5C,D). Thus, apoptosis was induced in vivo in association with intraneuronal accumulation of Aβ oligomers. Collectively, these results indicate that intraneuronal Aβ oligomers caused cell death by inducing ER stress, endosomal/lysosomal leakage, and mitochondrial dysfunction in vivo.

DISCUSSION

We previously demonstrated that the APP Osaka mutation induces the accumulation of Aβ oligomers in the ER and subsequent ER stress in COS-7 cells (Nishitsuji et al., 2009). ER stress elicits the UPR, which includes three responses that protect the cell against toxic buildup of misfolded proteins (Yoshida, 2007): 1) up-regulation of molecular chaperones to assist in protein refolding, 2) halt of further protein synthesis, and 3) ubiquitination of misfolded proteins and their translocation to proteasomes to degrade them (i.e., ERAD). We previously detected the first two responses in APP_{OSK}-transfected cells, i.e., enhanced induction of the molecular chaperone Grp78 and increased phosphorylation (i.e., inactivation) of the translation initiation factor eIF2α (Nishitsuji et al., 2009). In the present study, we demonstrated the occurrence of the last response, ERAD, by showing up-regulation of an E3 ubiquitin ligase, HRD1, in COS-7 cells. Consistent with these in vitro observations, we also observed accumulation of Aβ oligomers in ER and up-regulation of Grp78 and HRD1 in APP_{OSK}-Tg mouse brain. The occurrence of ERAD indicates that Aβ oligomers are transported from the ER into the cytoplasm, which may be a source of mitochondrial Aβ.

In addition to the ubiquitin-proteasome system, autophagy also participates in clearance of misfolded intracellular proteins. Autophagosomes engulf damaged organelles and aggregated proteins in the cytoplasm and sort them to lysosomes. The sorted materials are subsequently digested within autolysosomes that are formed by fusion of autophagosomes and lysosomes. Thus, the autophagy-lysosome system compensates for the deficiency of the ubiquitin-proteasome system, the latter of which is reportedly caused by excessive accumulation of intracellular Aβ (Almeida et al., 2006; Park et al., 2009). We previously reported that autophagy was markedly induced in APP_{OSK}-transfected cells (Nishitsuji et al., 2009). In spite of these protective mechanisms, severe and prolonged ER stress ultimately induces apoptosis. ER stress-induced apoptosis is mediated by activation of caspase-12 in rodents (Nakagawa et al., 2000) and caspase-4 in humans (Hitomi et al., 2004). We previously detected activation of both caspase-3 and −4 and DNA fragmentation in APP_{OSK}-transfected cells (Nishitsuji et al., 2009). Here we presented further evidence of apoptosis in APP_{OSK}-transfected cells using the annexin V and propidium iodide markers. In accordance with these in vitro observations, we revealed the occurrence of apoptosis in APP_{OSK}-Tg mice at 18 months of age with increased levels of activated caspase-3. These findings suggest that the APP Osaka mutation causes severe ER stress by producing Aβ oligomers beyond the capacity of UPR/autophagy cell survival systems. We supposed that the observed ER stress and apoptosis were attributed to intracellular accumulation of Aβ oligomers, rather than other APP-derived fragments such as soluble APP and the C-terminal fragments, because the control APP_{WT}-Tg mice displayed only slight increases in Grp78, HRD1, and activated caspase-3, despite the fact that they expressed human APP twice as much as APP_{OSK}-Tg mice (Tomiyama et al., 2010). Previously, we demonstrated that APP_{OSK}-Tg mice showed apparent neuronal loss in the hippocampus (particularly the CA3 region) at 24 months of age (Tomiyama et al., 2010). The present study showed the induction of apoptosis in several hippocampal and cortical neurons of APP_{OSK}-Tg mice at 18 months of age. As speculated previously, intracellular Aβ oligomer-induced neuronal death may require a long period of time in vivo and may occur in neurons vulnerable to Aβ oligomers.

Accumulation of Aβ oligomers was also observed in the endosomes/lysosomes of both APP_{OSK}-transfected cells and APP_{OSK}-Tg mouse brain. Exogenously applied Aβ has been shown to disrupt endosomal/lysosomal membrane impermeability and cause the leakage of the contents into the cytoplasm (Yang et al., 1998; Ditaranto et al., 2001; Ji et al., 2002). The acidic environment within endosomal/lysosomal vesicles accelerates Aβ aggregation, and the leakage likely occurs during this process. Aβ oligomers may assemble with one another to form pore-like structures in the membrane (Quist et al., 2005; Singer and Dewji, 2006; Kayed et al., 2009), and Aβ fibrils may grow during crossing of the membrane into the cytoplasm (Friedrich et al., 2010); both of these mechanisms should break down membrane impermeability. The present study demonstrated that endogenously generated Aβ oligomers induced endosomal/lysosomal leakage in both APP_{OSK}-transfected cells and APP_{OSK}-Tg mouse brain. Endosomal/lysosomal leakage is known to affect cellular functions profoundly and to cause cell death (Yang et al., 1998; D'Andrea et al., 2001; Ditaranto et al., 2001; Ji et al., 2002; Friedrich et al., 2010) and may be another source of mitochondrial Aβ. There are three possible origins or pathways of endosomal/lysosomal Aβ: 1) Aβ is secreted from cells by the secretary pathway and subsequently taken up from the extracellular space by endocytosis, 2) Aβ is generated within the endosomes by local processing of APP that is internalized from the plasma membrane via endocytosis, and 3) Aβ is transported from other intracellular compartments by the autophagic pathway. In the present study, we demonstrated that the first pathway contributed to endosomal/lysosomal leakage. Nevertheless, we conclude that the third pathway may also play a crucial role in endosomal/lysosomal leakage, because excessive accumulation of intraneuronal Aβ has been shown to cause both impairment of the autophagy-lysosome system (Yu et al., 2005; Ling et al., 2009) and eventually autolysosomal leakage that leads to cell death (Ling et al., 2009).

We found in the present study that Aβ oligomers also accumulated in mitochondria in both APP_{OSK}-transfected cells and APP_{OSK}-Tg mouse brain. Mitochondria are not typically thought to be a site of Aβ generation; it is assumed that mitochondrial Aβ is transported from other intracellular compartments (Hansson Petersen et al., 2008; Reddy, 2009). The potential origin of mitochondrial Aβ is possibly the ER and/or the endosomes/lysosomes, as mentioned above. However, it has been shown that both APP and γ-secretase components, including presenilin, nicastrin, Aph-1, and Pen-2, localize to mitochondria (Anandatheerthavarada et al., 2003; Hansson et al., 2004) and that APP has a mitochondrial targeting signal sequence (Anandatheerthavarada et al., 2003). These observations indicate the possibilities that at least a portion of mitochondrial Aβ is generated in situ and that Aβ may play a physiological, beneficial role for mitochondrial function. However, there is no evidence to date to confirm the physiological role of mitochondrial Aβ; instead, Aβ has been demonstrated to impair mitochondrial function (Keil et al., 2004; Caspersen et al., 2005; Crouch et al., 2005; Manczak et al., 2006; Eckert et al., 2008; Park et al., 2009; Yang et al., 2009; Yao et al., 2009). Interestingly, a portion of mitochondrial Aβ has been shown to form oligomers in AD, transgenic mouse brains and APP-transfected cells (Caspersen et al., 2005; Manczak et al., 2006). Furthermore, when it was applied exogenously, oligomeric and/or fibrillar Aβ, but not monomeric Aβ, displayed toxic effects (Crouch et al., 2005; Eckert et al., 2008; Yang et al., 2009). For APP_{OSK}-transfected cells, we demonstrated that mitochondrial accumulation of Aβ oligomers caused altered mitochondrial membrane potential and cytochrome c release from mitochondria. The latter was also observed in APP_{OSK}-Tg mouse brain. Aβ oligomer-induced mitochondrial dysfunction may be caused by the same mechanism at play in Aβ oligomer-induced endosomal/lysosomal leakage, whereby Aβ oligomers form pore-like structures in the membranes. Alternatively, Aβ may generate free radicals during aggregation and therefore cause oxidative damage to mitochondrial membranes and proteins. In either case, disruption of mitochondrial membrane impermeability allows passage of ions across the membranes and leads to their depolarization, the uncoupling of the respiratory chain from oxidative phosphorylation, and subsequently the release of cytochrome c. Injured mitochondria are also disposed by autophagy, which could overload the autophagy-lysosome system, resulting in enhanced lysosomal leakage.

It has been shown that there is cross-talk between cellular organelles (Ferri and Kroemer, 2001). In particular the ER and mitochondria are well known to interact via Ca^{2+} transfer and to regulate apoptosis (Pinton et al., 2008). Specifically, increased Ca^{2+} release from the ER causes excessive entry of this ion into mitochondria, which leads to apoptosis. Endosomal/lysosomal leakage has also been implicated in mitochondria-dependent apoptosis (Ferri and Kroemer, 2001). Both cathepsin D and a cathepsin-L-related protease released from late endosomes/lysosomes induce the proteolytic activation of Bid, a Bcl-2 family member, which generates a truncated Bid (t-Bid) that causes release of cytochrome c from mitochondria and activation of caspases-9 and −3 (Stoka et al., 2001; Heinrich et al., 2004). Thus, the enhanced release of cytochrome c from mitochondria in APP_{OSK}-transfected cells and in APP_{OSK}-Tg mouse brain may reflect, at least in part, both ER stress-induced Ca^{2+} release and lysosomal enzyme-mediated Bid activation (Fig. 6).

