9-26-2012

Identification of an Amino-Terminal Fragment of Apolipoprotein E4 that Localizes to Neurofibrillary Tangles of the Alzheimer's Disease Brain

Troy T. Rohn
Boise State University

Lindsey W. Catlin
Boise State University

Kendra G. Coonse
University of Washington School of Medicine

Jeffrey W. Habig
Boise State University

Identification of an Amino-Terminal Fragment of Apolipoprotein E4 that Localizes to Neurofibrillary Tangles of the Alzheimer's Disease Brain

Troy T. Rohn and Lindsey W. Catlin
Boise State University

Kendra G. Coonse
University of Washington School of Medicine

Jeffrey W. Habig
Boise State University

Abstract

Although the risk factor for harboring the apolipoprotein E4 (apoE4) allele in late-onset Alzheimer's disease (AD) is well known, the mechanism by which apoE4 contributes to AD pathogenesis has yet to be clarified. Preferential cleavage of the ApoE4 isoform relative to other polymorphic forms appears to be significant, as the resulting fragments are associated with hallmarks of AD. To examine the possible role of apoE4 proteolysis in AD, we designed a site-directed antibody directed at position D172, which would yield a predicted amino-terminal fragment previously identified in AD brain extracts. Western blot analysis utilizing this novel antibody, termed the amino-terminal apoE4 cleavage fragment (nApoE4CF) Ab consistently identified the predicted amino-terminal fragment (~18 kDa) in several commercially available forms of human recombinant apoE4 purified from *E. coli*. Mass spectrometry confirmed the identity of this 18 kDa fragment as being an amino-terminal fragment of apoE4. Immunohistochemical experiments indicated the nApoE4CF Ab specifically labeled neurofibrillary tangles (NFTs) in AD frontal cortex sections that colocalized with the mature tangle marker PHF-1. Taken together, these results suggest a novel cleavage event of apoE4, generating an amino-terminal fragment that localizes within NFTs of the AD brain.

Funded by a grant through the MSRT program, University of Washington to KGC, an NSF Major Research Instrumentation award (CHE-0923535) to JWH and by the KO Dementia Foundation, Boise Idaho and a NASA grant, NNX10AN29A to TTR

Keywords: Apolipoprotein E4, ApoE4, protease, Alzheimer's disease, Neurofibrillary tangles, Matrix Metalloproteinase, Thermolysin, Immunohistochemistry, Western Blot Analysis, PHF-1

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extensive neuronal loss leading to cognitive impairment and dementia. AD is diagnosed based upon the extent of senile plaques composed of beta-amyloid and NFTs containing abnormally phosphorylated tau (Golde et al., 2006). Despite exhaustive efforts, there are few genetic risk factors associated with late-onset AD that have been identified, with the exception of the apolipoprotein (apo) E4 allele. Inheritance of one copy of the apoE4 allele increases disease risk fourfold, while two copies raises the risk tenfold (Eisenstein, 2011). Although apoE4 clearly enhances the risk for AD, the mechanism by which apoE4 contributes to AD pathogenesis is not known. ApoE4 is a 34 kDa protein composed of 299 amino acids that plays a major role in cholesterol transport in the CNS, and may also help remove beta-amyloid from the brain (Eisenstein, 2011). Impairment in these two processes following inactivation of apoE4 may help drive the underlying pathology associated with AD. In this regard, apoE4 is highly susceptible to proteolysis compared to apoE3, and apoE4 fragments (14-20 kDa) have been identified in the AD brain (Harris et al., 2003). Thus, apoE4 may promote AD pathogenesis through a loss of function. On the other hand, emerging data suggests that apoE4 fragments may provide a gain of function by promoting the formation of both plaques and tangles. In particular, the

C-terminal portion of apoE has been implicated in binding to beta-amyloid and is localized to plaques (Marques et al., 2004). The N-terminal domain conversely, preferentially localizes within neurofibrillary tangles (NFTs) and is neurotoxic in culture (Harris et al., 2003). More recently, in a mouse model of AD, expression of a N-terminal fragment of apoE4 resulted in accumulation of pathogenic beta-amyloid oligomers and led to neuronal as well as behavioral defects in mice (Bien-Ly et al., 2011). Taken together, these data suggest the link between apoE4 and AD pathogenesis may be proteolytic cleavage of the protein leading to the production of fragments that enhance pathology. However, to date, the exact nature of the protease involved in cleaving apoE4 is unknown although several candidates have been reported including cathepsin D (Zhou et al., 2006), a chymotrypsin-like protease (Harris et al., 2003), and aspartic proteases (Marques et al., 2004).

Several studies have identified the presence of ~18 kDa band in human AD brain extracts, suggesting cleavage of apoE4 near position D172 (Harris et al., 2003; Huang et al., 2001). To determine if this site within apoE4 is cleaved by proteases in the AD brain, we developed and characterized a site-directed neoepitope antibody directed towards the amino-terminal fragment that would be generated following cleavage at D172. Application of this antibody, *in situ*, revealed specific localization within NFTs that co-localized with PHF-1. Thus, this antibody may have utility in future studies examining the role of apoE4 proteolysis in AD.

