but not diagnosis is the main predictor of plasma amyloid β-protein levels. Arch. Neurol. 60, 958–964.
Gaskin, F., Finley, J., Fang, Q., Fu, S.M., 1993. Human antibodies reactive with β-amyloid protein in Alzheimer's disease. J. Exp. Med. 177, 1181–1186.
Georganopoulou, D.G., Chang, L., Nam, J.M., Thaxton, C.S., Mufson, E.J., Klein, W.L., Mirkin, C.A., 2005. Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 102, 2273–2276.
Gilman, S., Koller, M., Black, R.S., Jenkins, L., Griffith, S.G., Fox, N.C., Eisner, L., Kirby, L., Rovira, M.B., Forette, F., Orgogozo, J.M., AN1792(QS-21)-201 Study Team., 2005. Clinical effects of Aβ immunization (AN1792) in patients with AD in an interrupted trial. Neurology 64, 1553–1562.
Graff-Radford, N.R., Crook, J.E., Lucas, J., Boeve, B.F., Knopman, D.S., Ivnik, R.J., Smith, G.E., Younkin, L.H., Petersen, R.C., Younkin, S.G., 2007. Association of low plasma Aβ42/Aβ40 ratios with increased imminent risk for mild cognitive impairment and Alzheimer disease. Arch. Neurol. 64, 354–362.
Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353–356.
Harigaya, Y., Shoji, M., Kawarabayashi, T., Kanai, M., Nakamura, T., Iizuka, T., Igeta, Y., Saido, T.C., Sahara, N., Mori, H., Hirai, S., 1995. Modified amyloid β protein ending at 42 or 40 with different solubility accumulates in the brain of Alzheimer's disease. Biochem. Biophys. Res. Commun. 211, 1015–1022.
Hock, C., Konietzko, U., Papassotiropoulos, A., Wollmer, A., Streffer, J., von Rotz, R.C., Davey, G., Moritz, E., Nitsch, R.M., 2002. Generation of antibodies specific for β-amyloid by vaccination of patients with Alzheimer disease. Nat. Med. 8, 1270–1275.
Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., Wollmer, M.A., Umbricht, D., de Quervain, D.J., Hofmann, M., Maddalena, A., Papassotiropoulos, A., Nitsch, R.M., 2003. Antibodies against β-amyloid slow cognitive decline in Alzheimer's disease. Neuron 38, 547–554.
Hyman, B.T., Smith, C., Buldyrev, I., Whelan, C., Brown, H., Tang, M.X., Mayeux, R., 2001. Autoantibodies to amyloid-β and Alzheimer's disease. Ann. Neurol. 49, 808–810.
Janus, C., Pearson, J., McLaurin, J., Mathews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., Mount, H.T., Nixon, R.A., Mercken, M., Bergeron, C., Fraser, P.E., St George-Hyslop, P., Westaway, D., 2000. Aβ peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 408, 970–982.
Kanai, M., Matsubara, E., Isoe, K., Urakami, K., Nakashima, K., Arai, H., Sasaki, H., Abe, K., Iwatsubo, T., Kosaka, T., Watanabe, M., Tomidokoro, Y., Shizuka, M., Mizushima, K., Nakamura, T., Igeta, Y., Ikeda, Y., Amari, M., Kawarabayashi, T., Ishiguro, K., Harigaya, Y., Wakabayashi, K., Okamoto, K., Hirai, S., Shoji, M., 1998. Longitudinal study of cerebrospinal fluid levels of tau, Aβ1-40, and Aβ1-42(43) in Alzheimer's disease: a study in Japan. Ann. Neurol. 44, 17–26.
Kawarabayashi, T., Younkin, L.H., Saido, T.C., Shoji, M., Ashe, K.H., Younkin, S.G., 2001. Age-dependent changes in brain, CSF, and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J. Neurosci. 21, 372–381.
Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., Glabe, C.G., 2003. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 300, 486–489.
Lesné, S., Koh, M.T., Kotilinek, L., Kayed, R., Glabe, C.G., Yang, A., Gallagher, M., Ashe, K.H., 2006. A specific amyloid-β protein assembly in the brain impairs memory. Nature 440, 352–357.
Lewis, J., Dickson, D.W., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., McGowan, E., 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293, 1487–1491.
Li, Q., Gordon, M., Cao, C., Ugen, K.E., Morgan, D., 2007. Improvement of a low pH antigen-antibody dissociation procedure for ELISA measurement of circulating anti-Aβ antibodies. BMC Neurosci. 8, 22.
Lombardo, J.A., Stern, E.A., McLellan, M.E., Kajdasz, S.T., Hickey, G.A., Bacskai, B.J., Hyman, B.T., 2003. Amyloid-β antibody treatment leads to rapid normalization of plaque-induced neuritic alterations. J. Neurosci. 23, 10879–10883.
Matsubara, E., Ghiso, J., Frangione, B., Amari, M., Tomidokoro, Y., Ikeda, Y., Harigaya, Y., Okamoto, K., Shoji, M., 1999. Lipoprotein-free amyloidogenic peptides in plasma are elevated in patients with sporadic Alzheimer's disease and Down's syndrome. Ann. Neurol. 45, 537–541.
Mayeux, R., Tang, M.X., Jacobs, D.M., Manly, J., Bell, K., Merchant, C., Small, S.A., Stern, Y., Wisniewski, H.M., Mehta, P.D., 1999. Plasma amyloid β-peptide 1–42 and incipient Alzheimer's disease. Ann. Neurol. 46, 412–416.
Mayeux, R., Honig, L.S., Tang, M.X., Manly, J., Stern, Y., Schupf, N., Mehta, P.D., 2003. Plasma Aβ40 and Aβ42 and Alzheimer's disease: relation to age, mortality, and risk. Neurology 61, 1185–1190.
McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., Stadlan, E.M., 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34, 939–944.
Mirra, S.S., Heyman, A., McKeel, D., Sumi, S.M., Crain, B.J., Brownlee, L.M., Vogel, F.S., Hughes, J.P., van Belle, G., Berg, L., 1991. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41, 479–486.
Moir, R.D., Tseitlin, K.A., Soscia, S., Hyman, B.T., Irizarry, M.C., Tanzi, R.E., 2005. Autoantibodies to redox-modified oligomeric Aβ are attenuated in the plasma of Alzheimer's disease patients. J. Biol. Chem. 280, 17458–17463.
Monsonego, A., Maron, R., Zota, V., Selkoe, D.J., Weiner, H.L., 2001. Immune hyporesponsiveness to amyloid β-peptide in amyloid precursor protein transgenic mice: implications for the pathogenesis and treatment of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 98, 10273–10278.
Monsonego, A., Zota, V., Karni, A., Krieger, J.I., Bar-Or, A., Bitan, G., Budson, A.E., Sperling, R., Selkoe, D.J., Weiner, H.L., 2003. Increased T cell reactivity to amyloid β protein in older humans and patients with Alzheimer disease. J. Clin. Invest. 112, 415–422.
Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M., Arendash, G.W., 2000. Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature 408, 982–985.
Nath, A., Hall, E., Tuzova, M., Dobbs, M., Jons, M., Anderson, C., Woodward, J., Guo, Z., Fu, W., Kryscio, R., Wekstein, D., Smith, C., Markesbery, W.R., Mattson, M.P., 2003. Autoantibodies to amyloid β-peptide (Aβ) are increased in Alzheimer's disease patients and Aβ antibodies can enhance Aβ neurotoxicity:

implications for disease pathogenesis and vaccine development. Neuromolecular Med. 3, 29–39.
Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., Weller, R.O., 2003. Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. Nat. Med. 9, 448–452.
Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., Michel, B.F., Boada, M., Frank, A., Hock, C., 2003. Subacute meningoencephalitis in a subset of patients with AD after Aβ42 immunization. Neurology 61, 46–54.
Saido, T.C., Iwatsubo, T., Mann, D.M., Shimada, H., Ihara, Y., Kawashima, S., 1995. Dominant and differential deposition of distinct β-amyloid peptide species, Aβ N3(pE), in senile plaques. Neuron 14, 457–466.
Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D., Seubert, P., 1999. Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400, 173–177.
Selkoe, D.J., 2002. Deciphering the genesis and fate of amyloid β-protein yields novel therapies for Alzheimer disease. J. Clin. Invest. 110, 1375–1381.
Shoji, M., Kanai, M., Matsubara, E., Tomidokoro, Y., Shizuka, M., Ikeda, Y., Ikeda, M., Harigaya, Y., Okamoto, K., Hirai, S., 2001. The levels of cerebrospinal fluid Aβ40 and Aβ42(43) are regulated age-dependently. Neurobiol. Aging 22, 209–215.
Shoji, M., 2002. Cerebrospinal fluid Aβ40 and Aβ42: natural course and clinical usefulness. Front. Biosci. 7, d997–d1006.
van Oijen, M., Hofman, A., Soares, H.D., Koudstaal, P.J., Breteler, M.M., 2006. Plasma Aβ1-40 and Aβ1-42 and the risk of dementia: a prospective case-cohort study. Lancet Neurol. 5, 655–660.
Weiner, H.L., Lemere, C.A., Maron, R., Spooner, E.T., Grenfell, T.J., Mori, C., Issazadeh, S., Hancock, W.W., Selkoe, D.J., 2000. Nasal administration of amyloid-β peptide decreases cerebral amyloid burden in a mouse model of Alzheimer's disease. Ann. Neurol. 48, 567–579.
Weksler, M.E., Relkin, N., Turkenich, R., LaRusse, S., Zhou, L., Szabo, P., 2002. Patients with Alzheimer disease have lower levels of serum anti-amyloid peptide antibodies than healthy elderly individuals. Exp. Gerontol. 37, 943–948.