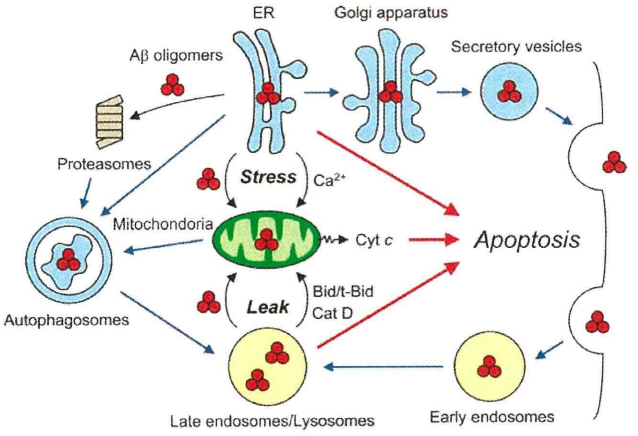

Fig. 6. Proposed mechanism underlying intracellular Aβ oligomer-induced cell death. Accumulation of Aβ oligomers in the ER causes ER stress that elicits the unfolded protein responses, including up-regulation of molecular chaperones, halt of further protein synthesis, and induction of ERAD. In ERAD, the Aβ oligomers are translocated from the ER into the cytoplasm to be degraded by proteasomes. Damaged ER and undegradable Aβ oligomers in the cytoplasm are engulfed by autophagosomes and sorted to lysosomes to be digested. In spite of these protective mechanisms, severe and prolonged ER stress activates caspase-4 in humans and initiates apoptosis. ER stress also induces Ca^{2+} release from the ER and causes excessive entry of Ca^{2+} into mitochondria, which elicits cytochrome c (Cyt c) release and apoptosis. Excessive formation of Aβ oligomers causes impairment of the autophagy-lysosome system, which results in disruption of endosomal/lysosomal membrane impermeability. Membrane disruption is also caused by Aβ taken up from the extracellular space by endocytosis and/or by Aβ generated within the endosomes via local processing of APP. Endosomal/lysosomal leakage profoundly affects cellular functions to cause cell death. Simultaneously, cathepsin D (Cat D) leaked from late endosomes/lysosomes activates Bid in the cytoplasm to generate t-Bid, which acts on mitochondria to induce cytochrome c release and apoptosis. A portion of Aβ oligomers in the cytoplasm, which presumably originate from the ER and endosomes/lysosomes, is sorted to mitochondria and directly damages their membranes, ultimately leading to apoptosis. Injured mitochondria are also disposed by autophagy, which may overload the autophagy-lysosome system, resulting in enhanced endosomal/lysosomal leakage. Thus, intracellular Aβ oligomers cause cell death by inducing ER stress, endosomal/lysosomal leakage, and mitochondrial dysfunction, both directly and indirectly.

Similar mechanisms have been proposed for mutant α-synuclein-induced cell death. It has been demonstrated

that expression of A53T-mutant α-synuclein in PC12 cells decreases proteasome activity, increases the reactive oxygen species level, and eventually causes cell death, which is accompanied by mitochondrial dysfunction (decreased membrane potential and increased cytochrome c release); ER stress (increased phosphorylated eIF2α and Grp78); and activation of caspases-12, −9, and −3 (Smith et al., 2005). In addition, it has been shown that proteosomal dysfunction in A53T-mutant α-synuclein-expressing cells is caused by α-synuclein oligomers (Emmanouilidou et al., 2010). Our finding in the present study that intracellular accumulation of peptide oligomers causes cell death by inducing ER stress, endosomal/lysosomal leakage, and mitochondrial dysfunction is not restricted to the APP Osaka mutation or AD but instead seems also to be plausible for other neurodegenerative disorders.

REFERENCES

Almeida CG, Takahashi RH, Gouras GK. 2006. β-amyloid accumulation impairs multivesicular body sorting by inhibiting the ubiquitin-proteasome system. J Neurosci 26:4277–4288.

Anandatheerthavarada HK, Biswas G, Robin MA, Avadhani NG. 2003. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. J Cell Biol 161:41–54.

Caspersen C, Wang N, Yao J, Sosunov A, Chen X, Lustbader JW, Xu HW, Stern D, McKhann G, Yan SD. 2005. Mitochondrial Aβ: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. FASEB J 19:2040–2041.

Crouch PJ, Blake R, Duce JA, Ciccotosto GD, Li QX, Barnham KJ, Curtain CC, Cherny RA, Cappai R, Dyrks T, Masters CL, Trounce IA. 2005. Copper-dependent inhibition of human cytochrome c oxidase by a dimeric conformer of amyloid-β1–42. J Neurosci 25:672–679.

D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH. 2001. Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38:120–134.

Ditaranto K, Tekirian TL, Yang AJ. 2001. Lysosomal membrane damage in soluble Aβ-mediated cell death in Alzheimer's disease. Neurobiol Dis 8:19–31.

Eckert A, Hauptmann S, Scherping I, Meinhardt J, Rhein V, Dröse S, Brandt U, Fändrich M, Müller WE, Götz J. 2008. Oligomeric and fibrillar species of β-amyloid (Aβ42) both impair mitochondrial function in P301L tau transgenic mice. J Mol Med 86:1255–1267.

Emmanouilidou E, Stefanis L, Vekrellis K. 2010. Cell-produced α-synuclein oligomers are targeted to, and impair, the 26S proteasome. Neurobiol Aging 31:953–968.

Fernández-Vizarra P, Fernández AP, Castro-Blanco S, Serrano J, Bentura ML, Martínez-Murillo R, Martínez A, Rodrigo J. 2004. Intra- and extracellular Aβ and PHF in clinically evaluated cases of Alzheimer's disease. Histol Histopathol 19:823–844.

Ferri KF, Kroemer G. 2001. Organelle-specific initiation of cell death pathways. Nat Cell Biol 3:E255–E263.

Friedrich RP, Tepper K, Rönicke R, Soom M, Westermann M, Reymann K, Kaether C, Fändrich M. 2010. Mechanism of amyloid plaque formation suggests an intracellular basis of Aβ pathogenicity. Proc Natl Acad Sci U S A 107:1942–1947.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR. 2000. Intraneuronal Aβ42 accumulation in human brain. Am J Pathol 156:15–20.

Gouras GK, Tampellini D, Takahashi RH, Capetillo-Zarate E. 2010. Intraneuronal β-amyloid accumulation and synapse pathology in Alzheimer's disease. Acta Neuropathol 119:523–541.

Gyure KA, Durham R, Stewart WF, Smialek JE, Troncoso JC. 2001. Intraneuronal Aβ-amyloid precedes development of amyloid plaques in Down syndrome. Arch Pathol Lab Med 125:489–492.

Hansson CA, Frykman S, Farmery MR, Tjernberg LO, Nilsberth C, Pursglove SE, Ito A, Winblad B, Cowburn RF, Thyberg J, Ankarcrona M. 2004. Nicastrin, presenilin, APH-1, and PEN-2 form active γ-secretase complexes in mitochondria. J Biol Chem 279:51654–51660.

Hansson Petersen CA, Alikhani N, Behbahani H, Wiehager B, Pavlov PF, Alafuzoff I, Leinonen V, Ito A, Winblad B, Glaser E, Ankarcrona M. 2008. The amyloid β-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. Proc Natl Acad Sci U S A 105:13145–13150.

Hashimoto M, Rockenstein E, Crews L, Masliah E. 2003. Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases. Neuromolecular Med 4:21–36.

Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, Wickel M, Schneider-Brachert W, Trauzold A, Hethke A, Schütze S. 2004. Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and −3 activation. Cell Death Differ 11:550–563.

Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M. 2004. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death. J Cell Biol 165:347–356.

Hoozemans JJ, Veerhuis R, Van Haastert ES, Rozemuller JM, Baas F, Eikelenboom P, Scheper W. 2005. The unfolded protein response is activated in Alzheimer's disease. Acta Neuropathol 110:165–172.

Hu X, Crick SL, Bu G, Frieden C, Pappu RV, Lee JM. 2009. Amyloid seeds formed by cellular uptake, concentration, and aggregation of the amyloid-β peptide. Proc Natl Acad Sci U S A 106:20324–20329.

Ji ZS, Miranda RD, Newhouse YM, Weisgraber KH, Huang Y, Mahley RW. 2002. Apolipoprotein E4 potentiates amyloid β peptide-induced lysosomal leakage and apoptosis in neuronal cells. J Biol Chem 277:21821–21828.

Kayed R, Pensalfini A, Margol L, Sokolov Y, Sarsoza F, Head E, Hall J, Glabe C. 2009. Annular protofibrils are a structurally and functionally distinct type of amyloid oligomer. J Biol Chem 284:4230–4237.

Keil U, Bonert A, Marques CA, Scherping I, Weyermann J, Strosznajder JB, Müller-Spahn F, Haass C, Czech C, Pradier L, Müller WE, Eckert A. 2004. Amyloid β-induced changes in nitric oxide production and mitochondrial activity lead to apoptosis. J Biol Chem 279:50310–50320.

Kikkert M, Doolman R, Dai M, Avner R, Hassink G, van Voorden S, Thanedar S, Roitelman J, Chau V, Wiertz E. 2004. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J Biol Chem 279:3525–3534.

Klein WL, Krafft GA, Finch CE. 2001. Targeting small Aβ oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci 24:219–224.

LaFerla FM, Green KN, Oddo S. 2007. Intracellular amyloid-β in Alzheimer's disease. Nat Rev Neurosci 8:499–509.

Lambert MP, Velasco PT, Chang L, Viola KL, Fernandez S, Lacor PN, Khuon D, Gong Y, Bigio EH, Shaw P, De Felice FG, Krafft GA, Klein WL. 2007. Monoclonal antibodies that target pathological assemblies of Aβ. J Neurochem 100:23–35.

Ling D, Song HJ, Garza D, Neufeld TP, Salvaterra PM. 2009. Aβ42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in *Drosophila*. PLoS One 4:e4201.

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH. 2006. Mitochondria are a direct site of Aβ accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. Hum Mol Genet 15:1437–1449.

Matsuyama S, Teraoka R, Mori H, Tomiyama T. 2007. Inverse correlation between amyloid precursor protein and synaptic plasticity in transgenic mice. Neuroreport 18:1083–1087.

Mori C, Spooner ET, Wisniewsk KE, Wisniewski TM, Yamaguch H, Saido TC, Tolan DR, Selkoe DJ, Lemere CA. 2002. Intraneuronal Aβ42 accumulation in Down syndrome brain. Amyloid 9:88–102.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature 403:98–103.

Nishitsuji K, Tomiyama T, Ishibashi K, Ito K, Teraoka R, Lambert MP, Klein WL, Mori H. 2009. The E693Δ mutation in amyloid precursor protein increases intracellular accumulation of amyloid β oligomers and causes endoplasmic reticulum stress-induced apoptosis in cultured cells. Am J Pathol 174:957–969.

