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Original Article
Apolipoprotein E pathology in vascular dementia

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Abstract: Vascular dementia (VaD) is the second most common form of dementia and is currently defined as a cerebral vessel vascular disease leading to ischemic episodes. Apolipoprotein E (apoE) gene polymorphism has been proposed as a risk factor for VaD, however, to date there are few documented post-mortem studies on apoE pathology in the VaD brain. To investigate a potential role for the apoE protein, we analyzed seven confirmed cases of VaD by immunohistochemistry utilizing an antibody that specifically detects the amino-terminal fragment of apoE. Application of this antibody, termed N-terminal, apoE cleavage fragment (nApoECF) revealed consistent labeling within neurofibrillary tangles (NFTs), blood vessels, and reactive astrocytes. Labeling occurred in VaD cases that had confirmed APOE genotypes of 3/3, 3/4, and 4/4, with respect to NFTs, staining of the nApoECF co-localized with PHF-1 and was predominantly localized to large, stellate neurons in layer II of the entorhinal cortex. Quantitative analysis indicated that approximately 38.4% of all identified NFTs contained the amino-terminal fragment of apoE. Collectively, these data support a role for the proteolytic cleavage of apoE in the VaD and support previous reports that APOE polymorphism is significantly associated with susceptibility in this disease.

Keywords: Vascular dementia, apoE, apolipoprotein E, neurofibrillary tangles, PHF-1, immunohistochemistry, plaques, astrocytes

Introduction

Vascular dementia (VaD) is currently the second leading cause of dementia in the USA, only trailing Alzheimer's disease (AD) [1]. Diagnosing VaD is often difficult owing to the common co-occurrence of VaD together with Alzheimer's disease (AD), with an estimated 25-80% of all dementia cases showing mixed pathologies [2]. An additional confounding factor is that there are currently no widely accepted neuropathological criteria for VaD [3]. VaD is classified as a cerebral vessel vascular disease characterized by both large and small infarcts, lacunes, hippocampal sclerosis, and white matter lesions [4]. The cognitive decline associated with VaD is thought to be the result of cerebral ischemia secondary to the vascular pathologies aforementioned.

Behaviorally, patients with VaD show loss in executive functions as an initial symptom, whereas in AD memory loss is often associated with the earliest known symptoms. Other important symptoms of VaD include confusion, lan-guage deficits, restlessness and agitation, gait disturbances, and depression [5]. VaD predominantly affects patients with cardiovascular risk factors including hypertension [6, 7], hyperlipidemia [8], atherosclerosis [9], and diabetes [10-12]. Stroke is also an important risk factor for dementia [13, 14] and the most common stroke subtype associated with VaD is lacunar stroke [15].

Human apolipoprotein E (apoE) is polymorphic with three major isoforms, apoE2, apoE3, and apoE4, which differ by single amino acid substitutions involving cysteine-arginine replacements at positions 112 and 158 [16]. In AD, inheritance of the *APOE4* allele greatly increases risk up to 10 fold if both alleles are present [17]. Human apoE has a major function in the CNS as a cholesterol transporter and therefore, would seem to be a logical risk factor for VaD. The loss of apoE function perhaps following proteolytic cleavage is thought to be one potential mechanism by which apoE4 confers disease risk in AD [18]. However, whether apoE4 con-

Table 1. Case Demographics

Case	Age	Sex	PMI	ApoE Genotype	NPD	Braak and Braak	Plaque Stage
1	83	M	3.75	3/4	VaD	Stage 1	Stage A
2	75	F	10.5	3/3	VaD	Stage 2	None
3	83	M	12.4	4/4	VaD	Stage 3	None
4	74	M	2.6	3/4	VaD	Stage 2	Stage A
5	73	M	4.3	3/3	VaD	Stage 0	None
6	88	M	9.9	3/4	VaD	ND	Stage A
7	85	F	3.4	3/3	VaD	Stage 3	Stage B

PMI, postmortem interval in hours; NPD, neuropathological diagnosis, ND, not Determined.

fers a similar risk for the VaD is currently unclear and despite the large number of studies examining whether harboring apoE4 is a risk factor for VaD, an exhaustive literature search failed to yield a single study examining apoE4 pathology in the post-mortem VaD brain. The purpose of the present study was to determine the extent of apoE distribution in VaD using both full-length antibodies to apoE as well as an in house antibody that specifically detects the amino-terminal fragment of apoE [19]. Three consistent findings were observed using our cleavage apoE antibody in the VaD brain and included labeling within NFTs, blood vessels, and reactive astrocytes. These findings suggest a potential role for apoE in contributing to the disease process underlying VaD.

