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Influence of Lipoproteins on Microglial Degradation of Alzheimer's Amyloid Beta-Protein

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ABSTRACT Amyloid β-protein (Aβ), the major component of plaques in Alzheimer's disease, is a small hydrophobic protein that is carried on apolipoprotein E (ApoE)- and ApoJ-containing lipoprotein particles in plasma and cerebrospinal fluid (CSF). Microglia, the scavenger cells of the CNS, take up and degrade Aβ via lipoprotein receptors including scavenger receptors A and B, and possibly via other receptors. Lipoproteins, ApoE, and ApoJ influence the uptake and degradation of Aβ in vitro and in vivo. Differences in ApoE-E4, -E3, and -E2 isoforms with respect to Aβ binding to lipoproteins and delivery to cells, including microglia, may contribute to the increased risk of Alzheimer's disease for people with an *APOE*4 genotype and to risk reduction with *APOE*2. *Microsc. Res. Tech. 50:316–324, 2000.* © 2000 Wiley-Liss, Inc.

INTRODUCTION

Extracellular plaques of amyloid β-protein (Aβ) and other associated proteins, distributed in specific brain regions, are a definitive characteristic of Alzheimer's disease, along with intracellular neurofibrillary tangles and loss of synapses. Aβ is a normally secreted byproduct of amyloid precursor protein metabolism that is normally rapidly degraded in a steady-state equilibrium with production (Savage et al., 1998). Accumulations of aggregated Aβ in solution or deposited in plaques may be damaging to neurons, either by direct toxicity or through inflammatory, neurotoxic secretions of microglia cells. Accrual of plaques appears to reach an equilibrium during the disease, based in part on the demonstration by Hyman et al. (1993) that whereas a higher total area of Aβ immunoreactivity in temporal cortex distinguishes Alzheimer's disease from normal brain tissue, there is no correlation of amount of Aβ immunoreactivity with the duration of illness. Rather than plaque deposition continuing unchecked throughout the course of the disease, it appears that turnover occurs, so that as amyloid is deposited, a percentage of it is also removed. Consistent with the idea of turnover of plaque material during the disease, the form of Aβ found in plaques changes from predominantly Aβ1–42 at early stages (diffuse plaques) to predominantly Aβ1–40 at later stages (senile plaques) (Dickson, 1997; Nakamura et al., 1997). In contrast, SDS insoluble, formic acid extractable amyloid, accumulates progressively and correlates with cognitive decline (Naslund et al., 2000).

Microglia are likely agents of Aβ aggregation and amyloid removal. Activated, HLA-DR positive microglia are closely associated with plaques (Dickson et al., 1993; Mattiace et al., 1990; Styren et al., 1990; Tooyama et al., 1990; for review see Kalaria, 1999). They are the phagocytic scavenger cells of the CNS, and they have multiple receptors for and take up both soluble and fibrillar Aβ in vitro (Ard et al., 1996; Cole et al., 1999; Paresce et al., 1996, 1997; Shaffer et al., 1995). Similarly, in vivo after injection of Aβ into hippocampus, microglia loaded with Aβ-immunoreactive material are found migrating toward ventricles and blood vessels (Frautschy et al., 1992).

Increasing amyloid removal by microglia is a potential therapeutic target in Alzheimer's disease. This will require understanding of both microglial endocytosis and degradation of Aβ, which may serve as a salutary clearance mechanism, and microglial inflammatory reactions to Aβ uptake. This review focuses on endocytosis and degradation of Aβ and its modulation by lipoproteins.

Lipoproteins are carriers of Aβ in biological fluids (Biere et al., 1996; Fagan et al., 1999; Koudinov et al., 1994, 1996; Koudinov and Koudinova, 1997; LaDu et al., 1995), and this discovery has led to interest in the influence of lipoprotein on the microglial-Aβ interaction. The observed effects of the E4 allele of apolipoprotein E (*APOE*4) on risk for Alzheimer's disease, reducing the age of onset (Saunders et al., 1993) and increasing the amount of amyloid plaque burden in Alzheimer's brain tissue (Gearing et al., 1996; Mann et al., 1997; Schmechel et al., 1993), could result directly from a lipoprotein role in Aβ trafficking and clearance. In keeping with such a role, polymorphism in an ApoE receptor, LRP, has recently been shown to be associated with Alzheimer's disease (Hollenbach et al., 1998). Lipoproteins may act at several levels as they affect aggregation of soluble Aβ into fibrils, binding of Aβ to cell surface receptors for endocytosis, lysosomal degradation of Aβ, and the inflammatory response.