Park HJ, Kim SS, Kang S, Rhim H. 2009. Intracellular Aβ and C99 aggregates induce mitochondria-dependent cell death in human neuroglioma H4 cells through recruitment of the 20S proteasome subunits. Brain Res 1273:1–8.

Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. 2008. Calcium and apoptosis: ER-mitochondria Ca^{2+} transfer in the control of apoptosis. Oncogene 27:6407–6418.

Quist A, Doudevski I, Lin H, Azimova R, Ng D, Frangione B, Kagan B, Ghiso J, Lal R. 2005. Amyloid ion channels: a common structural link for protein-misfolding disease. Proc Natl Acad Sci U S A 102:10427–10432.

Reddy PH. 2009. Amyloid β, mitochondrial structural and functional dynamics in Alzheimer's disease. Exp Neurol 218:286–292.

Selkoe DJ. 2002. Alzheimer's disease is a synaptic failure. Science 298:789–791.

Singer SJ, Dewji NN. 2006. Evidence that Perutz's double-β-stranded subunit structure for β-amyloids also applies to their channel-forming structures in membranes. Proc Natl Acad Sci U S A 103:1546–1550.

Smith WW, Jiang H, Pei Z, Tanaka Y, Morita H, Sawa A, Dawson VL, Dawson TM, Ross CA. 2005. Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant α-synuclein-induced toxicity. Hum Mol Genet 14:3801–3811.

Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V, Salvesen GS. 2001. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem 276:3149–3157.

Suga K, Tomiyama T, Mori H, Akagawa K. 2004. Syntaxin 5 interacts with presenilin holoproteins, but not with their N- or C-terminal fragments, and affects β-amyloid peptide production. Biochem J 381:619–628.

Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK. 2002. Intraneuronal Alzheimer Aβ42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am J Pathol 161:1869–1879.

Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK. 2004. Oligomerization of Alzheimer's β-amyloid within processes and synapses of cultured neurons and brain. J Neurosci 24:3592–3599.

Tomiyama T, Nagata T, Shimada H, Teraoka R, Fukushima A, Kanemitsu H, Takuma H, Kuwano R, Imagawa M, Ataka S, Wada Y, Yoshioka E, Nishizaki T, Watanabe Y, Mori H. 2008. A new amyloid β variant favoring oligomerization in Alzheimer's-type dementia. Ann Neurol 63:377–387.

Tomiyama T, Matsuyama S, Iso H, Umeda T, Takuma H, Ohnishi K, Ishibashi K, Teraoka R, Sakama N, Yamashita T, Nishitsuji K, Ito K, Shimada H, Lambert MP, Klein WL, Mori H. 2010. A mouse model of amyloid β oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. J Neurosci 30:4845–4856.

Wirths O, Multhaup G, Bayer TA. 2004. A modified β-amyloid hypothesis: intraneuronal accumulation of the β-amyloid peptide—the first step of a fatal cascade. J Neurochem 91:513–520.

Yang AJ, Chandswangbhuvana D, Margol L, Glabe CG. 1998. Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Aβ1–42 pathogenesis. J Neurosci Res 52:691–698.

Yang TT, Hsu CT, Kuo YM. 2009. Cell-derived soluble oligomers of human amyloid-β peptides disturb cellular homeostasis and induce apoptosis in primary hippocampal neurons. J Neural Transm 116:1561–1569.

Yao J, Irwin RW, Zhao L, Nilsen J, Hamilton RT, Brinton RD. 2009. Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 106:14670–14675.

Yoshida H. 2007. ER stress and diseases. FEBS J 274:630–658.

Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, Mohan PS, Mercken M, Farmery MR, Tjernberg LO, Jiang Y, Duff K, Uchiyama Y, Näslund J, Mathews PM, Cataldo AM, Nixon RA. 2005. Macroautophagy—a novel β-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol 171:87–98.

Hirosaki Med. J. 61(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. **61, 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

[1)]Department of Neuroscience and Neurology, Osaka City University Medical School, Osaka, Japan, [2)]Department of Pharmaceutical Sciences, Himeji Dokkyo University, Hyogo, Japan, [3)]Department of Behavior Science, Hyogo University of Health Sciences, Kobe, Japan, [4)]Department of Physiology, Hyogo College of Medicine, Nishinomiya, Japan, [5)]Department of Physiology, Osaka City University Medical School, Osaka, Japan, [6)]Molecular Imaging Research Program, FRS, RIKEN, Kobe, Japan.
Correspondence should be addressed to H.M. (mori@med.osaka-cu.ac.jp, Fax: +81-6-6645-3922).

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 A β1-42/ 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

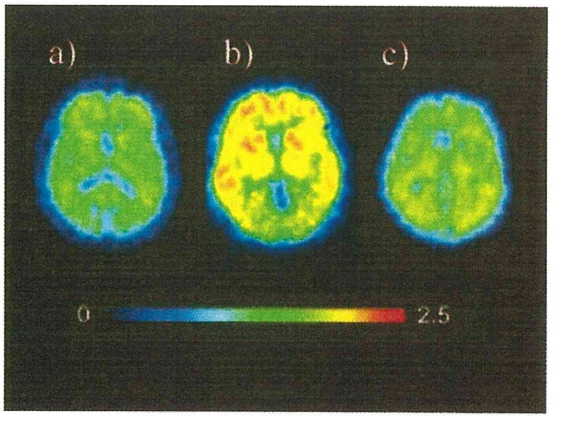

Figure 1 PiB-PET analysis
Amyloid imaging of the patient's brain with [^{11}C]PiB. PiB standardized uptake value images summed over 40 to 60 minutes are shown. (a) aged control (81-yr, female); (b) sporadic AD (71-yr, female); (c), the present case (62-year-old, female).

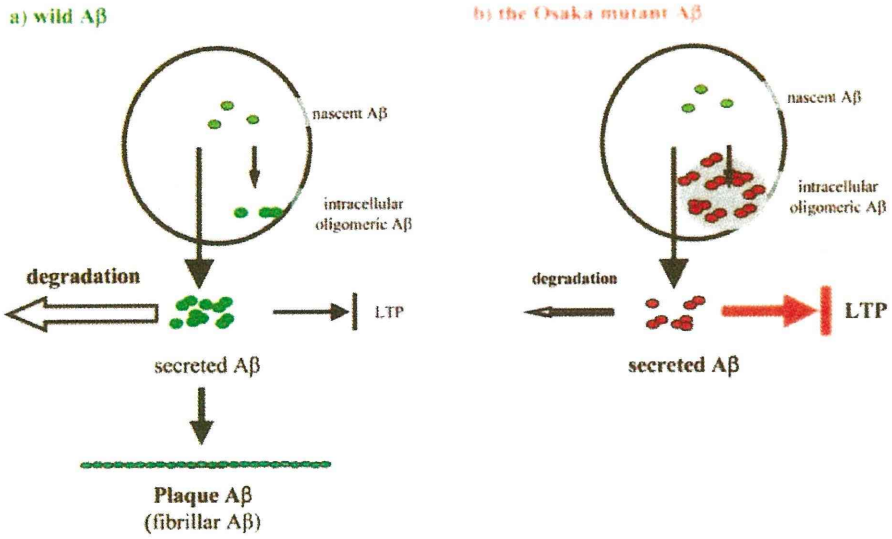

Figure 2 Scheme of Aβ pathway

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.

Acknowledgments

This study was supported by Grants-in-aid for Scientific Research from MEXT of Japan to T.T. and H.M., a Health and Labour Sciences Research Grant on Dementia and in part by the Alzheimer's Association (IIRG-09-132098) to H.M.

References

1) Selkoe DJ. Alzheimer's disease is a synaptic failure. Science 2002;298:789-91.

2) Klein WL, Krafft GA, Finch CE. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci 2001;24: 219-24.

3) Walsh, D.M., Klyubin, I., Fadeeva, J.V. *et al.* Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation *in vivo*. Nature 2002;416:535-9.

4) Lambert MP, Barlow AK, Chromy BA. *et al.* Diffusible, nonfibrillar ligands derived from $Abeta_{1-42}$ are potent central nervous system neurotoxins. Proc Natl Acad Sci USA 1998;95:6448-53.

5) Cleary JP, Walsh DM, Hofmeister JJ. *et al.* Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nature Neurosci 2005;8:79-84.

6) Lesné S, Koh MT, Kotilinek L. *et al.* A specific amyloid-beta protein assembly in the brain impairs memory. Nature 2006;440:352-7.

7) Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. J Lipid Res 1990;31:545-8.

8) Tomiyama T, Asano S, Suwa Y. *et al.* Rifampicin prevents the aggregation and neurotoxicity of amyloid beta protein *in vitro*. Biochem Biophys Res Commun 1994;204:76-83.

9) Klunk WE, Engler H, Nordberg A *et al.* Imaging brain amyloid in Alzheimer's disease with Pittsburgh compound-B. Ann Neurol 2004;55:306-19.

10) Morimoto A, Irie K, Murakami K. *et al.* Analysis of the secondary structure of beta-amyloid (Abeta42) fibrils by systematic proline replacement. J Biol Chem 2004;279:52781-8.

11) Oddo S, Caccamo A, Shepherd JD. *et al.* Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Abeta and synaptic dysfunction. Neuron 2003;39:409-21.

12) Billings LM, Oddo S, Green KN. *et al.* Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 2005;45:675-88.

13) Takahashi RH, Milner TA, Li F. *et al.* Intraneuronal Alzheimer Abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am J Pathol 2002;161:1869-79.

14) Yu WH, Cuervo AM, Kumar A. *et al.* Macroautophagy – a novel beta-amyloid peptide- generating pathway activated in Alzheimer's disease. J Cell Biol 2005;171:87-98.

15) Chui D.-H, Tanahashi H, Ozawa K. *et al.* Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaques formation. Nature Med 1999;5: 560-4.

16) Casas C, Sergeant N, Itier J.-M. *et al.* Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated $Abeta_{42}$ accumulation in a novel Alzheimer transgenic model. Am J Pathol 2004;165:1289-300.

17) Oakley H, Cole SL, Logan S. *et al.* Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: Potential factors

in amyloid plaque formation. J Neurosci 2006; 26:10129-40.