Material and methods

Immunohistochemistry

Autopsy brain tissue from seven neuropathologically confirmed VaD cases were studied. Case demographics are presented in **Table 1**. Fixed hippocampal tissue sections used in this study were provided by the Institute for Memory Impairments and Neurological Disorders at the University of California, Irvine. Free-floating 40 µm-thick sections were used for immunohistochemical studies as previously described [20]. No approval from Boise State University Institutional Review Board was obtained due to the exemption granted that all tissue sections were fixed and received from University of California, Irvine. Sections from the hippocampus were selected for immunohistochemical analysis.

For single labeling, all sections were washed with 0.1 M Tris-buffered saline (TBS), pH 7.4, and then pretreated with 3% hydrogen peroxide in 10% methanol to block endogenous peroxidase activity. Sections were subsequently washed in TBS with 0.1% Triton X-100 (TBS-A) and then blocked for thirty minutes in TBS-A with 3% bovine serum albumin (TBS-B). Sections were further incubated overnight at room temperature with the nApoECF antibody. Following two washes with TBS-A and a wash in TBS-B, sections were incubated in anti-rabbit or mouse biotinylated anti-IgG (1 hour) and then in avidin biotin complex (1 hour) (ABC, Elite Immunoperoxidase, Vector Laboratories, Burlingame, CA, USA). The primary antibody was visualized using brown DAB substrate (Vector Laboratories).

Immunofluorescence microscopy

Primary antibodies utilized included the nApoECF (rabbit, 1:100), PHF-1 (mouse monoclonal, 1:1,000), anti-apoE4 C-terminal (rabbit, 1:100), anti-apoE4 N-terminal (rabbit, 1:500), and anti-Ab mAb 1560 (clone 6E10, 1:400). The anti-Ab mAb 1560 (clone 6E10) was purchased from Covance (Dedham, MA). 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).