2. Results

Examination of the amino acid sequence of apoE4 that would generate an approximate 18 kDa fragment revealed the presence of several ideal caspase-cleavage consensus sites at position D128 (DMED) and D172 (DADD). Therefore, we hypothesized that apoE4 may be a suitable substrate for caspase-3 cleavage. As an initial approach, experiments were performed utilizing human recombinant apoE4 from *E. coli* incubated with active caspase-3 for 24 hours at room temperature and analyzed by western blot analysis using a full-length amino-terminal apoE4 antibody. Overall, there was very little difference in the appearance of apoE4 in the presence or absence of caspase-3. However, the results did indicate a unique band in the digested sample running at ~16 kDa (Fig. 1A, lane marked '+Casp-3'). This unique band is close to the predicted molecular weight of the amino-terminal fragment of apoE4 that would occur following cleavage at position D172 (predicted fragment ~18.2 kDa). These results suggested that apoE4 is a substrate for caspase-3 cleavage. A somewhat surprising result of this experiment was that in addition to an intense band labeling at 34 kDa, corresponding to full-length apoE4, there were several apoE4 amino-terminal bands present in the control lane, suggesting that proteolytic cleavage of apoE4 occurred following purification of apoE4 from *E. coli*, where is was expressed (Fig. 1A, lane marked 'Ctl'). The presence of these amino-terminal fragments of apoE4 under control conditions, particularly the band running at ~18 kDa was confirmed using an additional commercial source of recombinant apoE4 from MBL International (data not shown). This confirms the sensitivity of apoE4 to proteolytic cleavage and suggests that in addition to being a substrate for caspase-3, apoE4 may also be subjected to proteolysis by other proteases present in *E. coli*.

As previously mentioned, caspases are unique in that they show an absolute requirement for cleavage after aspartic acid (Rohn and Head, 2009). Based upon the sequence of human apoE4, there are several potential caspase-3 sites at position D128 (DMED) and D172 (DADD). The predicted amino terminal fragments following cleavage of caspase-3 at these two positions are 13 and 18.2 kDa, respectively. Because our results in Fig. 1A suggested the caspase-3 fragment was closer to 18 kDa, and was larger than 13 kDa, we chose the sequence at position D172 to synthesize a site-directed antibody. We chose the 7-mer peptide LLRDADD, which represents the upstream neoepitope fragment of apoE4 that would be generated following cleavage after the terminal aspartic acid residue. Following synthesis, this peptide was coupled to KLH and injected into rabbits. The resulting sera (verified by ELISAs) were used to affinity purify antibodies. Western blot analysis was performed to confirm whether this antibody, herein termed the amino-terminal apoE4 cleavage fragment (nApoE4CF Ab), recognized the predicted amino-terminal fragment following incubation of human recombinant apoE4 with activated caspase-3. As shown, the nAPoE4CF Ab strongly labeled a band running at ~18 kDa following incubation of apoE4 with caspase-3 (Fig. 1B, right lane). However, surprisingly, this same band was also evident in the control lane representing apoE4 incubated in the absence of caspase-3 (Fig. 1B, lane marked 'Ctl'). Several conclusions can be drawn from this experiment: the first is that this novel antibody did not label the full-length form of apoE4, a critical requisite for a site-directed antibody. Second, these data suggest that caspase-3 or some other protease already cleaved apoE4 following its purification from *E. coli*. Based on the results from Fig. 1A, the caspase-3 generated fragment ran slightly smaller (~16 kDa) than the nAPoE4CF-labeled band of ~18 kDa (Fig. 1B). Therefore, our data suggest the nApoE4CF Ab did not detect the 16 kDa caspase-3-generated fragment that was immunolabeled with the full-length apoE4 antibody (Fig. 1A).

To confirm that the 18 kDa fragment represented an amino-terminal cleavage fragment of apoE4, similar experiments were repeated using His-tagged human recombinant apoE4. As in Fig. 1B, the nAPoE4CF antibody again labeled an ~18 kDa band of apoE4 under control conditions (Fig. 1C, left panel). An amino-terminal His-tagged antibody immunolabeled the same 18 kDa band in addition to full-length apoE4 as well as smaller amino-terminal fragments (Fig. 1C, right panel).

To determine whether this 18 kDa band was unique to recombinant apoE4 purified from *E. coli*, similar experiments were performed using apoE4 purified from human plasma. Because this form of apoE4 had not been expressed in *E. coli*, we predicted that the 18 kDa band would no longer be present following western blot analysis using the nApoE4CF antibody. Results from Fig. 1D supported this idea, and revealed a complete lack of immunoreactivity to an 18 kDa band, whereas the 18 kDa band as well as two other higher molecular weight bands were immunolabeled under identical experimental conditions in apoE4 from *E. coli*. Moreover, these data confirmed the high specificity of this antibody to the fragment of apoE4, as no reactivity to full-length apoE4 was observed even though there were ample levels of the full-length protein present in the transferred gel (Fig. 1E). Experiments were also undertaken looking at whether the 18 kDa band detected using recombinant apoE4 is also present in recombinant apoE3. In this case, we pushed the sensitivity of the system by doubling the amount of apoE3 loaded onto gels as compared to apoE4 and by increasing the level of nApoE4CF antibody to 1:100 instead of 1:500. In this case, there was a total lack of immunoreactivity of the nApoE4CF Ab to apoE3 and this was supported by the absence of other bands in the Coomassie blue-stained gel (Fig. 1E).