18) Citron M, OLTERSDORF T, HAASS C. *et al.* Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 1992;360:672-4.

19) Suzuki N, Cheung TT, Cai XD. *et al.* An increased percentage of long amyloid protein secreted by familial amyloid beta protein precursor (beta-APP_{717}) mutants. Science 1994; 264:1336-40.

20) Tamaoka A, Odaka A, Ishibashi Y. *et al.* APP717 missense mutation affects the ratio of amyloid beta protein species (Abeta1-42/43 and Abetra1-40) in familial Alzheimer's disease brain. J Biol Chem 1994;269:32721-4.

21) De Jonghe C, Zehr C, Yager D. *et al.* Flemish and Dutch mutations in amyloid beta precursor protein have different effects on amyloid beta secretion. Neurobiol Dis 1998;5:281-6.

22) Murakami K, Irie K, Morimoto A. *et al.* Neurotoxicity and physicochemical properties of Abeta mutant peptides from cerebral amyloid angiopathy. J Biol Chem 2003;278:46179-87.

Journal of Neuroscience Research

Journal of Neuroscience Research 88:1985–1994 (2010)

Regulation of Cholesterol Efflux by Amyloid β Secretion

Tomohiro Umeda,[1] Hiroshi Mori,[1] Hui Zheng,[2] and Takami Tomiyama[1]*

[1]Department of Neuroscience, Osaka City University Graduate School of Medicine, Osaka, Japan
[2]Huffington Center on Aging, Departments of Molecular and Human Genetics, Molecular and Cellular Biology, and Neuroscience, Baylor College of Medicine, Houston, Texas

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

Contract grant sponsor: JSPS; Contract grant number: 21500352; Contract grant sponsor: MEXT; Contract grant number: 17300114; Contract grant number: 18023033; Contract grant number: 20023026.

*Correspondence to: Takami Tomiyama, PhD, Department of Neuroscience, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan.
E-mail: tomi@med.osaka-cu.ac.jp

Received 14 October 2009; Revised 9 November 2009; Accepted 1 December 2009

Published online 12 February 2010 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.22360

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

negative control, an antibody to COX-IV, a mitochondrial membrane protein, was used. The precipitates were subjected to SDS-PAGE, followed by Western blotting with AB.H10 and a monoclonal antibody to APP/Aβ (6E10; Signet Laboratories, Dedham, MA), as described previously (Nishitsuji et al., 2009).

^{14}C-Labeled Cholesterol/MβCD Complex

^{14}C-labeled cholesterol/MβCD complex was prepared using ^{14}C-cholesterol (Amersham Biosciences, Piscataway, NJ) essentially as described previously (Klein et al., 1995). In brief, 25 μCi (0.195 mg) of ^{14}C-cholesterol and 2.805 mg of nonlabeled cholesterol (Sigma) were mixed with 100 mg MβCD (Sigma) in 0.3 ml water and stirred for 60 min at room temperature. The clear solution was frozen at −80°C, and the solvent was evaporated under a vacuum. The dried complex was resuspended in 7.75 ml of DMEM (Sigma) to a cholesterol concentration of 1 mM. The calculated specific activity of the resulting complex was 3.33 mCi/mmol cholesterol.

^{14}C-Labeled Reconstituted Lipoproteins

^{14}C-labeled reconstituted lipoproteins were prepared using ^{14}C-cholesterol essentially as described previously (Tajima et al., 1983). In brief, 50 μCi (0.39 mg) of ^{14}C-cholesterol, 3.51 mg of nonlabeled cholesterol, 11.52 mg of cholesteryl oleate (Sigma), 18.59 mg of phosphatidylcholine (Sigma), and 3.16 mg of glyceryl trioleate (Sigma) were mixed in 3 ml of PBS, and sonicated 10 times for 3 min each with 1-min intervals at 12 W while being cooled in an ice bath. The proportion of lipids in this mixture reflects that in plasma HDL. The solution was centrifuged at 410,000*g* for 2 hr at 4°C. The floating fraction (about 1 ml) at the top of the solution was recovered as lipoprotein-like particles. The radioactive concentration of the particle solution was 55,000 dpm/μl. An aliquot of the solution was incubated with 10 μM Aβ40 in 5.4 ml of PBS for 30 min at room temperature. The ratio of Aβ to lipid particles in this mixture was 5:1, which reflects that of apoE to HDL particles in plasma. After being concentrated using a 50K-cut membrane device (Centricon YM-50; Millipore, Billerica, MA), the solution was diluted to 100,000 dpm/μl. ^{14}C-labeled control lipid particles without Aβ were also prepared. The Aβ/lipid particles complex prepared by the same method, except for use of nonlabeled cholesterol instead of ^{14}C-cholesterol, was examined for Aβ by immunoelectron microscopy with an anti-Aβ antibody, as described below. The resultant lipid particles were approximately 30–60 nm in diameter and thus larger than plasma HDL and similar to LDL-VLDL in size.

Cholesterol Efflux From Cells

Cholesterol loading onto cells was performed with 1 mM ^{14}C-labeled cholesterol/MβCD complex in serum-free DMEM at 4°C for 1 hr. At this temperature, cellular activities presumably declined, and Aβ production was probably inhibited. After cholesterol loading, the culture media were replaced with serum-free DMEM, and the cells were further incubated at 37°C for 1 hr to allow cholesterol efflux. The culture media and cells were harvested separately, and their radioactivity was counted with a liquid scintillation counter. In some experiments, the conditioned media were fractionated to VLDL, LDL, HDL, and VHDL fractions by sequential ultracentrifugation according to the method of Hatch (1968), with minor modifications. In brief, conditioned media were supplemented with 0.1% EDTA and 0.01% NaN_3, and 2 ml of the solution was overlaid with 1 ml of NaCl solution (d = 1.006) and centrifuged at 80,000 rpm for 4 hr at 16°C using a himac CS120 ultracentrifuge and a RP80AT rotor (Hitachi Koki Co., Ltd., Tokyo, Japan). The upper phase (1 ml) was harvested as the VLDL ($0.96 < d < 1.006$) fraction. The lower phase (1.8 ml) was mixed with 0.9 ml of NaCl-NaBr solution (d = 1.182) to make the relative density 1.063. Then, the solution was overlaid with 500 μl of NaCl-NaBr solution (d = 1.063) and centrifuged at 80,000 rpm for 5 hr. The upper phase (1 ml) was harvested as the LDL ($1.006 < d < 1.063$) fraction. The lower phase (2 ml) was mixed with 1 ml of NaCl-NaBr solution (d = 1.478) to make the relative density 1.21, overlaid with 200 μl of NaCl-NaBr solution (d = 1.21), and centrifuged at 80,000 rpm for 10 hr. The upper phase (1 ml) was harvested as the HDL ($1.063 < d < 1.21$) fraction. The lower phase (2.2 ml) was mixed with 386 μl of NaCl-NaBr solution (d = 1.478) to make the relative density 1.25, overlaid with 700 μl of NaCl-NaBr solution (d = 1.25), and centrifuged at 80,000 rpm for 4.5 hr. Finally, the upper phase (1 ml) was harvested as the VHDL ($1.21 < d < 1.25$) fraction. The radioactivity in each fraction was counted.

Cholesterol Efflux From the Brain

Twelve-month-old APP knockout mice were anesthetized with pentobarbital and placed on a stereotaxic apparatus. One microliter (100,000 dpm) of the ^{14}C-labeled reconstituted lipoproteins with or without Aβ was injected into the cerebral ventricle over 1 min using a Hamilton syringe. The syringe was left in place for 10 min to avoid regurgitation of the injectate. 10 or 60 min after injection, the mice were dissected into liver, spleen, kidney, heart, and lung, and then decapitated to harvest the brain. The collected tissues were homogenized in a Polytron PT3100 homogenizer (Kinematica, Bohemia, NY) in 9 vol of water, and 200 μl of the homogenate was mixed with 3 ml of water and added to 10 ml of liquid scintillation cocktail Insta-Gel Plus (PerkinElmer, Waltham, MA). The radioactivity in each sample was counted. All animal experiments were performed in accordance with the Guide for Animal Experimentation, Osaka City University.

Immunoelectron Microscopy

Samples were absorbed onto 200-mesh carbon-coated nickel grids (Stork Veco B.V., Eerbeek, The Netherlands). The grids were blocked with 1%BSA/PBS for 10 min and incubated with an anti-Aβ polyclonal antibody (β001; Lippa et al., 1999) for 1 hr. followed by 10 nm gold-labeled second antibody (AuroProbe EM GAR G10; Amersham) for 1 hr. After being washed with PBS and water, the specimens were negatively stained with 2% phosphotungstate (TAAB Laboratories Equipment Ltd., Berks, United Kingdom) and viewed