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Aβ CIRCULATES BOUND TO LIPOPROTEINS

Like other nonpolar or hydrophobic peptides, Aβ in aqueous body fluids attaches to a carrier protein or lipoprotein particle for solubilization. Studies of Aβ in plasma by LaDu et al. (1995) and by Koudinov et al. (1994) showed that Aβ circulating in plasma is bound to lipoprotein particles, mainly to HDL. In one set of experiments in which radiolabeled Aβ was added to plasma, serum albumin as well as lipoprotein was found to bind Aβ (Biere et al., 1996). Transthyretin has also been reported as an Aβ carrying protein (Schwarzman et al., 1994). The discovery of lipoprotein carriers for Aβ was quickly followed by fractionation of CSF and identification of native Aβ in lipoprotein fractions, leading to the conclusion that Aβ circulates in CSF as a component of lipoprotein particles, mainly ApoE- and ApoJ-containing lipoproteins similar in size and density to plasma HDL (Fagan et al., 1999; Koudinov et al., 1996).

Lipoprotein is produced by astrocytes, secreted as relatively lipid-poor apolipoprotein and lipid particles (LaDu et al., 1998). Aβ itself in a transfected cell line is secreted with lipid as part of a lipoprotein particle (Koudinov and Koudinova, 1997). Therefore, Aβ is most likely bound to lipoprotein in brain parenchyma, as in CSF. This is an important point since in Alzheimer's disease the level of soluble Aβ1–42 in brain tissue is about 50 times greater than that in CSF (Kuo et al., 1996). Furthermore, increases in levels of Aβ in Alzheimer's relative to control brains are found in brain tissue rather than in CSF (Kuo et al., 1996; Tabaton et al., 1994; Wang et al., 1999).

Within lipoproteins Aβ is bound to ApoJ and to ApoE (Ghiso et al., 1993; Golabek et al., 1995; Matsubara et al., 1996; Wisniewski et al., 1993) as well as to lipids (Koudinov et al., 1998). Binding of synthetic Aβ to ApoJ in its native lipidated form prevents fibril formation in vitro (Matsubara et al., 1996). This observation heightened interest in the possibility that *APOE* allelic differences in risk for Alzheimer's disease might be based on differences in direct interaction between ApoE and Aβ.

Aβ CAN BE ENDOCYTOSED VIA MICROGLIAL LIPOPROTEIN RECEPTORS

Activated microglia express most if not all macrophage cell surface markers including lipoprotein receptors. The standard tool for identifying microglia is a fluorescently tagged acetylated LDL probe for scavenger receptor; they also have LRP, LDL receptor, and presumably other macrophage receptors. These receptors allow macrophages to play a major role in lipid uptake, especially after injury. Therefore, microglia can be expected to avidly take up ApoE-containing lipoprotein particles (with or without Aβ) after brain injury, when the *APOE*4-related risk of Aβ deposits dramatically increases (Nicoll et al., 1995).

The macrophage scavenger receptor (class A, Yamada et al., 1998), which appears on microglia in response to activation (Bell et al., 1994) and in Alzheimer's plaques (Christie et al., 1996; Honda et al., 1998), binds and internalizes fibrillar Aβ in vitro (Chu et al., 1998; El Khoury et al., 1996; Paresce et al., 1996, 1997). Binding of Aβ fibrils to scavenger receptor stimulates microglial secretion of reactive oxygen species, which can be blocked by a synthetic peptide competitively inhibiting binding to the receptor (El Khoury et al., 1996). Uptake of Aβ microaggregates or fibrils is also partially inhibited by the scavenger receptor ligands acetylated LDL, maleylated bovine serum albumin, or fucoidan (Chu et al., 1998; Paresce et al., 1996).

Scavenger receptors of class B (SR-B) were also investigated by Paresce et al. (1996), using CHO cells transfected with the hamster SR-B1 receptor. These receptors also mediated uptake of Aβ microaggregates, which was competitively inhibited by acetylated LDL, maleylated bovine serum albumin, or, to a lesser extent, fucoidan. Ligands internalized via SR-B1, such as HDL, acetylated LDL, β-VLDL, and lipoprotein-bound ApoE, instead of entering the lysosomal degradation pathway, may alternatively be sequestered in surface-connected compartments formed by deep invaginations of the macrophage plasma membrane (Kruth et al., 1995). Aβ enters surface-connected compartments in microglia (Fig. 1; Cole et al., 1999). Entry into this compartment may delay proteolytic degradation and facilitate resecretion of undegraded protein. In fact, an exceptional function of SR-B1 is that it mediates delivery of HDL lipids to cells without entry of the associated apolipoproteins into the coated pit-endosome-lysosome pathway (Acton et al., 1996).

Apolipoproteins that are delivered into the lysosomal pathway by other receptors such as class A scavenger receptors, LDL receptor, or LRP may either separate from the lipid moiety, as ApoE does, or, like ApoB, remain attached to lipid in lysosomes as further separation of lipid and protein occurs. In our electron microscopical studies of cellular internalization of Aβ, immunolabeled Aβ is frequently seen attached to lipid in lysosomes (Fig. 1; Cole et al., 1999).