Further support that the 18-kDa band represented an amino-terminal fragment of apoE4 was provided by mass spectrometry. When recombinant human apoE4 from *E. coli* was separated by SDS-PAGE a number of bands were evident following staining (Fig. 2), including bands corresponding to the full-length protein (34 kDa) and the presumptive amino-terminal cleavage product (18 kDa). The 34-kDa and 18-kDa bands were isolated, digested, and analyzed by HPLC-coupled mass spectrometry. As expected, nearly complete sequence coverage of apoE4 was obtained from the 34-kDa band with a very high Sequest confidence score (Fig. 2, top right). The peptides from the 18-kDa band also corresponded to apoE4, but were not localized solely to the amino terminus (Fig. 2, lower right) as would be expected if this band were purely the amino-terminus cleavage product. Two pieces of evidence suggest that the 18-kDa band was a combination of the amino-terminal cleavage product that co-migrated with apoE4 degradation and/or cleavage products, accounting for the carboxy-terminal peptides in the analysis. First, a similar analysis of the region located between the 18- and 34-kDa bands resulted in abundant apoE4 peptides and complete sequence coverage similar to the full-length product, consistent with apoE4 degradation and/or cleavage. Second, the Sequest confidence score associated with spectral matches to the amino-terminal fragment was six-fold higher than the carboxy-terminal fragment for the 18-kDa band, whereas the amino- and carboxy-terminal scores were roughly equivalent for the 34-kDa band. Taken together the results presented in Figs. 1 and 2 suggested the presence of a unique amino-terminal proteolytic fragment of apoE4 generated in *E. coli* that did not appear to be the result of caspase-3-mediated cleavage. Furthermore, none of the low abundant bacterial contaminants associated with the purification process appeared to possess a suitable epitope for the nApoE4CF antibody (data not shown).

These initial findings suggest a novel cleavage event of apoE4 *in vitro*. To test whether a similar cleavage event occurs in the AD brain, immunohistochemistry experiments were undertaken using postmortem frontal cortex brain sections from AD subjects. As an initial approach, we first characterized the staining profile in sections using commercial full-length antibodies to either the extreme N-terminal or C-terminal region of apoE4. Previous studies have indicated a preferential localization of apoE4 antibodies such that N-terminal antibodies immunolabel both plaques and tangles, while C-terminal antibodies immunolabel plaques only (Huang et al., 2001). Figure 3 displays representative results following immunohistochemical analysis with N- and C-terminal full-length antibodies to apoE4. As depicted for the N-terminal antibody (Fig. 3A), both full-length antibodies strongly labeled blood vessels. However, the N-terminal antibody preferentially localized to both plaques and tangles (Fig. 3B), while the C-terminal antibody predominantly labeled plaques only (Fig. 3C). These results confirmed previous findings (Huang et al., 2001). We next performed experiments utilizing our novel cleavage antibody to apoE4 under identical experimental conditions. The apoE4CF antibody exclusively labeled NFTs in the AD brain (Fig. 3D). Of interest was the general lack of staining in both blood vessels and within senile plaques. Because the nApoE4CF antibody localized exclusively within NFTs, this supports the hypothesis that the antibody is specific for the N-terminal region of apoE4. Additional experimentation was carried out on specific AD cases in which the exact apoE allele type was known. Previous studies have indicated that the risk of AD is highest with the inheritance of the apoE4 allele, while the apoE3 allele is neutral and the apoE2 allele is actually protective (Verghese et al., 2011). We

quantified the number of nApoE4CF-positive NFTs in AD cases possessing both apoE3 alleles (3/3), one apoE4 allele, (3/4), and both apoE4 alleles (4/4). Our results were intriguing in that the highest number of nApoE4CF-positive NFTs were found in 3/4 and 4/4 cases, both of which were significantly higher than 3/3 cases (Fig. 3E). These data suggest a potential relationship between inheritance of the apoE4 allele, the cleavage of apoE4 and the localization of this N-terminal fragment within NFTs. In support of these findings, we also demonstrated the presence of nApoE4CF in AD frontal cortex extracts by western blot analysis (Fig. 3F).

In an attempt to further characterize the labeling of the nAPoE4CF Ab, experiments were performed comparing the staining in AD versus age-matched control sections. Specific staining within NFTs was only observed in AD frontal cortex sections (Fig. 4A) and was absent in age-matched controls (Fig. 4B). We also confirmed the specificity of the nAPoE4CF Ab by performing control experiments with preimmune versus immunized serum in serial AD frontal cortex sections. No specific staining was observed when preimmune serum was used in place of immunized serum (Fig. 4C and D). In addition, staining with the nAPoE4CF Ab was prevented after preadsorption with free peptide that was used as the immunogen (Fig. 4E and F).