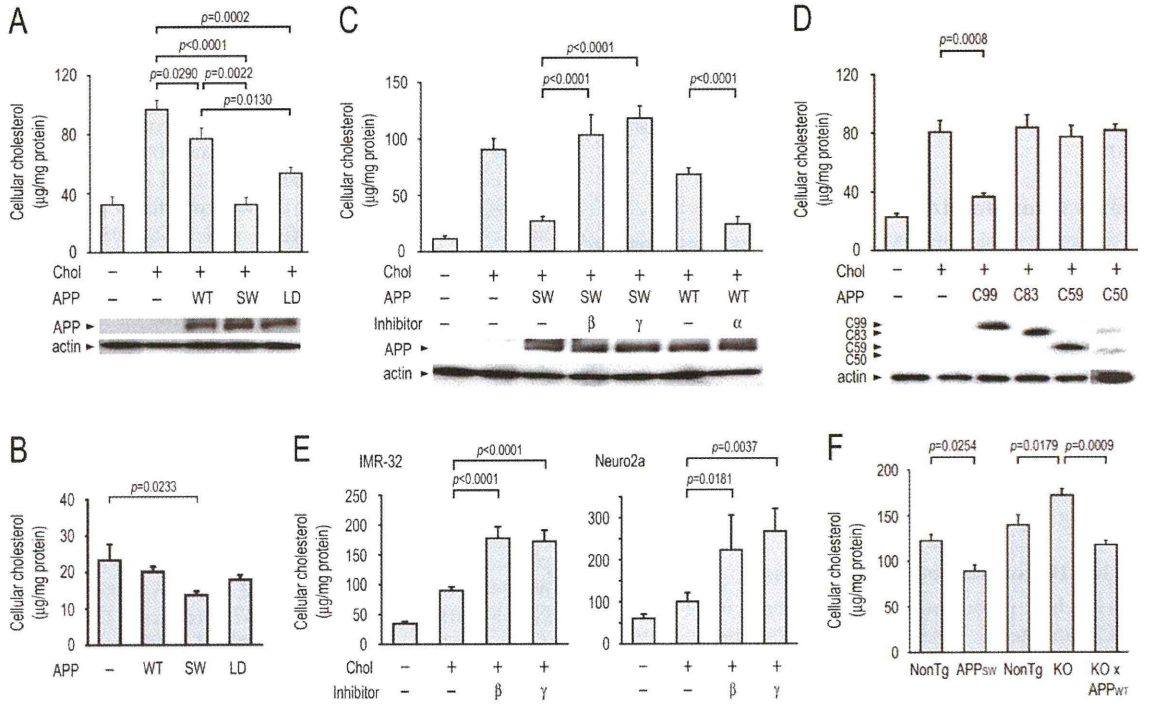

Fig. 1. Aβ regulates cellular cholesterol levels. **A:** APP-expressing HEK293 cells exhibited lower levels of cellular cholesterol than mock-trasfected cells after cholesterol loading. APP_{SW} and APP_{LD} exhibited more potent effects than APP_{WT}. **B:** Cholesterol-lowering effects of APP expression were also observed under physiological conditions without cholesterol loading. **C:** Inhibition of Aβ production with β- and γ-secretase inhibitors attenuated the effect of APP expression, whereas enhancement of Aβ production with α-secretase inhibitor exhibited the opposite effect. **D:** C99, but not C83, C59, or C50, exhibited an effect similar to that of APP. **E:** Aβ-mediated regulation of cellular cholesterol was also observed in nontransfected neuronal cells. Inhibition of Aβ production with β- and γ-secretase inhibitors increased cellular cholesterol levels in both IMR-32 and Neuro2a cells. **F:** APP_{SW} transgenic (Tg2576) mice (6 months) exhibited lower, whereas APP knockout mice (12 months) exhibited higher cellular cholesterol in the brain than the age-matched control mice. Cross-breeding of APP knockout mice with APP_{WT} transgenic mice resulted in lower cellular cholesterol levels. NonTg, nontransgenic littermates of the Tg2576 mice or nontransgenic wild-type mice; KO, APP knockout mice. Comparison between APP_{SW} and NonTg was performed using the unpaired Student's *t*-test, whereas those among KO, NonTg, and KO × APP_{WT} were performed using Fisher's PLSD test following ANOVA. All columns and bars represent the mean ± SEM (A–E, n = 3; F, n = 4).

under a JEM-1200EX2 electron microscope (JEOL Ltd., Akishima, Japan).

Statistical Analysis

All values obtained are expressed as the mean ± SEM. Comparisons of means between two groups were performed using the unpaired Student's *t*-test, whereas those among multiple groups were performed using Fisher's PLSD test following ANOVA.

RESULTS

Aβ Regulates Cellular Cholesterol Levels

We initially examined the effect of Aβ production on cellular cholesterol levels in vitro. HEK293 cells transfected with APP were cholesterol-loaded by incubation with cholesterol/ MβCD complex. Compared with mock-transfected cells, APP-expressing cells exhibited significantly lower levels of cellular cholesterol (Fig. 1A). APP with the Swedish mutation (K670N/M671L; APP_{SW}), which increases total Aβ production (Table I), and that with the London mutation (V717I; APP_{LD}), which selectively enhances Aβ42 production (Table I), were more effective than wild-type APP (APP_{WT}). The cholesterol-lowering effect of APP expression was also observed under physiological conditions without cholesterol loading (Fig. 1B). When Aβ production was inhibited by β-secretase inhibitor II or γ-secretase inhibitor L-685,458 (Table I), the effect of APP expression on cellular cholesterol was attenuated (Fig. 1C). In contrast, in the presence of α-secretase inhibitor TAPI-I, which leads to increased Aβ production (Table I), the effect of APP was enhanced (Fig. 1C). Thus, cellular cholesterol level was shown to be regulated depending on Aβ (particularly Aβ42) production but not expression of APP holoprotein. A cholesterol-lowering effect was also displayed by C99, an APP carboxyl-terminal fragment that can produce Aβ (Table I), but not by C83, another

TABLE I. Aβ Secretion From Cells

	Aβ1–40 (pg/ml)	Aβ1–42 (pg/ml)
HEK293 cells		
Mock	33 ± 2	0 ± 0
APP_{WT}	258 ± 25	14 ± 0
+ α-Inhibitor	829 ± 14	32 ± 1
APP_{SW}	4,078 ± 1,420	137 ± 21
+ β-Inhibitor	26 ± 3	2 ± 1
+ γ-Inhibitor	53 ± 15	ND
APP_{LD}	161 ± 14	31 ± 2
C99	2,142 ± 428	106 ± 1
C83	58 ± 3	3 ± 1
C59	75 ± 4	3 ± 1
IMR-32 cells		
Control	159 ± 3	10 ± 0
+ β-Inhibitor	46 ± 1	6 ± 1
+ γ-Inhibitor	31 ± 3	4 ± 1
HEK293 cells		
APP_{SW}	175 ± 7	12 ± 1
+ Cholesterol	261 ± 15	14 ± 1

*HEK293 cells were transfected with APP constructs and cultured in serum-free medium for 1 day in the presence or absence of secretase inhibitors. Aβ concentrations in culture media were determined by ELISA. IMR-32 and Neuro-2a cells were also cultured in serum-free medium for 1 day in the presence or absence of secretase inhibitors. Aβ concentrations of IMR-32 cells are shown, but those of Neuro-2a cells could not be measured because the ELISA kits used are for human Aβ alone and not mouse Aβ. In another experiment, HEK293 cells transfected with APP_{SW} were loaded with cholesterol in serum-free medium for 1 hr, and culture media were immediately removed without further incubation to determine Aβ concentrations. Values are means ± SEM for triplicate transfections. ND, not detected.

APP carboxyl-terminal fragment that produces p3 peptide instead of Aβ, or APP intracellular domains (AICDs) such as C59 and C50 (Fig. 1D). These findings exclude the possibility of contribution of p3 peptide and AICD to the cholesterol-lowering effect. Aβ-mediated regulation of cellular cholesterol was also observed in two types of neuronal cells, IMR-32 and Neuro2a, both of which were not transfected with APP but produce endogenous human and mouse Aβ, respectively. Inhibition of Aβ production by β- and γ-secretase inhibitors induced higher levels of cellular cholesterol in these cells (Fig. 1E).

APP Expression Influences Cellular Cholesterol Levels in the Brain

If Aβ participates in the regulation of cellular cholesterol, APP expression levels should influence cellular cholesterol levels in vivo. Thus we examined cellular cholesterol levels in the brains of APP_{SW} transgenic (Tg2576) mice at 6 months and APP knockout mice at 12 months of age. The Tg2576 mice are known to possess no amyloid pathology at this age. Cellular cholesterol levels in Tg2576 mice were lower than those in age-matched nontransgenic littermates, whereas those in APP knockout mice were higher than those in nontransgenic wild-type mice (Fig. 1F). Importantly, the high cholesterol levels in APP knockout mice were reversed to levels beneath those in wild-type mice by cross-breeding with APP_{WT} transgenic mice. This suggests that APP is involved in physiological regulation of cellular cholesterol probably through Aβ production.

Aβ Promotes Cholesterol Efflux From Cells

We assumed that Aβ reduced cellular cholesterol levels by enhancing cholesterol efflux from cells probably via its apolipoprotein-like function. Thus we examined efflux of cellular cholesterol in APP-expressing cells. For this purpose, we prepared radiolabeled cholesterol/MβCD complex using ^{14}C-cholesterols and loaded it onto HEK293 cells. Cholesterol efflux was measured by counting radioactivities of the culture media and cells. In mock-transfected cells, approximately 13% of the loaded ^{14}C-cholesterol was excreted into the media, whereas excretion increased up to 39% in APPsw-expressing cells (Fig. 2A). In parallel with the cholesterol efflux, increased Aβ secretion from cells was observed (Table I). When Aβ production was inhibited by γ-secretase inhibitor, cholesterol efflux decreased to levels in mock-transfected cells (Fig. 2A). Aβ-mediated cholesterol efflux was also observed in nontransfected IMR-32 cells, in which β- and γ-secretase inhibitors attenuated the efflux again. (Fig. 2B).