Cells may also resecrete intact Aβ after uptake, as first convincingly shown by Chung et al. (1999). In agreement with earlier reports (Ard et al., 1996; Cole et al., 1999; Frackowiak et al., 1992; Paresce et al., 1996, 1997; Shaffer et al., 1995), the authors found that microglia accumulated fibrillar Aβ. While some Aβ was degraded, a substantial proportion was retained undegraded for up to 7 days, and a part of the ingested fibrillar Aβ was resecreted into the chase medium for up to 12 days after loading for 1 hour. Soluble Aβ, in contrast to fibrillar, was taken up by microglia and almost entirely resecreted into the medium over a period of hours, with little degradation. Uptake of soluble Aβ was not saturable and was not competitively inhibited by scavenger receptor ligands; therefore, it appeared to occur by non-receptor-mediated fluid-phase endocytosis. Like fibrillar Aβ, soluble Aβ appeared to enter lysosomes, based on light microscopic colocalization with fluorescently labeled α2-macroglobulin.

Aβ entering cells via lipoprotein receptors may ultimately affect the lipid content of membranes. Lysosomal membrane integrity was compromised by cellular uptake of Aβ1–42, but not Aβ1–40, in a cell line (Yang et al., 1998), and in synaptosome preparations treated with Aβ in vitro, changes in membrane phospholipids were observed (Mattson et al., 1998). Aβ has been shown to increase free cholesterol in neurons, altering trafficking of intracellular transport vesicles (Liu et al., 1998). The increase in cholesterol, due to Aβ or lipopro-

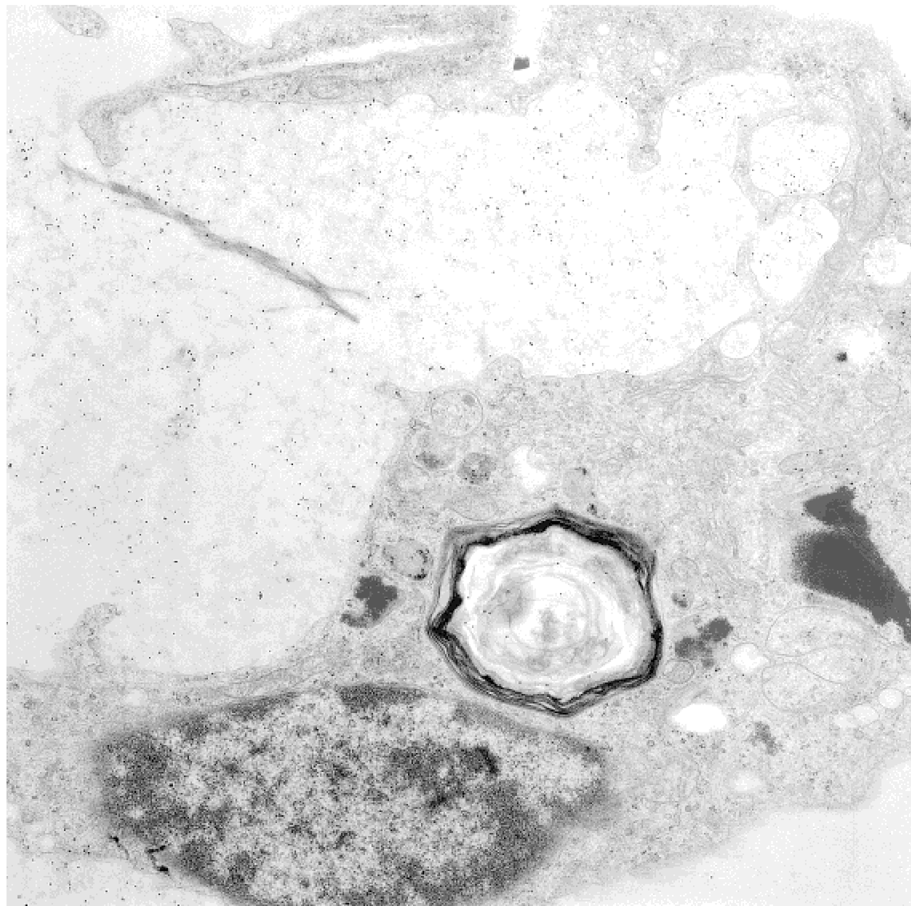

Fig. 1. Electron micrograph of a microglia cell cultured from adult rat, incubated overnight with Aβ1-42 and HDL. Postembedding immunolabeling with 15 nm gold shows aggregated Aβ in a large phagocytic compartment that remains open to the extracellular space (at upper left). The single arrow indicates sequestration of the partially ingested Aβ into smaller, surface-connected compartments that retain continuity with the phagocytic compartment and extracellular space. Within the cytoplasm, a large secondary lysosome containing lipid lamellae is also immunolabeled for Aβ.