To further examine the distribution of nApoE4CF in AD tissue, double-labeling experiments were carried out using an anti beta-amyloid antibody as a marker for plaques and PHF-1 as a marker of late NFTs. By bright-field immunohistochemistry, no co-localization was observed between nApoE4CF and beta-amyloid, suggesting nApoE4CF is absent in plaques (Fig. 5A). However, using immunofluorescence it was demonstrated that nApoE4CF co-localized with PHF-1 in merged images (Fig 5B and C, yellow). Quantitative experiments indicated that nApoE4CF was present in ~60% of PHF-1-positive NFTs (Fig. 5D). Although single PHF-1-positive NFTs were identified (arrowheads, Fig 5C), no nApoE4CF single-positive NFTs were observed.

3- Discussion

Human apolipoprotein E (apoE) is a 34 kDa glycoprotein that exists in three isoforms, E2, E3, and E4, which differ by single amino acid variations. Inheritance of one copy of apoE4 increases the risk of Alzheimer's disease (AD) fourfold, while inheritance of two copies raises the risk about tenfold (Eisenstein, 2011). The molecular mechanisms by which apoE4 contributes to AD pathogenesis is unknown, however, emerging data suggests that apoE4 is highly unstable and a loss of function may impair the movement of cholesterol and beta-amyloid within the brain (Eisenstein, 2011). Recent evidence suggests that the instability of apoE4 is a result of a susceptibility to proteolytic cleavage. Compared to other isoforms of apoE, apoE4 is highly susceptible to proteolytic cleavage (Harris et al., 2003) and apoE N-terminal fragments (14-20 kDa) are present at much greater levels in the AD brain as compared to age-matched controls (Harris et al., 2003). Besides a possible loss of function of apoE4 in cholesterol and beta-amyloid transport within the CNS following proteolysis, emerging data suggests that apoE4 fragments may provide a toxicity gain of function by promoting the formation of both plaques and NFTs. For example, employing a mouse model of AD, expression of a N-terminal fragment of apoE4 resulted in accumulation of pathogenic beta-amyloid oligomers and leads to neuronal as well as behavioral deficits in mice (Bien-Ly et al., 2011). Taken together, these data suggest the link between apoE4 and AD pathogenesis may be proteolytic cleavage leading to the generation of fragments that enhance pathology. However, to date, the exact nature of the protease involved in cleaving apoE4 is unknown although several candidates have been reported including cathepsin D (Zhou et al., 2006), a chymotrypsin-like protease (Harris et al., 2003), and aspartic proteases (Marques et al., 2004). The goal of the current study was to examine in further detail the mechanisms underlying proteolytic cleavage of apoE4 in the AD brain.

Because of the well documented role that caspases play in promoting the pathology underlying AD, including proteolysis of tau and the amyloid precursor protein (APP), which may promote the formation of NFTs and beta-amyloid formation, respectively, (Rohn, 2010), we hypothesize that this family of proteases may be involved in the proteolysis of apoE4. Caspases are critical to the execution of apoptosis and represent a family of cysteine proteases, which are unique in their specificity of cleavage after aspartyl residues (Rohn and Head, 2009). Examination of the amino acid sequence of apoE4 reveals several candidate caspase-cleavage consensus cleavage sites, including one in particular at D172 (DADD), that would produce a predicted ~18 kDa amino-terminal fragment of apoE4, similar in size to what has been previously reported to be present in the AD brain (Harris et al., 2003). We chose the 7-mer peptide LLRDADD, which represents the upstream neoepitope fragment of apoE4 that would be generated following cleavage after the terminal aspartic acid residue. Following affinity purification of this antibody, which we termed the amino-terminal apoE4 C-terminal fragment (nApoE4CF) antibody, we tested the antibody in fixed frontal brain sections from AD subjects using immunohistochemistry. As an initial approach, we first characterized

the staining profile in sections using commercial full-length antibodies to either the extreme N-terminal or C-terminal region of apoE4. Previous studies have indicated a preferential localization of apoE4 antibodies such that N-terminal antibodies immunolabel both plaques and tangles, while C-terminal antibodies immunolabel plaques only (Huang et al., 2001). Results in Fig. 3 confirmed these findings whereby application of our novel antibody specifically labeled NFTs not plaques and, in addition, was significantly dependent upon the apoE4 allele status of selected cases. Taken together these data suggest the nApoE4CF antibody is specific for a N-terminal fragment of apoE4, which may not be produced until full-length apoE4 exits blood vessels and enters in the extravascular space. This suggests that the protease involved in producing this fragment is either present extracellularly or within neurons.