Immunofluorescence studies were performed by incubating sections with primary antibodies overnight at a room temperature, followed by secondary anti-rabbit or mouse biotinylated anti-IgG (1 hour) and then in ABC (1 hour). Visualization was accomplished by using a tyramide signal amplification kit (Molecular Probes, Eugene, OR) consisting of Alexa Fluor 488-labeled tyramide (green, Ex/Em = 495/519). To visualize beta-amyloid staining, sections were pretreated for 5 minutes in 95% formic acid. For immunofluorescence co-localization studies, antigen visualization was accomplished using an Alexa fluor 488-labeled tyramide (green, Ex/Em = 495/519) for one label and streptavidin Alexa fluor 555 (red, Ex/
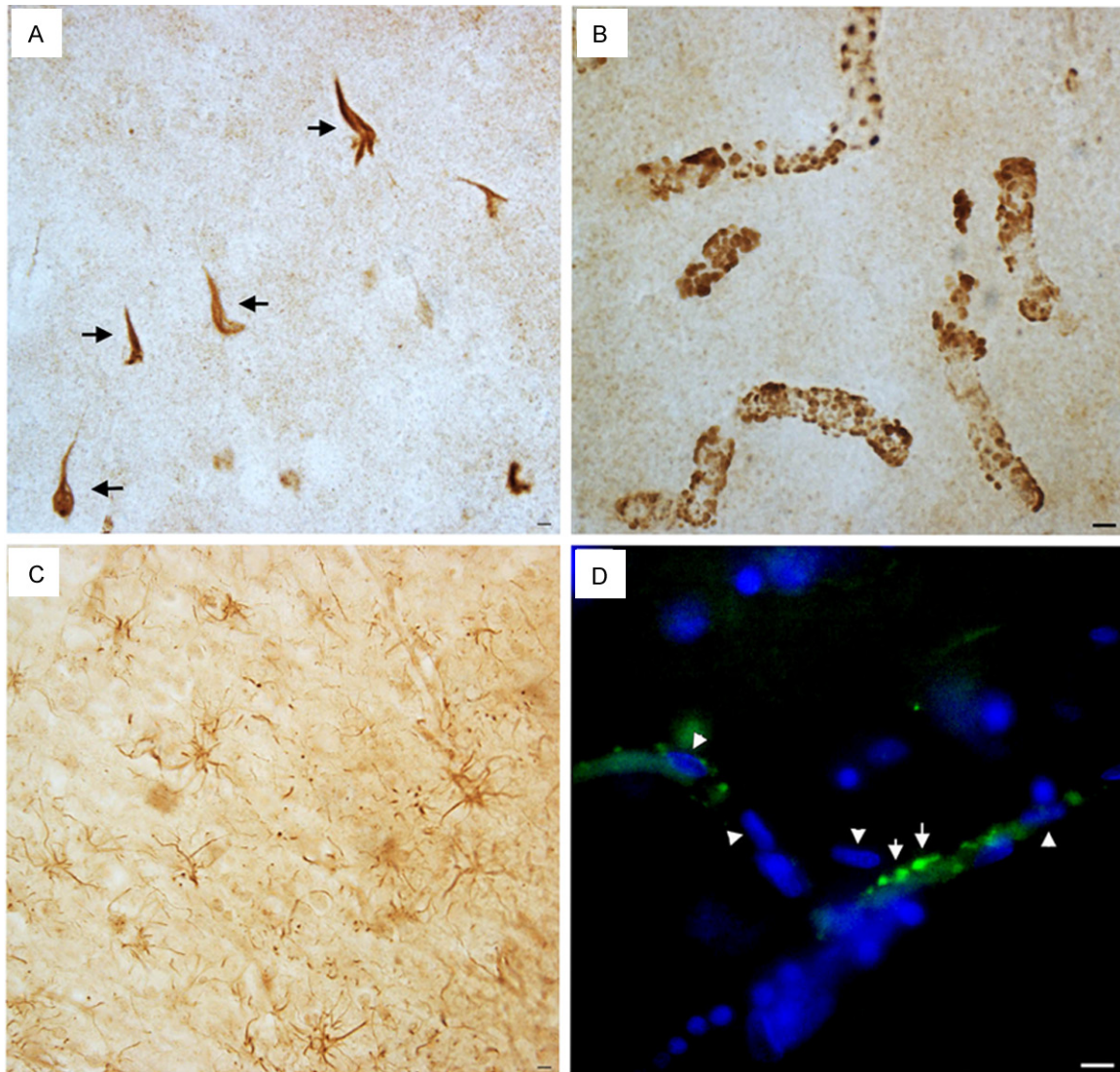
Figure 1. Localization of an amino-terminal fragment of apoE in vascular disease. Application of the nApoECF antibody in hippocampal tissue sections from VaD cases revealed labeling predominantly within NFTs (arrows, A), blood vessels (B), and reactive astrocytes (C). For Panel A, Case #7 was utilized, which had an APOE genotype of 3/3. Representative staining within blood vessels indicated a punctate labeling pattern following application of the nApoECF antibody (B, Case #5, APOE genotype, 3/3). For Panel C, Case #2 was use, which had a confirmed APOE genotype of 3/3. (D) Double-label immunofluorescence experiment utilizing the nApoECF antibody (arrows, green) together with Hoechst nuclear staining (arrowheads, blue) confirmed the punctate, irregular staining pattern of nApoECF within blood vessels. Note the appearance of cuboid, elongated nuclei that typically morphologically define endothelial cell nuclei (arrowheads). Rounder nuclei within the same regions most likely represent staining of nuclei from pericytes. For Panel D, Case #3 was utilized, which had a confirmed APOE genotype of 4/4. All scale bars represent 10 μm.

Em = 555/565) for the second label, both from Invitrogen (Carlsbad, CA).

Statistical analysis

To determine the percent co-localization, quantitative analysis was performed as described previously [19, 20] by taking 20X immunofluorescence, overlapping images from three differ-

ent fields in the hippocampus of four separate VaD cases. Capturing was accomplished by using a 2.5x photo eyepiece, a Sony high resolution CCD video camera (XC-77). As an example, to determine the percent co-localization between nApoECF and PHF-1, photographs were analyzed by counting the number of nApoECF-, PHF-1-positive NFTs alone per 20X

Case	NFTs	Reactive Astrocyts	Blood Vessels
1	+++	++	++
2	-	+++	+++
3	+++	++	+
4	+	-	++
5	-	-	+++
6	+++	++	+++
7	+++	-	+

(-) Denotes absence of any labeling with nApoECF.

field for each case, and the number of cells labeled with both PHF-1 and nApoECF. Data are representative of the average number (±S.D.) of each antibody alone or co-localized with both antibodies in each 20X field (3 fields total for 4 different cases). Statistical differences in this study were determined using Student's two-tailed T-test employing Microsoft Office Excel.