tein mediated cholesterol uptake into surface-connected compartments (caveolae) can alter APP processing by reducing alpha secretase activity (Bodovitz et al., 1996) and increasing Aβ generation (Simons et al., 1998). Aβ1–42 at less than nanomolar concentrations activates the enzyme phospholipase A_2 in vitro, with the effect dependent on the composition of the phospholipid substrate (Lehtonen et al., 1996). In the HepG2 cell line, Aβ1–40 decreases synthesis of esterified cholesterol and phospholipids (Koudinova et al., 1996). Phospholipid changes also occur in brain tissue from Alzheimer's disease cases, with a reduction in long-chain fatty acids in phosphatidylcholine and phosphatidylethanolamine (Corrigan et al., 1998).

In addition to lipoprotein receptor binding, Aβ has also been shown to bind to the RAGE receptor (receptor for advanced glycation end products, a member of the immunoglobulin superfamily) on microglia, neurons, and endothelial cells in a dose-dependent manner (Yan et al., 1996). Soluble Aβ or Aβ immobilized on a substrate binds to RAGE; it does not appear to be a receptor for fibrils. However, Aβ activation of immortalized BV-2 microglia, measured by stimulation of cell migration, secretion of TNFα, increase of TNFα mRNA, and activation of NF-κB, could be inhibited by anti-RAGE $F(ab')_2$. Soluble Aβ, but not fibrillar, is also reported to bind to the serpin-enzyme complex receptor (receptor for serine protease inhibitor-enzyme complexes) (Boland et al., 1995).

A caveat about receptors comes from the studies of signal transduction mechanisms by McDonald et al. (1997, 1998). These investigators confirmed previous reports of activation of microglia by fibrillar Aβ, and in addition assayed not only inflammatory mediators (superoxide radicals and IL-1β) secreted by the cells but also signal transduction molecules of the mitogen-activated protein kinase superfamily. They found that whereas fibrillar Aβ activated kinases in this cascade, other scavenger receptor ligands and RAGE ligands were ineffective or much less effective, indicating that Aβ acted at least partially through a different, unknown receptor pathway. These experiments used a large amount of Aβ, 50 μm, leaving open the possibility that phagocytosis of fibrils might have contributed to activation of the cells in a way that was chemically nonspecific (Yates et al., 1999).

LIPOPROTEIN DEPENDENCE OF Aβ-RECEPTOR INTERACTION IS UNTESTED

Studies of Aβ binding to microglial receptors are difficult to interpret for at least three reasons. First, Aβ is almost always presented in free form rather than associated with a lipoprotein or other carrier as it is in CSF and probably in brain. Indeed, this was the method used in all of the studies by El Khoury et al. (1996), Paresce et al. (1996, 1997), Chung et al. (1999), Yan et al. (1996), Boland et al. (1995), and McDonald et al. (1997, 1998) discussed above.

Second, even though exogenous lipoprotein is not added to the incubation medium, microglia may secrete lipoprotein or ApoE; this secretion may vary from experiment to experiment and it is not controlled for. Although astrocytes are believed to be the major producers of ApoE in the CNS, macrophages are known to have robust ApoE synthesis (Deng et al, 1995), and microglia in culture synthesize as much ApoE as astrocytes (fig. 6 in Cole et al., 1999; unpublished observations). The major pool of macrophage ApoE is retained on the cell surface bound to heparan sulfate proteoglycans (Lucas and Mazzone, 1996). This ApoE can be secreted or reinternalized, either directly or via higher affinity receptors on the cell surface such as LRP, LDL receptor, or scavenger receptors. Potentially, then, endogenous ApoE may affect Aβ binding to microglia in vitro.

Third, lipoprotein receptors share a common low-affinity co-receptor, cell-surface heparan sulphate proteoglycan (HSPG). Cell surface HSPGs are now thought to initially bind lipoproteins (Mahley, 1996), serving as high capacity, low affinity receptors for ApoE-containing particles, which are then passed on to LRP, LDL, scavenger or other high affinity receptors. This trafficking is ApoE isoform-dependent so that in tests with neuronal cells, ApoE3 accumulates 3–6-fold greater than ApoE4 (Mahley, 1996, 1997; Mahley et al., 1996; Weisgraber and Mahley, 1996). New data on biochemical differences show that ApoE3 and E4 differ in that Arg 61 forms an intramolecular salt bridge in E3, but not in E4 (2). This appears to result in differential association with cell surface HSPGs. Cell surface HSPGs are essential for selective accumulation of ApoE via lipoprotein receptors and may also regulate accumulation of Aβ bound to ApoE and subsequent uptake and degradation or resecretion and deposition.