We verified whether our nApoE4CF antibody could detect the predicted caspase-cleavage fragment of 18 kDa following cell-free experiments using human recombinant apoE4 and activated caspase-3. Although we were able to confirm that apoE4 is indeed a substrate for caspase-3 proteolysis, our data clearly indicated that the nApoE4CF antibody does not recognize a caspase-cleaved fragment of apoE4 by western blot analysis. Instead we observed a consistent immunoreactivity to a 18 kDa band in commercial preparations of human recombinant apoE4 under control conditions (Fig. 1). These data suggest some other protease already cleaved apoE4 following its purification from *E. coli*. Based on the results from Fig. 1A, the caspase-3 generated fragment ran slightly smaller (~16 kDa) than the predicted fragment of 18.2 kDa. Therefore, it is possible we chose the incorrect cleavage site to synthesize our antibody. Indeed, further examination of the amino acid sequence of apoE4 by CASVM (server for SVM prediction of caspase substrate cleavage sites) (Wee et al., 2006), indicated another potential caspase-cleavage consensus site at position D169, just upstream to D172, would yield a predicted cleavage fragment of ~16 kDa, which would correspond to the size fragment present in Fig. 1A following incubation of apoE4 with caspase-3. Taken together our data support the conclusion that the nApoE4CF antibody recognizes a novel cleavage site generated by an unknown protease and that this site is cleaved *in vivo*.

Future studies utilizing our novel nApoE4CF antibody should aid in identifying the protease responsible for the *in vivo* cleavage of apoE4 in the AD brain. Examination of the apoE4 sequence indicates the next amino acid after D172 to which our nApoE4CF antibody was designed towards, is leucine at position D173. There are several potential candidate proteases that could be cleaving apoE4 between residues D172 and D173 generating an amino-terminal fragment of 18 kDa. These include the family of matrix metalloproteinases (MMPs) that accept large hydrophobic amino acids at the S1' site. We hypothesize that a member of this MMP family may be cleaving apoE4 at the N-terminal side of leucine at position D173. Cleavage of the peptide bond between these two the aspartate and leucine residues would generate an amino-terminal fragment that could now be recognized by our nApoE4CF antibody. Examining the MMP family, several candidate proteases stand out including MMP-3 (also known as stromelysin-1), MMP-7 (also known as matrilysin) and MMP-9. Of particular interest is MMP-3 and MMP-9 both of which have been implicated in the pathogenesis associated with AD. For example, MMP-3 has a selective distribution in the AD brain being found in astrocytes and within senile plaques (Yoshiyama et al., 2000). For MMP-9, studies have shown an increase in MMP-9 activity in patients with mild cognitive impairment compared to normal age-matched controls (Bruno et al., 2009). In addition, there was an inverse correlation between Global Cognitive Score and Mini-Mental State Examination score and MMP-9 activity (Bruno et al., 2009). More intriguing is a recent study that has linked MMP-9 together with apoE4 in the breakdown and loss of integrity of the blood-brain barrier associated with AD (Bell et al., 2012).

In conclusion, in the present study we examined the proteolytic cleavage of apoE4 both *in vitro* and *in situ* using an epitope site-directed antibody. Our results demonstrate a novel cleavage event of apoE4 at position D172 generating an amino-terminal fragment that localizes to NFTs of the AD brain. Further studies are warranted to identify the nature of this protease and our novel nApoE4CF antibody should aid in identifying potential proteases. Our present results suggest the focus should be on examining on the family of MMPs such as MMP-3 and MMP-9.

4. Experimental Procedure

Materials.

The anti-Aβ mAb 1560 (clone 6E10) was purchased from Covance (Dedham, MA). The beta-actin rabbit polyclonal antibody was from AbCam (Cambridge, MA). Human apolipoprotein E purified from plasma was purchased from Millipore (Billerica, MA). Human recombinant apoE4 and E3 proteins purified from *E. coli* were purchased from

ProSci Inc. (Poway, CA). An additional source of human recombinant apoE4 was purchased from MBL International Corporation (Woburn, MA). His-tagged human recombinant apoE4 purified from *E. coli* was purchased from Biovision Inc. (Milpitas, CA). Activated human recombinant caspase-3 was purchased from Calbiochem (San Diego, CA). The anti-apoE4 C-terminal rabbit polyclonal antibody was purchased from Abgent (San Diego, CA). The anti-apoE4 N-terminal rabbit polyclonal antibody was purchased from Aviva Systems Biology Corp. (San Diego, CA). PHF-1 was a generous gift from Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY).

Generation of the polyclonal site-directed cleavage antibody to ApoE4. Polyclonal antibodies were synthesized based upon a putative caspase cleavage consensus site ($DADD^{172}$) within apoE4. We chose the 7-mer peptide LLRDADD, which represents the N-terminal upstream neoepitope fragment of apoE4 that would be generated following cleavage after the terminal aspartic acid residue. Following synthesis, this peptide was coupled to KLH and injected into rabbits. The resulting sera (verified by ELISAs) were used to affinity purify antibodies using a sulfolink column (Thermo Scientific) coupled with the peptide used as an immunogen. For this antibody, synthesis of peptides, injections of immunogens, and collection of antisera were contracted out to Bethyl laboratories (Montgomery, TX).