Aβ Assembles Lipoprotein-Like Particles During Its Secretion

Cholesterol efflux from cells is usually accompanied by lipoprotein formation (Yokoyama, 2005). To determine whether lipoprotein formation occurs in Aβ-mediated cholesterol efflux and which lipoproteins are formed, if any, we fractionated conditioned media of APP_{SW}-expressing HEK293 cells by sequential ultracentrifugation into the VLDL ($0.96 < d < 1.006$), LDL ($1.006 < d < 1.063$), HDL ($1.063 < d < 1.21$), and VHDL ($1.21 < d < 1.25$) fractions. Approximately 50% of ^{14}C-cholesterol in the media was collected in the HDL fraction (Fig. 2C). With immunoelectron microscopy, the HDL fraction was found to contain many Aβ-positive lipoprotein-like particles with diameters of 10–50 nm, ranging from HDL to VLDL in size, whereas very few lipoprotein-like particles were observed in the HDL fraction in the presence of γ-secretase inhibitor (Fig. 2D). These findings indicate that Aβ promoted cholesterol efflux by assembling lipoprotein-like particles with cellular cholesterol during its secretion. It is unlikely that these particles were formed after Aβ secretion by extracellular Aβ to pull out cholesterol from the plasma membrane (Michikawa et al., 2001), insofar as addition of synthetic Aβ peptide to culture medium after cholesterol loading did not reduce cellular cholesterol levels (Fig. 2E). Furthermore, the possibility was ruled out that Aβ secreted from cells bound to cholesterol in the media to prevent their cellular uptake (Yao and Papadopoulos, 2002), because the presence of Aβ in

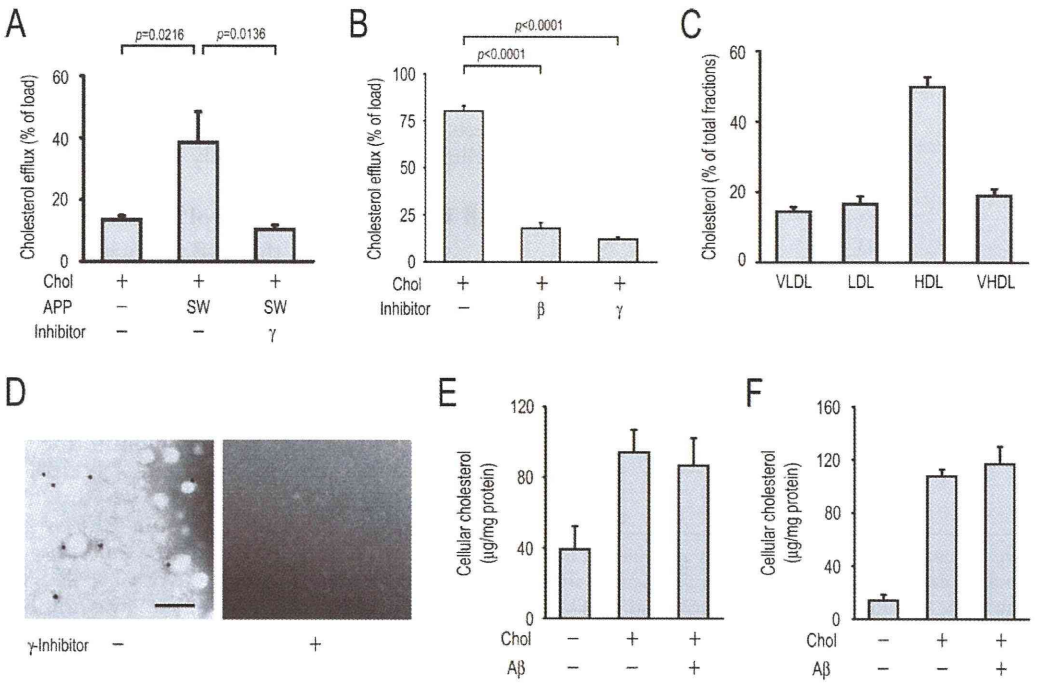

Fig. 2. Aβ promotes cholesterol efflux from cells by assembling lipoprotein-like particles during its secretion. **A:** APP_{SW}-expressing HEK293 cells exhibited higher cholesterol efflux into medium than mock-transfected cells. Inhibition of Aβ production with γ-secretase inhibitor attenuated the cholesterol efflux. **B:** Cholesterol efflux and its inhibition by β- and γ-secretase inhibitors were also observed in nontransfected IMR-32 cells. **C:** Conditioned media of APP_{SW}-expressing HEK293 cells were fractionated by sequential ultracentrifugation. Approximately 50% of cholesterol in the media was collected in the HDL fraction. **D:** The HDL fraction contained many Aβ-positive lipoprotein-like particles with diameters of 10–50 nm. In contrast, very few lipoprotein-like particles were observed in the HDL fraction in the presence of γ-secretase inhibitor. Aβ was probed with 10-nm gold particles. **E:** Addition of Aβ to culture medium after cholesterol loading did not affect cellular cholesterol levels. **F:** The presence of Aβ in culture medium during cholesterol loading did not affect cholesterol levels loaded into cells. All columns and bars represent the mean ± SEM (n = 3). Scale bar = 100 nm.

culture medium during cholesterol loading did not affect cholesterol levels loaded onto cells (Fig. 2F).

Aβ-Induced Cholesterol Efflux Is Mediated by ABCA1

It has been proposed that ABCA1 plays a key role in apolipoprotein-mediated cholesterol efflux from cells (Yokoyama, 2005). ABCA1, a transmembrane protein, interacts with extracellular lipid-poor apolipoproteins, such as apoA-I and apoE, and transports cellular cholesterol to those proteins. To examine whether Aβ-mediated cholesterol efflux depends on ABCA1, we knocked down ABCA1 of IMR-32 cells with a short interfering RNA (siRNA). This treatment resulted in increased cellular cholesterol levels (Fig. 3A) and decreased cholesterol efflux from cells (Fig. 3B). In addition, direct interaction between ABCA1 and APP/Aβ was demonstrated in nontransfected IMR-32 and transfected HEK293 cells by immunoprecipitation (Fig. 3C). Notably, their interaction was apparently promoted by cholesterol loading onto cells. APP and Aβ coprecipitated with ABCA1 increased up to 254% ($P = 0.0243$ vs. without cholesterol) and 206% ($P = 0.0483$), respectively, whereas ABCA1 coprecipitated with APP increased up to 333% ($P = 0.0049$) by cholesterol loading (n = 3 for each group). These findings indicate that Aβ-mediated cholesterol efflux requires ABCA1. The presence of anti-Aβ antibodies in culture medium did not affect cholesterol efflux from cells (Fig. 3D), suggesting that ABCA1-Aβ interaction does not occur between the cell surface and the extracellular space, where antibodies to Aβ can interfere with the interaction (see, for example, Tsujita et al., 2005), but does occur within the plasma membrane or intracellular compartments.

Aβ Accelerates Cholesterol Transport From the Brain to the peripheral tissues

It is known that Aβ is transported from the brain into the circulation by receptor-mediated transcytosis across the blood–brain barrier (BBB; Shibata et al., 2000) and the choroids plexus (Crossgrove et al., 2005). This mechanism is presumed to function to clear toxic Aβ from the brain. However, we speculate that Aβ is exported from the brain to discharge cerebral cholesterol. To test this hypothesis, we examined whether intracranial cholesterol can be transported into the

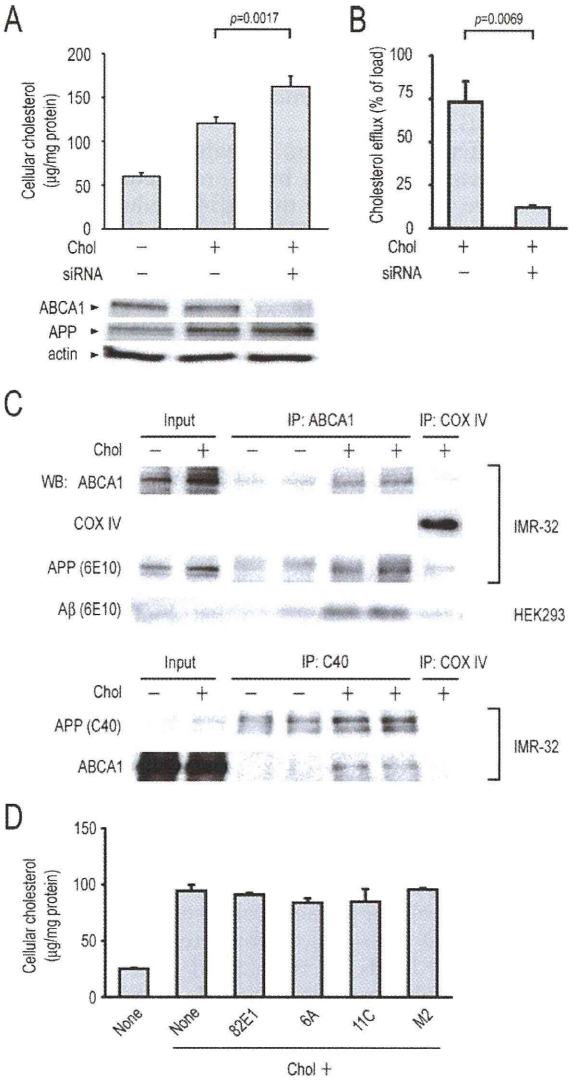

Fig. 3. Aβ-induced cholesterol efflux is mediated by ABCA1. Knocking down ABCA1 by siRNA increased cellular cholesterol levels (**A**) and decreased cholesterol efflux (**B**) in IMR-32 cells. **C:** ABCA1 and APP were immunoprecipitaed from cell lysates of non-transfected IMR-32 and APP_{SW}-transfected HEK293 cells with or without cholesterol loading and stained with antibodies to these proteins. Antibodies C40 and 6E10 recognize the C-terminal and Aβ regions of APP, respectively. A cholesterol-promoted interaction between ABCA1 and APP/Aβ was demonstrated. As a negative control, an antibody to COX-IV, a mitochondrial membrane protein, was used. **D:** IMR-32 cells were cholesterol loaded in the presence of an antibody to the N-terminus (82E1) or C-terminus (6A for Aβ40 and 11C for Aβ42) of Aβ or an antibody to FLAG (M2). None of the antibodies affected cellular cholesterol levels, suggesting that ABCA1-Aβ interaction does not occur between the cell surface and the extracellular space. All columns and bars represent the mean ± SEM (n = 3).

peripheral tissues and, if so, whether Aβ has any effect on such efflux. For this purpose, we reconstituted lipoproteins with or without Aβ using ^{14}C-cholesterols (Fig. 4A) and injected them into the cerebral ventricle of APP knockout mice. Ten or sixty minutes after injection, mice were sacrificed and dissected into tissues including the brain, heart, kidney, liver, lung, and spleen. The distribution of the injected ^{14}C-cholesterol in tissues was determined by measurement of the radioactivity of tissue homogenates. The injected cholesterol was found to be excreted rapidly from the brain into the peripheral tissues (Fig. 4B). Compared with control lipoproteins without Aβ, lipoproteins with Aβ exhibited accelerated transport from the brain to the liver (Fig. 4C). This finding supports our hypothesis that Aβ plays a role in maintenance of cerebral cholesterol homeostasis as well as of cellular cholesterol homeostasis. Even in the absence of Aβ, however, a certain amount of the injected cholesterol was excreted into the peripheral tissues. This might have occurred as a result of the function of endogenous brain apolipoproteins, such as apoE, or as a result of the conversion of cholesterol to membrane-diffusible oxysterols (Björkhem, 2006), which may have proceeded during preparation of lipoprotein-like particles.