LIPOPROTEINS AFFECT DEGRADATION OF Aβ BY MICROGLIA IN VITRO

Considering that there is good evidence that Aβ in CSF is carried by lipoprotein, it is surprising that so few studies of Aβ cell binding and degradation have incorporated lipoprotein or serum into the incubation medium. We have found profound differences in microglial uptake and degradation of Aβ1–42 depending on the presence or absence of lipoprotein in the incubation medium (Cole et al., 1999). The different lipoproteins showed distinct differences in the ultimate amount of the Aβ removed from the medium, accumulated in the cells or degraded.

Lipoproteins in 2% fetal bovine serum or human plasma HDL increased microglial removal of Aβ from the medium, compared to control medium containing 0.1% bovine serum albumin without exogenous lipoprotein (Table 1). At the same time that Aβ was removed from the medium, very little accumulated in the cell pellet, suggesting degradation was stimulated by these lipoproteins. Plasma HDL has been used as a model for CSF lipoproteins in some of our own and other studies because of their similarities including particle size and apolipoprotein content (Borghini et al., 1995; Koudinov et al., 1996; Pitas et al., 1987). However, there are functional differences between them, as noted by Rebeck et al. (1998), who observed that CSF lipoprotein, but not plasma HDL, competitively inhibits LDL degradation by fibroblasts. Whether CSF HDL also stimulates Aβ degradation remains unknown.

TABLE 1. Effect of apolipoproteins on microglial Aβ accumulation[1]

Lipoprotein	Aβ loss from medium	Aβ accumulation in cell pellet	Aβ degradation (net loss)	ApoE accumulation by cells
Aβ alone	+	+	+	—
FBS	—	<<	—	—
HDL	++	<<<	++	—
ApoJ	<	<<	—	—
ApoJ + E4	<	+	—	++
ApoJ + E3	<<	+	—	++
ApoJ + E2	<<	<	—	+

[1]+ = positive result in the parameter indicated;
++ = larger positive result than +;
< = less than seen with Aβ alone, without lipoprotein; << = less than <;
— = values not measured for comparison with this set of experiments.

The apolipoproteins reconstituted in liposomes did not mimic complete HDL purified from pooled human plasma, which is not surprising as HDL (unlike ApoE or ApoJ) is known to be a good scavenger receptor B1 ligand on macrophage lineage cells. Nevertheless, use of the liposomes did show differences between ApoE isoforms, as displayed in Table 1. In contrast to plasma HDL, dimyristoylphosphatidylcholine liposomes with reconstituted ApoJ and recombinant ApoE2, -E3, or -E4 decreased Aβ removal from the media and increased total Aβ remaining, suggesting reduced Aβ clearance. These results were taken from Western blotting, which allowed densitometric comparisons of Aβ monomer and aggregates remaining in the medium at the end of the incubation and Aβ accumulated by the cells. Simultaneous reductions in both pools indicated net loss, which was interpreted as degradation based on sensitivity to protease inhibitors.

Compared to ApoE3 and ApoE2, ApoE4 resulted in more Aβ removal from the medium. This is consistent with the reduced affinity of ApoE4 for Aβ, since it resulted in a clearance pattern more similar to that seen for free Aβ. ApoE3 and ApoE2 had equivalent levels of Aβ removed from the medium, but with ApoE2 there was less accumulation in the cell pellet, indicating more effective net Aβ degradation, a phenomenon that could help account for reduced Aβ deposits with *APOE*2 genotype.

In addition to microglia, neurons and smooth muscle cells also respond to the combination of Aβ with lipoproteins. In rat hippocampal neurons in vitro, artificial liposomes containing ApoE4 increased the net internalization of Aβ–40, compared to ApoE3-containing liposomes (Beffert et al., 1998). In the same study, Aβ increased uptake of ApoE by neurons, with a greater increase in uptake of ApoE4 than of ApoE3 liposomes; however, ApoE3 was degraded more effectively than ApoE4, and degradation was not altered by Aβ.

In smooth muscle cells, lipoproteins from either human serum or CSF promoted uptake of Aβ1–40 and Aβ1–42; Aβ internalization was reduced with lipoprotein-deficient serum (Urmoneit et al., 1997). Lipoprotein receptors on these cells appear to mediate Aβ uptake, since it can be inhibited by the 39 kDa receptor-associated protein that blocks LRP and other lipoprotein receptors. Furthermore, Aβ colocalizes im-

munocytochemically with both ApoE and LRP. Degradation of Aβ was not addressed in this study.