Cell-free digestion of ApoE4 with caspase-3

To determine whether apoE4 is subject to proteolytic digestion, 40 μg of purified human recombinant apoE4 from two sources (MBL International and Prosci Inc.) was incubated with active human recombinant caspase-3 at 37°C overnight in 2x reaction buffer containing 10 mM DTT. Reactions were terminated by the addition of 5x sample buffer and stored at −20°C until analyzed.

Mass Spectrometry

Gel slices were excised from SDS-PAGE gels and digested with trypsin as previously described (Shevchenko et al., 2006). Briefly, excised bands were destained in 25 mM ammonium bicarbonate/50% acetonitrile overnight at 4°C. Gel slices were dehydrated in 100% acetonitrile for 10 minutes at room temperature, followed by reduction in 10 mM DTT for 60 minutes at 37°C. Next, alkalation was performed in 55 mM iodoacetamide for 60 minutes at room temperature in the dark. Protein samples were digested with trypsin in 10 mM ammonium bicarbonate overnight at 30°C. Digested peptides were separated using reversed phase chromatography (Thermo Scientific Easy-nLC II) and infused into a Velos Pro Dual-Pressure Linear Ion Trap Mass Spectrometer for MS/MS using CID fragmentation. Results were analyzed with Thermo Scientific's Proteome Discoverer software (v1.3) and the Mascot search engine probing the human SwissProt database. Search parameters included carbamidomethyl (C) fixed modification, oxidation (M) variable modification, maximum of 2 missed cleavages, and 1.5 Da peptide mass tolerance.

Western blot analysis

Western blot analysis was performed as previously described (Rohn and Catlin, 2011). Proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose. Transferred slabs were stained in coomassie blue to verify equal loading between samples. Membranes were incubated in nApoE4CF (1:500) or a full-length N-terminal apoE4 antibody (1:1,000) overnight at 4°C and primary antibodies were visualized using goat anti-rabbit HRP-linked secondary, incubated for 1 hour at room temperature (1:5,000; Jackson's Laboratory, West Grove, PA), followed by ECL detection.

Human subjects

Autopsy brain tissue from the front cortex of eight neuropathologically confirmed AD cases with known ApoE isoform genotype and three age-matched control cases were studied. Human brain tissues used in this study were provided by the Institute for Brain Aging and Dementia Tissue Repositories at the University of California, Irvine.

Immunohistochemistry and immunofluorescence microscopy

Free-floating 40 μm-thick serial sections were used for immunohistochemical and immunofluorescence studies as previously described (Kokoulina and Rohn, 2010). Antibody dilutions were the following: nApoE4CF (1:500), full-

length N-terminal ApoE4 (1:500), full-length C-terminal ApoE4 (1:100), anti beta-amyloid mAb 1560 clone 6E10 (1:400), and PHF-1 (mouse monoclonal, 1:1000). To visualize beta-amyloid staining, sections were pretreated for 5 minutes in 95% formic acid. Antigen visualization was determined using ABC complex (ABC Elite immunoperoxidase kit, Vector labs), followed by DAB (brown) or Vector SG substrate (blue) (Vector Labs). For immunofluorescence co-localization studies, antigen visualization was accomplished using an Alexa fluor 488-labeled tyramide (green, Ex/Em = 495/519) or streptavidin Alexa Fluor 555 (red, Ex/Em = 555/565), both from Invitrogen (Carlsbad, CA).

Quantification and statistical analysis

Statistical difference between the average number of nApoE4CF-positive NFTs in 3 AD cases from each allelic combination of ApoE3/ApoE3, ApoE3/ApoE4, and ApoE4/ApoE4A was determined using Student's two-tailed T-test. To determine the percent co-localization, a semi-quantitative analysis was performed as described previously (Kokoulina and Rohn, 2010; Rohn et al., 2011; Rohn and Kokoulina, 2009) by taking 20X immunofluorescence, overlapping images from three different fields in frontal cortex brain sections of three separate AD cases. Capturing was accomplished by using a 2.5x photo eyepiece and a Sony high resolution CCD video camera (XC-77). As an example, to determine the percent co-localization between PHF-1 and nApoE4CF, photographs were analyzed by counting the total number of PHF-labeled NFTs per 20X field for each case, and the number of NFTs labeled with both PHF-1 and nApoE4CF. Data are representative of the average number (±S.E.M.) of NFTs or NFTs that co-localized with nApoE4CF in each 20X field (3 fields total for 3 different cases). Statistical differences in this study were determined using Student's two-tailed T-test employing Microsoft Office Excel.

Acknowledgements

This work was funded by KO Dementia Foundation, Boise Idaho and a NASA grant, NNX10AN29A. Mass spectrometry was made possible through an NSF Major Research Instrumentation award (CHE-0923535) to Boise State University.