Molecular Brain

BioMed Central

Research

Open Access

Comprehensive behavioral phenotyping of calpastatin-knockout mice

Ryuichi Nakajima*[1,5], Keizo Takao[2,3,4], Shu-Ming Huang[1,6], Jiro Takano[1], Nobuhisa Iwata[1], Tsuyoshi Miyakawa[2,3,4] and Takaomi C Saido*[1]

Address: [1]Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan, [2]Genetic Engineering and Functional Genomics Group, Frontier Technology Center, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [3]Division of Systems Medicine, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan, [4]JST, CREST, Hon-chou, Kawaguchi, Saitama, Japan, [5]Current Address: Department of Physiology & Biophysics, Dalhousie University Faculty of Medicine, Halifax, Nova Scotia, Canada and [6]Current Address: Department of Neuroscience, Institute for Chinese Medicine, Heilongjiang University of Chinese Medicine, Harbin, PR China

Email: Ryuichi Nakajima* - RNakajima@dal.ca; Keizo Takao - keizo@behav.hmro.med.kyoto-u.ac.jp; Shu-Ming Huang - shuminghuang@hotmail.com; Jiro Takano - jtakano@brain.riken.jp; Nobuhisa Iwata - iwatan@brain.riken.jp; Tsuyoshi Miyakawa - miyakawa@fujita-hu.ac.jp; Takaomi C Saido* - saido@brain.riken.jp

* Corresponding authors

Published: 15 September 2008

Molecular Brain 2008, 1:7 doi:10.1186/1756-6606-1-7

Received: 8 August 2008
Accepted: 15 September 2008

This article is available from: http://www.molecularbrain.com/content/1/1/7

© 2008 Nakajima et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Calpastatin is an endogenous inhibitor of calpain, intracellular calcium-activated protease. It has been suggested to be involved in molecular mechanisms of long-term plasticity and excitotoxic pathways. However, functions of calpastatin in vivo are still largely unknown. To examine the physiological roles of calpastatin, we subjected calpastatin-knockout mice to a comprehensive behavioral test battery.

Results: Calpastatin-knockout mice showed decreased locomotor activity under stressful environments, and decreased acoustic startle response, but we observed no significant change in hippocampus-dependent memory function.

Conclusion: These results suggest that calpastatin is likely to be more closely associated with affective rather than cognitive aspects of brain function.

Background

Calpastatin (CS) is the endogenous inhibitor of intracellular cysteine protease calpain. CS inhibits the Ca^{2+}-activated form of calpain. In other words, calpain is bidirectionally regulated by Ca^{2+} and CS, and this is called the "calpain-CS system". CS inhibits two forms of calpain: μ-calpain (calpain I) and m-calpain (calpain II), which are activated by micromolar and millimolar Ca^{2+} in vitro, respectively [1].