ApoJ as well as ApoE binds to Aβ and promotes its uptake via the ApoJ receptor LRP-2 followed by lysosomal degradation (Hammad et al., 1997). In those experiments, formation of the ApoJ/Aβ complex was necessary for LRP-2 receptor recognition of Aβ. In our microglia experiments described in Table 1, particles with ApoJ alone resulted in greater Aβ clearance (removal from the medium, without accumulation in the cells) than particles with both ApoJ and any of the ApoE isoforms (Cole et al., 1999). In the absence of ApoE, ApoJ may contribute to more effective degradation of Aβ, consistent with observations of reduced plaque formation in PDAPP transgenics crossed with *APOE* null mice (Bales et al., 1997).

LIPOPROTEINS AND Aβ DEGRADATION IN VIVO

Although a number of proteases can contribute to Aβ degradation in vitro, there is very limited information about what regulates Aβ degradation in vivo. Evidence for a role of matrix metalloproteases has been developed, but remains inconclusive (Lim et al., 1997). Results of metabolic labeling studies in APP transgenic mice with a 30-minute labeling pulse suggested a rough estimate of t1/2 for the immunoprecipitable pool of endogenous Aβ between 1 and 2.5 hours based on assumptions of a steady state equilibrium with the rate of synthesis (Savage et al., 1998). Injection of small amounts of soluble radiolabeled Aβ into rat hippocampus is followed by a rapid degradation of the Aβ by extracellular proteases, including the metalloprotease, neprilysin (Iwata et al., 2000). The t1/2 for Aβ in these experiments is less than 1 hour. Chronic inhibition of neprilysin results in Aβ deposit formation in rat brain demonstrating the physiological importance of this pathway. Thus, Aβ is normally rapidly degraded and this rapid degradation plays a critical role in preventing Aβ deposit formation.

Interest in Aβ degradation has increased dramatically following reports of a promising Aβ vaccine. Immunization of plaque-forming APP transgenic mice with aggregated Aβ and adjuvant can both prevent Aβ deposit formation and actually reduce previously formed Aβ deposits (Schenk et al., 1999). This report links the efficacy of vaccination to the formation of high titers of anti-Aβ antibodies and the promotion of increased Aβ clearance by Fc receptor–bearing microglia and receptor-mediated endosomal/lysosomal clearance. Increasing Aβ clearance thus shows real therapeutic potential. However, additional vaccine effects are possible, for example, anti-Aβ antibodies are also capable of directly inhibiting Aβ aggregation in vitro (Solomon et al., 1996).

Like Aβ antibodies, lipoproteins are relatively high affinity Aβ binding proteins with Kd in the nanomolar range capable of regulating Aβ aggregation and receptor-mediated Aβ degradation. Further, unlike Fc receptor concentrated on microglia, multiple lipoprotein receptors linked to endocytosis and lysosomal degradation are present not only on microglia but other cell types including neurons and astrocytes. The lower affinity of lipoproteins relative to antibodies may be compensated by CNS micromolar levels of lipoproteins that are likely much higher than the level of Aβ antibodies reaching the brain, which have relatively poor access to the CNS. Thus, the normal regulation of Aβ aggregation and degradation by CNS lipoproteins may be analogous to that of antibodies in preventing self-aggregation and promoting receptor-mediated endocytosis and clearance.

APOE CONTROLS AMYLOID DEPOSITION IN VIVO AND IN VITRO

Whether via directly regulating degradation or indirectly regulating degradation by promoting aggregation and deposition, ApoE clearly controls the ultimate fate of Aβ in vivo. Whereas ApoE had been previously discovered in Aβ deposits (Namba et al., 1992), the identification of the *APOE*4 allele as a genetic risk factor for AD and the *APOE*2 allele as protective (Corder et al.,1994; Saunders et al., 1993) demonstrated some important role for ApoE in the pathogenesis of AD (Hyman, 1997; Mahley, 1997). ApoE deposits are reported to be numerous, not entirely coincident with Aβ and to occur at an early stage in diffuse plaque formation (Nishiyama et al., 1997). However, the ApoE4 isoform consistently increases Aβ1–40 (not 1–42) deposition (Gearing et al., 1996; Ishii et al., 1997; Mann et al., 1997). Direct evidence for a major role in controlling amyloid deposition comes from crosses between *APOE* knockout mice and amyloid plaque–forming APP transgenics. This work has provided compelling evidence that reducing mouse ApoE dramatically limits Aβ deposition and amyloid plaque formation (Bales et al., 1997). Crosses of human *APOE* allele transgenics with APP transgenics on a null background have further demonstrated that ApoE expression and isoform subtype influence amyloid deposit formation, but at early stages of Aβ deposition, human ApoE expression actually reduced deposits in the hippocampus (Holtzman et al., 1999).