Abbreviations:

AD- Alzheimer's disease
Aβ- beta amyloid
apoE4- apolipoprotein 4
APP- amyloid precursor protein
nApoE4CF- Amino-terminal apoE4 carboxyl-terminal fragment
MMP- matrix metalloproteinase
NFTs- neurofibrillary tangles
PHF- paired helical filaments

Fig. 1- Identification of an 18 kDa amino-terminal fragment of apoE4 that is caspase-3-independent. (A and B) Recombinant human apoE4 was incubated with or without active caspase-3 overnight at 37 °C. Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed with an amino-terminal full-length antibody to apoE4 (1:1,000), (A) or with affinity-purified nApoE4CF antibody (1:500), (B). Western blot analysis indicated the antibody, nApoE4CF, predominantly labeled an 18 kDa band in both the control sample (apoE4 alone) and in the digested sample (apoE4 + caspase-3). (C) Recombinant His-tagged apoE4 was subjected to western blot analysis and probed with the nApoE4CF antibody (left panel), again illustrating the presence of an 18 kDa band under control conditions. The presence of this amino-terminal fragment of apoE4 along with the full-length form of apoE4 at 34 kDa was confirmed utilizing an amino-terminal His-tagged antibody to apoE4 (right panel). (D) Western blot analysis comparing apoE4 purified from human plasma or recombinant apoE4 and E3 from *E. coli* under control conditions utilizing the nApoE4CF Ab (1:100). Notice the presence of several bands in addition to the 18 kDa band in lane marked 'apoE4 E. Coli', all of which were absent in apoE4 purified from human plasma or recombinant apoE3 from *E. coli*. (E) Depicts the transferred gel slab that was stained with Coomassie blue revealing the presence of numerous high and low molecular weight bands of recombinant apoE4 that were largely absent in the other two samples.

Fig. 2- Confirmation of recombinant apoE4 protein by mass spectrometry.
(Left Panel) Coomassie stained SDS-PAGE of purified recombinant apoE4 protein from *E. coli*. The 34-kDa and 18-kDa bands were independently excised from the gel and digested with trypsin prior to HPLC/MS/MS analysis. **(Right Panel)** Proteome Discoverer was used to search the mammalian SwissProt database for peptide spectral matches (PSMS) using the Mascot search engine. Upper panel: 86% coverage of ApoE4 (accession: 178853; score: 86,886; peptide spectral matches: 4410) from the 34 kDa band. Lower panel: 82% coverage of apoE4 (accession: 178853; score: 14,914; peptide spectral matches: 1009) from the 18 kDa band. Green, red, and yellow highlights indicate high, medium, and low peptide confidences, respectively. [The region between the 18 and 34 kDa bands was also analyzed and was found to consist of apoE4 protein fragments (data not shown).]

Fig. 3- Localization of the nApoE4CF antibody within NFTs of the Alzheimer's disease brain. In Panels A-D, apoE allele AD cases (4/4) were utilized for immunohistochemical analysis. (A and B) Representative staining in AD frontal cortex sections utilizing a full-length, N-terminal antibody to apoE4 indicated labeling of blood vessels (A), plaques (B, arrowheads), and NFTs (B, arrow). (C) Identical to above with the exception of employment of a C-terminal full-length antibody to apoE4 (. In this case staining was confined to senile plaques (arrowheads) with no staining evident within NFTs. (D) Representative labeling of our cleavage-specific antibody, nApoE4CF, revealed staining within NFTs of the AD brain (arrows). (E) Quantitative analysis of the number of nApoE4CF-positive NFTs in specific apoE allele AD cases: 3/3 (n=3), 3/4 (n=2), and 4/4 (n=3). (F) Western blot analysis with the nApoE4CF antibody in soluble human brain extracts from age-matched controls (Ctl) or Alzheimer's subjects (AD). Bottom panel F represents loading control blot using a rabbit antibody to beta-actin (1:400). For all experiments, data are representative of three independent experiments. *Denotes significance difference between 4/4 and 3/3 cases ($p = 0.04$). All error bars are equivalent to 10 μm.

Fig. 4- Specificity of the nApoE4CF antibody for NFTs in the Alzheimer's disease brain. (A) Representative staining with the nApoE4CF antibody in an AD frontal cortex section (apoE allele genotype, 4/4) showing numerous NFTs (arrows), whereas staining was largely absent in a representative age-matched control case (apoE allele genotype, 3/3) (B). (C and D) Serial AD frontal cortex sections were immunolabeled with immunized serum (C) or preimmune serum (D) with staining observed only following application of immunized serum (arrows, C). (E and F) Serial AD frontal cortex sections were incubated with nApoE4CF antibody alone (E), or following preadsorption with the peptide used as an immunogen (F). Staining was completely prevented following preadsorption with peptide (F). In panels C-F, AD cases with the apoE genotype of 4/4 were utilized. All scale bars represent 10 μm.