The physiological roles of the calpain-CS system have not yet been well understood, though limited proteolysis by calpain is known to modify the functions of various substrates. Calpains are widely distributed in mammalian organs [2], and some important functions are already well known. For instance, the cyclin-dependent kinase 5 (Cdk5) activator, p35, is cleaved to p25 by calpain [3,4], and the generated p25 hyperactivates Cdk5, possibly leading to neurodegeneration. Another calpain-mediated neuronal death pathway involves the cleavage of Bid to generate tBid, resulting in DNA fragmentation [5]. The

levels of CS in most organs of normal animals are sufficient to inhibit calpain [2], so CS can inhibit these calpain cascades.

The calpain-CS system is hypothesized to be involved in molecular processes of long-term potentiation (LTP) [6,7], which is considered to contribute to the synaptic changes associated with learning and memory [8-11]. One of the major calpain substrates in neurons is fodrin (spectrin), a cytoskeltal molecule that contributes to the post-synaptic structure, and this degradation of fodrin is inhibited by CS. Therefore, it is possible that the calpain-CS system contributes to the learning and memory processes, and there are several experiments that are related to the contributions of calpain-CS system on memory [12,13]. However, the calpain-CS system's involvement in learning and memory processes remains controversial.

To investigate the physiological roles of CS, we have generated CS knockout (KO) mice. In a previous study, CS-KO mice showed increased spectrin proteolysis following kainate administration, which suggested increased activity of calpain in such pathological conditions [5]. We also found increased LTP in CS-KO mice in both the hippocampal CA1 and dentate gyrus regions (Huang and Saido, unpublished data), even though no significant difference in LTP was detected in μ-calpain KO mice [12]. However, Grammer et al. also found a decreased paired pulse ratio in μ-calpain KO mice, suggesting a presynaptic modulatory role of μ-calpain [12]. In this report, we have subjected CS-KO mice to a systematic and well-defined comprehensive behavioral test battery [14-16], to clarify the physiological roles of CS in behavior.

Results

Physical features

Home cage behaviors and general health conditions of both the genotype groups, WT (wild-type) and CS-KO, appeared normal. Body weight and body temperature were not significantly different between the genotypes ($F_{1,36} = 2.75$, $P = 0.106$ for body weight, $F_{1,36} = 0.320$, $P = 0.575$ for body temperature). The appearance of fur and whiskers were not significantly different between the genotypes (Table 1).

Neurological reflexes

Neurological reflexes were essentially normal in the CS-KO mice as compared with WT mice. Key jangling, whisker twitch response to a whisker touch from behind, and righting reflex were similar across genotypes (Table 1). Ear twitch responses tend to be decreased in CS-KO mice, but narrowly failed to achieve conventional measures of significance ($P = 0.0594$, *Student's* t-test).

Pain sensation and motor abilities

In the hot plate test, latency to the first paw response was not affected by the lack of calpastatin ($F_{1,36} = 1.93$, $P = 0.174$). Muscular abilities appeared normal in terms of the wire hanging test across genotypes ($F_{1,36} = 0.269$, $P = 0.607$) and the grip strength test ($F_{1,36} = 2.46$, $P = 0.126$). (Table 1)

Acoustic startle response and prepulse inhibition (sensorimotor gating)

CS-KO mice displayed a significantly lower acoustic startle response than WT mice (repeated measures ANOVA, $F_{1,36} = 4.98$, $P = 0.032$; Figure 1A). Analysis of variance

Table 1: General characteristics of CS-KO mice.

	WT	CS-KO
Number of animals	20	18
Physical characteristics		
- Weight (g)	26.2 (± 0.33)	27.1 (± 0.40)
- Body temperature (°C)	36.9 (± 0.20)	37.1 (± 0.18)
- Whiskers (% with)	100	89
- Fur (% with normal fur)	100	100
Sensory and motor reflexes		
- Ear twitch (% with normal response)	100	83
- Key jangling (% with normal response)	95	89
- Whisker twitch	100	94
- Righting reflex	100	100
Pain test		
- Hot plate (latency; seconds)	6.75 (± 0.349)	6.05 (± 0.364)
Motor tests		
- Wire hang (% stayed up to 60 s)	95	94
- Grip strength (N)	0.893 (± 0.03)	0.825 (± 0.03)

No significant differences between genotypes were detected in physical characteristics (weight, body temperature, whiskers and fur), sensory and motor reflex (ear twitch response, key-jangling response and righting reflex), hot plate test (latency to the first paw response), and muscular abilities (number of animals that stayed up to 60 sec in wire hang test and grip strength).