There are two major theories to account for these observations. The first theory argues that ApoE4 directly promotes β-amyloid formation and is based on observations showing: (1) ApoE is found in many types of amyloid (Castano et al., 1995a) and, (2) the relative speed and extent of Aβ assembly into amyloid fibrils in vitro can be controlled by the addition of purified (and delipidated and therefore denatured) ApoE (Castano et al., 1995b; Ma et al., 1994; Sanan et al., 1994; Wisniewski et al., 1994). The amyloidogenic effects were in the order ApoE4>ApoE3>ApoE2. By itself, this theory is seemingly in conflict with the data showing increased ApoE4 actually reduced hippocampal Aβ deposition at initial stages (Holtzman et al., 1999), but the early hippocampal deposits may not be amyloid, but preamyloid. The theory is consistent with new data from the same group showing ApoE4 later increases Aβ deposition (see review by Fagan et al., pages xxx–xxx, this issue) and observations showing overexpression of human ApoE4 in APPsw transgenics on a mouse *APOE* background results in accelerated Aβ deposition (Carter et al., 1999).

A second theory is based on the finding that Aβ binds ApoE, ApoJ, and other lipoproteins suggesting these particles serve as chaperones or carriers for Aβ (Castano et al., 1995b; Ghiso et al., 1993; Koudinov et al., 1994; Wisniewski et al., 1993; Zlokovic et al., 1994).

TABLE 2. ApoE isoform Aβ interactions in cell-free systems

Isoform difference	Assay features	Reference
E4 > E3	w/o lipid, SDS stable ApoE/Aβ complex, DTT, aa244-272	Strittmatter et al. (1993)
E4 > E3 > E2	w/o lipid, promotion of Aβ fibril/amyloid formation	Ma et al. (1994); Sanan et al. (1994); Wisniewski et al. (1994); Castano et al. (1995b)
E3 > E4 (E2 = E3 = E4)	w/o lipid, kinetic inhibition of amyloid formation (blocking fibril seeding)	Evans et al. (1995); Wood et al. (1996)
E2 = E3 = E4	w/o lipid, *E. coli* derived, gel filtration in native buffer, ApoE/Aβ complex	Chan et al. (1996)
E3 > E4	Lipidated, SDS stable ApoE/Aβ complex	LaDu et al. (1994); Zhou et al. (1996)
E2 > E3 > E4	Lipidated cell lines, SDS stable ApoE/Aβ complex	LaDu et al. (1995); Yang et al. (1997); Aleshkov et al. (1997)

Native (lipidated) ApoE4 binds Aβ with 2–3 times lower affinity than native ApoE3 or ApoE2 (Aleshkov et al., 1997; LaDu et al., 1995; Yang et al., 1997). In this view, the higher affinity ApoE3 binding to Aβ is protective by preventing self-aggregation leading to amyloid formation and instead promoting receptor-mediated endocytosis followed by endosomal/ lysosomal degradation. The defect in ApoE4 leading to reduced Aβ binding could result in reduced endosomal/lysosomal degradation or extracellular degradation of Aβ on ApoE and enhanced Aβ aggregate formation.

Experimental evidence supporting both mechanisms can be obtained depending on important details of how experiments are performed. Injection of small amounts of soluble radiolabeled Aβ into rat hippocampus is followed by a rapid degradation of the Aβ by extracellular proteases (Iwata et al., 2000). Rapid disappearance of Aβ (detected by sandwich ELISA) also occurs after intrahippocampal injection of 400 ng of Aβ1–40 into WT and *APOE* knockout brains with a small pool of significantly more Aβ remaining in the wildtype ApoE producing animals 2.5 hours after injection (Chu et al., 1997). Similar data have been obtained in a separate set of experiments injecting 1–2 μg of Aβ1–40 and assaying 30 minutes after injection (Chu et al., unpublished data). These results suggest that Aβ binding by the wild-type mouse ApoE delays the rapid Aβ degradation of a small fraction of the Aβ by extracellular proteases, notably neprilysin. Under these conditions (using low levels of Aβ1–40 and no salt) self-aggregation is probably not a key factor determining the fate of the soluble Aβ1–40. The kinetics of degradation of ApoE-Aβ complexes may take longer because of the additional steps required for internalization and be dependent on the levels of cellular lipoprotein receptors such as LRP and LDL compared with extracellular matrix heparan sulfate proteoglycans or similar ApoE binding proteins.

With a different paradigm, Permanne and coworkers injected radiolabeled Aβ intraventricularly and found rapid clearance with no difference between *APOE*4, *E*3, and *APOE* knockout animals (Permanne et al., 1999). Much of this i.c.v. injected Aβ is not degraded, but rapidly leaves the brain (Ghersi-Egea et al., 1996). They also injected into the amygdala using 2 nm (10 μg) of Aβ1–42 in 1.5 μl (a level resulting in Aβ aggregation) in *APOE* knockout mice and transgenics expressing GFAP driven human ApoE3 or ApoE4. Animals were examined at 1 month for Aβ immunostaining and Congo red labeled amyloid deposits. Under these conditions, the amount of amyloid remaining was similar for *APOE*3 and *APOE*4 transgenics, but the *APOE* knockouts had significantly more. These results were interpreted to suggest a role for ApoE in both promoting Aβ clearance and/or promoting amyloid formation. One key factor determining the effect of ApoE appears to be whether or not there are robust Aβ aggregate forming conditions.