Fig. 5- nApoE4CF co-localizes with the mature tangle marker, PHF-1. (A) Representative bright field microscopy image from frontal cortex AD brain sections utilizing anti-Aβ antibody, clone 6E10 (blue) together with the nApoE4CF antibody (brown) revealed specific localization of nApoE4CF within NFTs (arrow). (B and C) Double-label immunofluorescence merged images utilizing PHF-1 (red) and nApoE4CF (green) at high (B) and low (C) magnification indicated co-localization between the two markers (yellow, arrows). The arrowheads in Panel C depict NFTs that were labeled with PHF-1 only. (D) Quantification of NFTs double-labeled by PHF-1 and nApoE4CF. Data show the total number of NFTs labeled with PHF-1 (bar labeled "Total NFTs") or together with

nApoE4CF (bar labeled "NFT + nApoE4CF"), identified in a 20X field in frontal cortex AD sections by immunofluorescence overlap microscopy (n=3 fields for 3 different AD cases) ±S.E.M, *$p < 0.05$. Data analysis indicated that roughly 60% of all identified tangles were immunolabeled with nApoE4CF Ab. All data were collected utilizing AD cases identified as having an apoE 4/4-allele genotype. All scale bars represent 10 µm.

References Cited

Bell, R.D., Winkler, E.A., Singh, I., Sagare, A.P., Deane, R., Wu, Z., Holtzman, D.M., Betsholtz, C., Armulik, A., Sallstrom, J., Berk, B.C., Zlokovic, B.V., 2012. Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. Nature. 485, 512-6.

Bien-Ly, N., Andrews-Zwilling, Y., Xu, Q., Bernardo, A., Wang, C., Huang, Y., 2011. C-terminal-truncated apolipoprotein (apo) E4 inefficiently clears amyloid-beta (Abeta) and acts in concert with Abeta to elicit neuronal and behavioral deficits in mice. Proc Natl Acad Sci U S A. 108, 4236-41.

Bruno, M.A., Mufson, E.J., Wuu, J., Cuello, A.C., 2009. Increased matrix metalloproteinase 9 activity in mild cognitive impairment. J Neuropathol Exp Neurol. 68, 1309-18.

Eisenstein, M., 2011. Genetics: finding risk factors. Nature. 475, S20-2.

Golde, T.E., Dickson, D., Hutton, M., 2006. Filling the gaps in the abeta cascade hypothesis of Alzheimer's disease. Curr Alzheimer Res. 3, 421-30.

Harris, F.M., Brecht, W.J., Xu, Q., Tesseur, I., Kekonius, L., Wyss-Coray, T., Fish, J.D., Masliah, E., Hopkins, P.C., Scearce-Levie, K., Weisgraber, K.H., Mucke, L., Mahley, R.W., Huang, Y., 2003. Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice. Proc Natl Acad Sci U S A. 100, 10966-71.

Huang, Y., Liu, X.Q., Wyss-Coray, T., Brecht, W.J., Sanan, D.A., Mahley, R.W., 2001. Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. Proc Natl Acad Sci U S A. 98, 8838-43.

Kokoulina, P., Rohn, T.T., 2010. Caspase-cleaved transactivation response DNA-binding protein 43 in Parkinson's disease and dementia with Lewy bodies. Neurodegener Dis. 7, 243-50.

Marques, M.A., Owens, P.A., Crutcher, K.A., 2004. Progress toward identification of protease activity involved in proteolysis of apolipoprotein e in human brain. J Mol Neurosci. 24, 73-80.

Rohn, T.T., 2010. The role of caspases in Alzheimer's disease; potential novel therapeutic opportunities. Apoptosis.

Rohn, T.T., Wirawan, E., Brown, R.J., Harris, J.R., Masliah, E., Vandenabeele, P., 2011. Depletion of Beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain. Neurobiol Dis. 43, 68-78.

Rohn, T.T., Head, E., 2009. Caspases as therapeutic targets in Alzheimer's disease: is it time to "cut" to the chase? Int J Clin Exp Pathol. 2, 108-18.

Rohn, T.T., Kokoulina, P., 2009. Caspase-cleaved TAR DNA-binding protein-43 in Pick's disease. Int J Physio Pathophysio Pharmacol. 1, 24-31.

Rohn, T.T., Catlin, L.W., 2011. Immunolocalization of influenza A virus and markers of inflammation in the human Parkinson's disease brain. PLoS One. 6, e20495.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M., 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 1, 2856-60.

Verghese, P.B., Castellano, J.M., Holtzman, D.M., 2011. Apolipoprotein E in Alzheimer's disease and other neurological disorders. Lancet Neurol. 10, 241-52.

Wee, L.J., Tan, T.W., Ranganathan, S., 2006. SVM-based prediction of caspase substrate cleavage sites. BMC Bioinformatics. 7 Suppl 5, S14.

Yoshiyama, Y., Asahina, M., Hattori, T., 2000. Selective distribution of matrix metalloproteinase-3 (MMP-3) in Alzheimer's disease brain. Acta Neuropathol. 99, 91-5.

Zhou, W., Scott, S.A., Shelton, S.B., Crutcher, K.A., 2006. Cathepsin D-mediated proteolysis of apolipoprotein E: possible role in Alzheimer's disease. Neuroscience. 143, 689-701.