DISCUSSION

High levels of plasma cholesterol are known to be a risk factor for AD (Solomon and Kivipelto, 2009; Stefani and Liguri, 2009). This epidemiological linkage could be explained by experimental evidence that cellular cholesterol influences the activities of both β- and γ-secretases and thereby affects Aβ production and amyloid pathology in the brain (Simons et al., 1998; Frears et al., 1999; Refolo et al., 2000; Petanceska et al., 2002; Shie et al., 2002; Grimm et al., 2008; Xiong et al., 2008). Nevertheless, it remains unclear why high levels of cholesterol increase Aβ production and what the physiological function of Aβ is.

With regard to the biochemical and physiological aspects of Aβ, there are several features common to Aβ and apoE. First, both proteins are amphiphilic and exist on HDL-like lipoproteins in the CSF (Koudinov et al., 1996). Second, each can function as a ligand for LDL receptor-related protein 1 (LRP1) and the VLDL receptor and is transported from the brain to the circulation across the BBB (Deane et al., 2008). Third, both are up-regulated in response to high cholesterol stimulation in vivo (Wu et al., 2003; Petanceska et al., 2003). These findings imply that Aβ may play a role in cholesterol transport and metabolism as a member of the apolipoproteins or in cooperation with them. In the present study, we showed that Aβ promoted efflux of cellular cholesterol during its secretion, which was mediated by ABCA1 and was accompanied by or with formation of lipoprotein-like particles. This mechanism resembles that of apolipoprotein-mediated cholesterol efflux, although apolipoproteins interact with ABCA1 from the

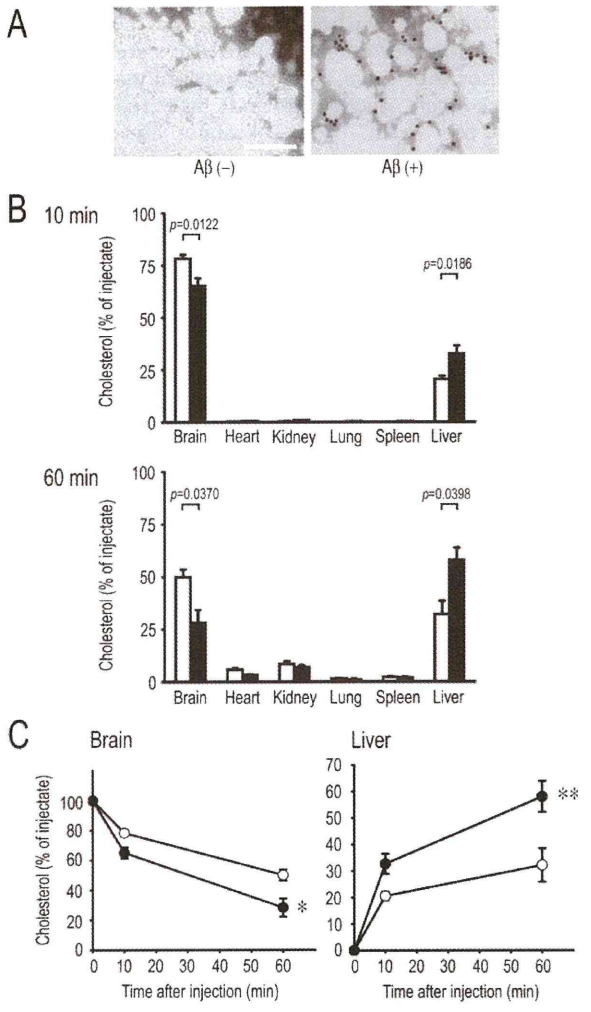

Fig. 4. Aβ accelerates cholesterol transport from the brain into the peripheral tissues. **A:** ^{14}C-labeled lipoprotein-like particles with or without Aβ were prepared. Aβ was probed with 10-nm gold particles. **B:** The reconstituted lipoproteins were injected into the cerebral ventricle of APP knockout mice (100,000 dpm/1 μl/head). At 10 and 60 min after injection, radioactivity in various tissues was measured. Approximately 60,000 dpm was successfully injected into the brain. Open columns, Aβ (−); solid columns, Aβ (+). Comparison between Aβ (+) and Aβ (−) in each tissue was performed using the unpaired Student's *t*-test. Columns and bars represent the mean ± SEM (10 min, n = 5; 60 min, n = 3). **C:** Aβ-containing lipoproteins exhibited accelerated excretion from the brain into the peripheral tissues compared with control lipoproteins. Values at each point were calculated assuming that total count of tissues represents 100% successful injection and that the counts in the brain and liver at 0 min account for 100% and 0% of the injectate, respectively. Open circles, Aβ (−); solid circles, Aβ (+). $*P = 0.0007$, $**P = 0.0011$ vs. Aβ (−), when values were compared 10–60 min after injection using two-way factorial ANOVA followed by Fisher's PLSD test. Scale bar = 100 nm.

extracellular space (Yokoyama, 2005), whereas the Aβ–ABCA1 interaction appeared to occur within the plasma membrane or intracellular compartments. Our findings suggest a novel, apolipoprotein-like function of Aβ by which Aβ participates in cellular cholesterol homeostasis. This is evidence for the biological significance of Aβ production.

A different mechanism by which Aβ regulates cellular cholesterol levels has been proposed. Grimm et al. (2005) have demonstrated that Aβ40 reduces cholesterol synthesis by inhibiting HMG-CoA reductase, whereas Aβ42 down-regulates sphingomyelin levels by activating sphingomyelinases. This finding is somewhat contrary to our finding that Aβ42 appeared to be more effective than Aβ40 in reduction of cellular cholesterol levels. Although the lower levels of cellular cholesterol we observed in APP-expressing cells likely reflect enhanced cholesterol efflux from cells rather than reduced cholesterol synthesis within cells, our results do not exclude the latter possibility. It may be that Aβ regulates cellular cholesterol levels by dual mechanisms, i.e., enhanced efflux by Aβ42 and reduced synthesis by Aβ40, both of which function as a negative feedback system against high levels of cellular cholesterol. On the other hand, Liu et al. (2007) have suggested that AICD, but not Aβ, regulates cellular and cerebral cholesterol levels through LRP1. AICD suppressed expression of the major apoE/lipoprotein receptor LRP1 and thereby reduced cellular uptake of apoE/lipoprotein. According to their hypothesis, the reduced cellular uptake of apoE/lipoprotein results in a decrease in their catabolism, which ends in increased apoE levels and decreased cholesterol levels in cells and in the brain. Consequently, cholesterol-enhanced γ-cleavage of APP, which generates not only AICD but also Aβ as a byproduct, would lead to down-regulation of cellular cholesterol levels. Our results indicate that AICD did not contribute to reduction of cellular cholesterol levels in transfected cells. However, in our assay, cholesterol was loaded onto cells not via LRP1 but by using MβCD. Thus, it is possible to assume that Aβ regulates cellular cholesterol by promoting its efflux and simultaneously AICD does so by inhibiting cellular cholesterol uptake. The lower and higher levels of cellular cholesterol that we observed in APP transgenic and knockout mouse brains may be, in part, attributable to the action of AICD. As an alternative mechanism, it is possible that Aβ or AICD induces expression of apoE, which in turn mediates cholesterol efflux from cells by interacting with ABCA1. This possibility remains to be addressed.

We also showed in the present study that Aβ accelerated transport of brain cholesterol to the peripheral tissues, at which cholesterol was injected into mouse cerebral ventricle as lipoprotein-like particles. Aβ has been demonstrated to be excreted from the brain to the circulation by receptor-mediated transcytosis at the BBB (Shibata et al., 2000) and the choroid plexus (i.e., the blood–CSF barrier; Crossgrove et al., 2005). LRP1 is the major receptor involved in this transport (Zlokovic,

2004; Johanson et al., 2008). This receptor also mediates the clearance of brain apoE to the circulation (Deane et al., 2008). Our finding implies an additional physiological function of Aβ by which Aβ transports brain cholesterol to the BBB and/or the blood–CSF barrier and discharges it into the circulation probably by a receptor-mediated mechanism. It is believed that cerebral cholesterol is excreted to the circulation at the BBB only after its conversion into 24S-hydroxycholesterol, which can cross the cell membranes more easily than cholesterol itself (Björkhem, 2006). The efflux of 24S-hydroxycholesterol to the circulation is presumed to occur by diffusion as a result of its concentration gradient between the brain and the circulation. Inversely, another oxysterol, 27-hydroxycholesterol, has been observed to be taken up by the brain from the circulation (Björkhem, 2006). This influx is also presumed to occur by diffusion dependent on its concentration gradient between the two compartments. Thus, cerebral cholesterol levels are thought to be regulated by de novo synthesis and by flux of these oxysterols and not by transport of cholesterol itself or lipoproteins. However, not a few reports have demonstrated that transcytosis of lipoproteins potentially occurs at the BBB (Dehouck et al., 1997; Balazs et al., 2004; Candela et al., 2008; Rohrer et al., 2009). In addition, 24S-hydroxycholestrol, as well as cholesterol, is not present free in plasma but is associated with acceptor proteins such as LDL and HDL (Babiker and Diczfalusy, 1998). Thus, it is likely that cerebral cholesterols including 24S-hydroxycholesterol are transported by apoE- and/or Aβ-containing lipoproteins to the BBB and/or the blood–CSF barrier and then are excreted to the circulation by transcytosis or by diffusion after dissociation from lipoproteins. Cerebral Aβ levels are regulated by de novo synthesis and degradation and also by influx and efflux at the BBB and the blood–CSF barrier (Zlokovic et al., 2000). Whereas the efflux is mediated mainly by LRP1, the influx is done primarily by the receptor for advanced glycation end products (RAGE; Zlokovic, 2004; Johanson et al., 2008). As well as the influx of oxysterols, the RAGE-mediated Aβ influx may also contribute to the regulation of cerebral cholesterol homeostasis.