Depending on experimental detail, ApoE isoform differences in interactions with Aβ have also been quite varied in vitro (Table 2).

These conflicting data show that depending on the Aβ and ApoE preparations and the assay used, one gets varying results. ApoE4 directly promotes (while ApoE2 inhibits) fibril formation (Table 2) (Ma et al., 1994; Wisniewski et al., 1994) in experiments using high levels of ApoE and Aβ prepared from acid (HCL or TFA), which results in many seeds and rapid aggregation. In contrast, inhibition of amyloid formation was obtained with lower levels of ApoE and Aβ prepared out of DMSO or HFIP, which resulted in fewer seeds and slower aggregation. ApoE isoform differences in Aβ binding are radically altered by delipidation and are presumably conformation-dependent. It is widely believed that interactions in the absence of lipid are questionably relevant in lipid-rich CNS. However, Permanne et al. (1999) have recently presented evidence that lipidated (native) ApoE isoforms have similar isoform-dependent effects on beta-amyloid formation in vivo as those seen with delipidated ApoE isoforms in vitro.

Because ApoE is likely to be lipidated in vivo and lipidation alters the affinity for Aβ, the results with lipidated ApoE are likely more relevant to its function as an Aβ carrier in vivo. Native, lipidated ApoE2 or E3 binds Aβ far better than ApoE4 (LaDu et al., 1994; Yang et al., 1997). However, the formation of SDS stable complexes is not necessarily physiologically relevant and one would like to see gel filtration assays in native buffer using lipidated ApoE to firmly establish the order of affinity for Aβ as E2>E3>E4. This difference in affinity may allow ApoE2 or E3 to inhibit Aβ fibrillogenesis by binding free Aβ and inhibiting aggregation. Consistent with this idea, lipidated ApoE3 (not E4) protects target cells from toxicity caused by aggregating Aβ (Farhangrazi et al., 1997; Jordán et al., 1998). ApoE4 or E4 particles with other apolipoproteins may also interact differently with receptors (LDL, LRP, HSPG) (Rebeck et al., 1995) or lipoprotein particles. For example, an ApoE isoform difference in HDL trafficking (Gregg et al., 1986; Hayek et al., 1994; Maz-

zone and Reardon, 1994) might result in an additional loss of Aβ trafficking function.

As discussed earlier, Aβ in plasma and CSF is associated with HDL particles containing ApoE and ApoJ (Castano et al., 1995b; Ghiso et al., 1993; Koudinov et al., 1994; Wisniewski et al., 1993; Zlokovic et al., 1994). SDS stable Aβ dimers accumulate in normal aging brain and may be an initial phase of amyloid deposition or clearance or related to neurotoxicity (Enya et al., 1999). Soluble dimer levels were much lower in PDAPP mice, which show limited neuron loss, but extensive deposits. Soluble Aβ dimer-ApoE complexes have been purified from AD brain while complexes with other Aβ binding proteins were not detected (Permanne et al., 1997). Another group found that soluble ApoE/Aβ complexes were significantly higher in normal brain than AD brain (Russo et al., 1998). They also reported that the Aβ in the soluble complexes with ApoE running on SDS gels at ~40 kD was more susceptible to proteinase K digestion in total homogenates. This data would be consistent with a role for ApoE in promoting the solubilization and clearance of monomeric Aβ and dimers in normal brain as in the early stages of deposition in PDAPP transgenics expressing human ApoE4 or E3 (Holtzman et al., 1999). In contrast, in conditions with many preformed seeds and rapidly aggregating levels of amyloid (e.g., AD cases and older APP transgenic mice), the role of ApoE4 may switch to pro-amyloidogenic. These data are consistent with the variable results with ApoE and Aβ in vitro reviewed above.

In conclusion, ApoE isoforms can influence Aβ degradation by multiple mechanisms through differential effects on Aβ binding, lipoprotein trafficking followed by receptor-mediated endocytosis, and direct effects on amyloid formation and susceptibility to proteolytic degradation.

SUMMARY

Aβ in vivo circulates bound to lipoprotein particles. Evidence from microglial cultures, from injection of Aβ into rat brain, and from Aβ deposition in transgenic and *APOE* knockout mice shows that lipoprotein affects clearance of Aβ from the cellular environment, and that different isoforms of ApoE incorporated into lipoprotein also affect Aβ clearance differentially. This is a mechanism by which allelic variation in *APOE* genotype can influence an individual's risk of Alzheimer's disease, since there is a relationship, though not a direct correspondence, between amyloid plaque formation and Alzheimer's dementia.

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