Results

Cleaved apoE immunoreactive pathology

Previous characterization of the nApoECF antibody indicated that it is highly specific for an 18 kDa amino-terminal fragment of apoE [19]. This in house antibody was synthesized based upon a putative caspase-cleavage site (DADD) at position D172 of the full-length protein. Application of this antibody to AD frontal cortex brain sections revealed specific localization within neurofibrillary tangles (NFTs) that was dependent upon the APOE genotype: 4/4≥3/4>3/3 [19]. However, *in vitro* cleavage of apoE4 by caspase-3 to generate an 18 kDa fragment detectable by the nApoECF antibody was unsuccessful [19] and the protease responsible for the *in vivo* cleavage of apoE4 is unknown at this time. To determine if amino-terminal fragments of apoE can be detected in VaD, an immunohistochemical study utilizing the nApoECF antibody was performed utilizing fixed hippocampal brain sections from seven VaD cases. Case demographics for the VaD cases used in this study are presented in **Table 1**. Notice that the APOE genotype was confirmed in all seven cases. All seven cases had a primary neuropathological diagnosis of VaD disease.

As an initial step, we screened all seven cases for nApoECF immunoreactivity using bright-field microscopy. Representative staining is depicted in **Figure 1** indicating consistent labeling of

nApoECF within NFTs (arrows, **Figure 1A**), blood vessels (**Figure 1B**), and within reactive astrocytes (**Figure 1C**). Labeling of nApoECF within blood vessels was punctate and irregular (**Figure 1B** and **1D**). The relative intensity and distribution of nApoECF in all seven cases is shown in **Table 2**. It is noteworthy that the nApoECF antibody cannot distinguish between the three different isoforms of apoE and will readily recognize the cleavage of apoE3 and E4 [19]. Although the general degree of nApoECF immunoreactive pathology appeared to be lower in those VaD cases defined as having *APOE* genotype of 3/3, strong immunolabeling was still observed in these cases (**Table 2**). However, overall it did appear that those cases determined to have the *APOE* genotype of either 3/4 or 4/4 exhibited a greater degree of nApoECF immunoreactive pathology (**Table 2**).

Full-length apoE4 immunoreactive pathology

Utilizing VaD Case #3 with a confirmed *APOE* genotype if 4/4, we screened this case using full-length antibodies to the N- or C-terminal regions of the protein. Previous studies in AD 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 [21]. We confirmed these results in VaD as depicted in **Figure 2**. Application of the full-length N-terminal antibody to apoE4 indicated staining within both NFTs (arrows, **Figure 2A**) and diffuse plaques (arrows, **Figure 2B**). In contrast, no staining within NFTs was observed utilizing the full-length C-terminal antibody to apoE but strong labeling was observed within blood vessels (**Figure 2C**). To confirm the co-localization of the full-length N-terminal antibody to apoE with our in house cleavage apoE antibody, double-label immunofluorescence studies were performed. Co-localization of both antibodies was evident within NFTs (**Figure 2D-F**). It is noteworthy that the nApoECF antibody did not label extracellular plaques in any VaD cases, as also previously demonstrated in the AD brain [19]. These data suggest that extracellular plaques are composed principally of full-length apoE4.

Full-length apolipoprotein E immunoreactivity co-localizes with beta-amyloid both extra- and intracellularly