The hypothesis presented here not only explains why cholesterol is a risk factor for AD but also provides new insights into the known link between apoE genotypes and AD (Corder et al., 1993). It is shown that the ability of apoE4 to mediate cholesterol efflux from cells is less than the abilities of apoE2 and apoE3 and that the presence of apoE4 therefore results in higher levels of cellular cholesterol in vitro (Michikawa et al., 2000). In addition, clearing of apoE4 from mouse brain across the BBB is less efficient than for apoE2, apoE3, and Aβ (Deane et al., 2008). Thus, the presence of apoE4 would result in enhanced Aβ production (Ye et al., 2005) to compensate for its lower cholesterol efflux, which might lead to increased accumulation of Aβ in the brain. Furthermore, compared with apoE2 and apoE3, apoE4 in the circulation is shown to be significantly taken up by cerebral microvessels and choroids plexus when allowed to form a complex with Aβ, which resulted in a moderate, but significant, transport of this complex into the brain (Martel et al., 1997). This may also contribute to enhanced amyloid pathology in patients with apoE4. Our findings also highlight a potential adverse effect of agents administered to reduce Aβ production, such as β- and γ-secretase inhibitors: inhibition of Aβ production may disturb its function in cholesterol homeostasis proposed in this study. Therefore, careful management of cholesterol may be required upon such treatments.

ACKNOWLEDGMENTS

We thank Hideki Nakagawa and Hiroo Nakagawa for their technical assistance.

REFERENCES

Babiker A, Diczfalusy U. 1998. Transport of side-chain oxidized oxysterols in the human circulation. Biochim Biophys Acta 1392:333–339.

Balazs Z, Panzenboeck U, Hammer A, Sovic A, Quehenberger O, Malle E, Sattler W. 2004. Uptake and transport of high-density lipoprotein (HDL) and HDL-associated α-tocopherol by an in vitro blood–brain barrier model. J Neurochem 89:939–950.

Björkhem I. 2006. Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. J Intern Med 260:493–508.

Candela P, Gosselet F, Miller F, Buee-Scherrer V, Torpier G, Cecchelli R, Fenart L. 2008. Physiological pathway for low-density lipoproteins across the blood–brain barrier: transcytosis through brain capillary endothelial cells in vitro. Endothelium 15:254–264.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. 1993. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921–923.

Crossgrove JS, Li GJ, Zheng W. 2005. The choroid plexus removes β-amyloid from brain cerebrospinal fluid. Exp Biol Med 230:771–776.

Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV. 2008. ApoE isoform-specific disruption of amyloid β peptide clearance from mouse brain. J Clin Invest 118:4002–4013.

Dehouck B, Fenart L, Dehouck MP, Pierce A, Torpier G, Cecchelli R. 1997. A new function for the LDL receptor: transcytosis of LDL across the blood–brain barrier. J Cell Biol 138:877–889.

Frears ER, Stephens DJ, Walters CE, Davies H, Austen BM. 1999. The role of cholesterol in the biosynthesis of β-amyloid. Neuroreport 10:1699–1705.

Grimm MOW, Grimm HS, Pätzold AJ, Zinser EG, Halonen R, Duering M, Tschäpe JA, De Strooper B, Müller U, Shen J, Hartmann T. 2005. Regulation of cholesterol and sphingomyelin metabolism by amyloid-β and presenilin. Nat Cell Biol 7:1118–1123.

Grimm MOW, Grimm HS, Tomic I, Beyreuther K, Hartmann T, Bergmann C. 2008. Independent inhibition of Alzheimer disease β- and γ-secretase cleavage by lowered cholesterol levels. J Biol Chem 283:11302–11311.

Hatch FT. 1968. Practical methods for plasma lipoprotein analysis. Adv Lipid Res 6:1–68.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. 1996. Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science 274:99–102.

Johanson CE, Duncan JA 3rd, Klinge PM, Brinker T, Stopa EG, Silverberg GD. 2008. Multiplicity of cerebrospinal fluid functions: new challenges in health and disease. Cerebrospinal Fluid Res 5:10.

Klein U, Gimpl G, Fahrenholz F. 1995. Alteration of the myometrial plasma membrane cholesterol content with b-cyclodextrin modulates

the binding affinity of the oxytocin receptor. Biochemistry 34:13784–13793.

Kontush A. 2004. Apolipoprotein Aβ: black sheep in a good family. Brain Pathol 14:433–447.

Koudinov AR, Koudinova NV, Kumar A, Beavis RC, Ghiso J. 1996. Biochemical characterization of Alzheimer's soluble amyloid β protein in human cerebrospinal fluid: association with high density lipoproteins. Biochem Biophys Res Commun 223:592–597.

Lippa CF, Ozawa K, Mann DM, Ishii K, Smith TW, Arawaka S, Mori H. 1999. Deposition of β-amyloid subtypes 40 and 42 differentiates dementia with Lewy bodies from Alzheimer disease. Arch Neurol 56:1111–1118.

Liu Q, Zerbinatti CV, Zhang J, Hoe HS, Wang B, Cole SL, Herz J, Muglia L, Bu G. 2007. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. Neuron 56:66–78.

Martel CL, Mackic JB, Matsubara E, Governale S, Miguel C, Miao W, McComb JG, Frangione B, Ghiso J, Zlokovic BV. 1997. Isoform-specific effects of apolipoproteins E2, E3, and E4 on cerebral capillary sequestration and blood–brain barrier transport of circulating Alzheimer's amyloid β. J Neurochem 69:1995–2004.

Matsuyama S, Teraoka R, Mori H, Tomiyama T. 2007. Inverse correlation between amyloid precursor protein and synaptic plasticity in transgenic mice. Neuroreport 18:1083–1087.

Michikawa M, Fan QW, Isobe I, Yanagisawa K. 2000. Apolipoprotein E exhibits isoform-specific promotion of lipid efflux from astrocytes and neurons in culture. J Neurochem 74:1008–1016.

Michikawa M, Gong JS, Fan QW, Sawamura N, Yanagisawa K. 2001. A novel action of Alzheimer's amyloid β-protein (Aβ): oligomeric Aβ promotes lipid release. J Neurosci 21:7226–7235.

Nishitsuji K, Tomiyama T, Ishibashi K, Ito K, Teraoka R, Lambert MP, Klein WL, Mori H. 2009. The E693Δ mutation in amyloid precursor protein increases intracellular accumulation of amyloid β oligomers and causes endoplasmic reticulum stress-induced apoptosis in cultured cells. Am J Pathol 174:957–969.

Petanceska SS, DeRosa S, Olm V, Diaz N, Sharma A, Thomas-Bryant T, Duff K, Pappolla M, Refolo LM. 2002. Statin therapy for Alzheimer's disease: will it work? J Mol Neurosci 19:155–161.

Petanceska SS, DeRosa S, Sharma A, Diaz N, Duff K, Tint SG, Refolo LM, Pappolla M. 2003. Changes in apolipoprotein E expression in response to dietary and pharmacological modulation of cholesterol. J Mol Neurosci 20:395–406.

Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, Sambamurti K, Duff K, Pappolla MA. 2000. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol Dis 7:321–331.

Rohrer L, Ohnsorg PM, Lehner M, Landolt F, Rinninger F, von Eckardstein A. 2009. High-density lipoprotein transport through aortic endothelial cells involves scavenger receptor BI and ATP-binding cassette transporter G1. Circ Res 104:1142–1150.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J, Zlokovic BV. 2000. Clearance of Alzheimer's amyloid-β_{1-40} peptide from brain by LDL receptor-related protein-1 at the blood–brain barrier. J Clin Invest 106:1489–1499.

Shie FS, Jin LW, Cook DG, Leverenz JB, LeBoeuf RC. 2002. Diet-induced hypercholesterolemia enhances brain Aβ accumulation in transgenic mice. Neuroreport 13:455–459.

Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, Simons K. 1998. Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons. Proc Natl Acad Sci U S A 95:6460–6464.

Solomon A, Kivipelto M. 2009. Cholesterol-modifying strategies for Alzheimer's disease. Expert Rev Neurother 9:695–709.

Stefani M, Liguri G. 2009. Cholesterol in Alzheimer's disease: unresolved questions. Curr Alzheimer Res 6:15–29.

Suga K, Tomiyama T, Mori H, Akagawa K. 2004. Syntaxin 5 interacts with presenilin holoproteins, but not with their N- or C-terminal fragments, and affects β-amyloid peptide production. Biochem J 381:619–628.

Tajima S, Yokoyama S, Yamamoto A. 1983. Effect of lipid particle size on association of apolipoproteins with lipid. J Biol Chem 258:10073–10082.

Tsujita M, Wu CA, Abe-Dohmae S, Usui S, Okazaki M, Yokoyama S. 2005. On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway. J Lipid Res 46:154–162.

Wu CW, Liao PC, Lin C, Kuo CJ, Chen ST, Chen HI, Kuo YM. 2003. Brain region-dependent increases in β-amyloid and apolipoprotein E levels in hypercholesterolemic rabbits. J Neural Transm 110:641–649.

Xiong H, Callaghan D, Jones A, Walker DG, Lue LF, Beach TG, Sue LI, Woulfe J, Xu H, Stanimirovic DB, Zhang W. 2008. Cholesterol retention in Alzheimer's brain is responsible for high β- and γ-secretase activities and Aβ production. Neurobiol Dis 29:422–437.

Yao ZX, Papadopoulos V. 2002. Function of β-amyloid in cholesterol transport: a lead to neurotoxicity. FASEB J 16:1677–1679.

Ye S, Huang Y, Müllendorff K, Dong L, Giedt G, Meng EC, Cohen FE, Kuntz ID, Weisgraber KH, Mahley RW. 2005. Apolipoprotein (apo) E4 enhances amyloid β peptide production in cultured neuronal cells: apoE structure as a potential therapeutic target. Proc Natl Acad Sci U S A 102:18700–18705.

Yokoyama S. 2005. Assembly of high density lipoprotein by the ABCA1/apolipoprotein pathway. Curr Opin Lipidol 16:269–279.

Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, Stevens KA, Slunt HH, Sisoda SS, Chen HY, Van der Ploeg LH. 1995. β-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81:525–531.

Zlokovic BV. 2004. Clearing amyloid through the blood–brain barrier. J Neurochem 89:807–811.

Zlokovic BV, Yamada S, Holtzman D, Ghiso J, Frangione B. 2000. Clearance of amyloid β-peptide from brain: transport or metabolism? Nat Med 6:718.