To confirm the presence of full-length apoE within extracellular plaques, double-label immunofluorescence experiments were performed.
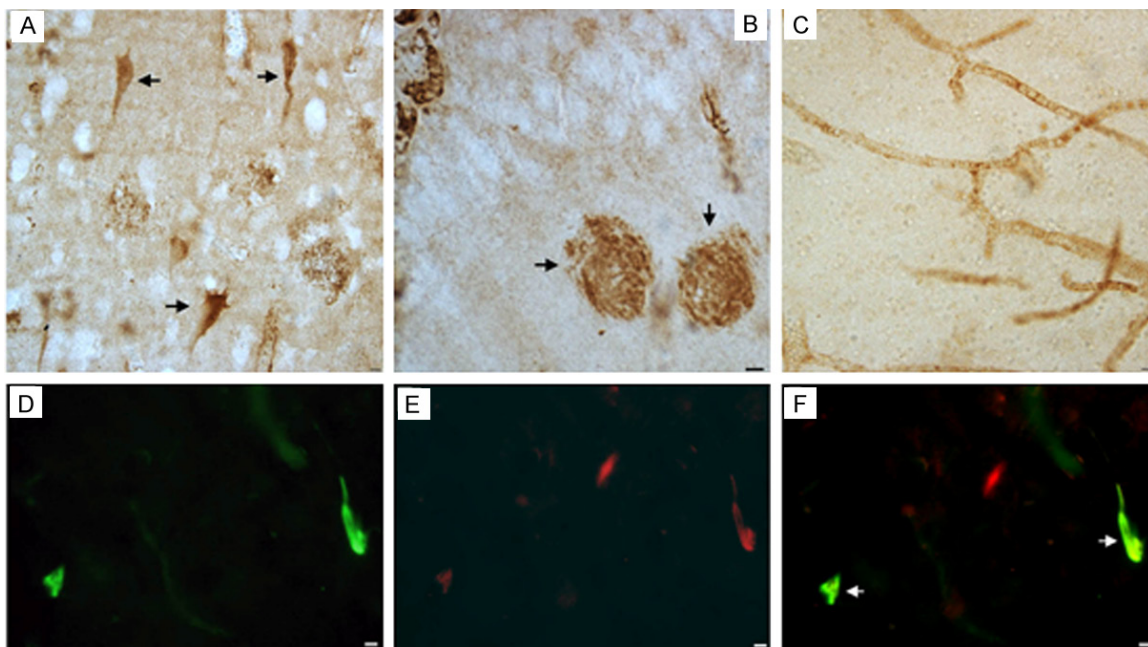
Figure 2. Characterization of apoE labeling in vascular disease utilizing full-length antibodies to both the C- and N-terminals regions to apoE. For all panels, representative labeling is shown utilizing Case #3, which had a confirmed APOE genotype of 4/4. (A and B) Representative staining following application of a full-length apoE antibody to the amino-terminal region of apoE revealed labeling within both NFTs (arrows, A) and diffuse plaques (arrows, B). (C) Identical to Panels A and B except for the use of a C-terminal full-length antibody to apoE indicating labeling predominantly within blood vessels. (D-F) Double-label immunofluorescence experiment employing our in house nApoECF antibody (green, D), amino-terminal full-length antibody to apoE (red, E), and the overlap image for the two antibodies indicating co-localization within apparent NFTs (arrows, F). All scale bars represent 10 μm.

As shown in **Figure 3**, co-localization between the full-length amino-terminal apoE4 antibody and 6E10 was evident within extracellular plaques in VaD (**Figure 3A-C**). A quantitative analysis of this co-localization revealed that approximately 76.5% of all beta-amyloid plaques contained the full-length apoE protein (**Figure 3H**). Besides plaques, co-localization of 6E10 and the full-length amino-terminal apoE antibody was also evident within blood vessels (**Figure 3D**), degenerating glial cells (**Figure 3F**), and within occasional NFTs (**Figure 3G**). Interesting, we also observed strong intracellular staining of the 6E10 antibody within neurons of the hippocampus that did not co-localize with the full-length apoE antibody (**Figure 3E**).

Co-localization of the amino-terminal fragment apoE antibody with PHF-1, a marker for NFTs

Experiments were undertaken to determine the extent of localization of the nApoECF antibody within NFTs in VaD. As depicted in **Figure 4**, co-localization between PHF-1 and nApoECF was evident within NFTs predominantly within layer II of the entorhinal cortex (**Figure 4A-C**). Interesting, co-localization was primarily reserved in Stage 3 and 4 NFTs, in contrast to Stage 2 NFTs that were only labeled with PHF-1 (**Figure 3A-C**). A quantitative analysis indicated that approximately 38.4% of all identified PHF-labeled NFTs also labeled with the nApoECF antibody (**Figure 4E**). Rarely, did we observe the labeling of NFTs with the nApoECF antibody alone (**Figure 4E**). In addition to NFTs, PHF-1 also strongly labeled neuritic plaques that consisted of large PHF-1 labeled strands (asterisk, **Figure 4D**). These structures were never labeled with the nApoECF antibody.

Identified NFTs in VaD were large and regionally localized to the entorhinal cortex

Application of the PHF-1 antibody in VaD revealed labeling of large NFTs in layer II of the entorhinal cortex (**Figure 5A**). It is possible that these are stellate cells as they were often locat-
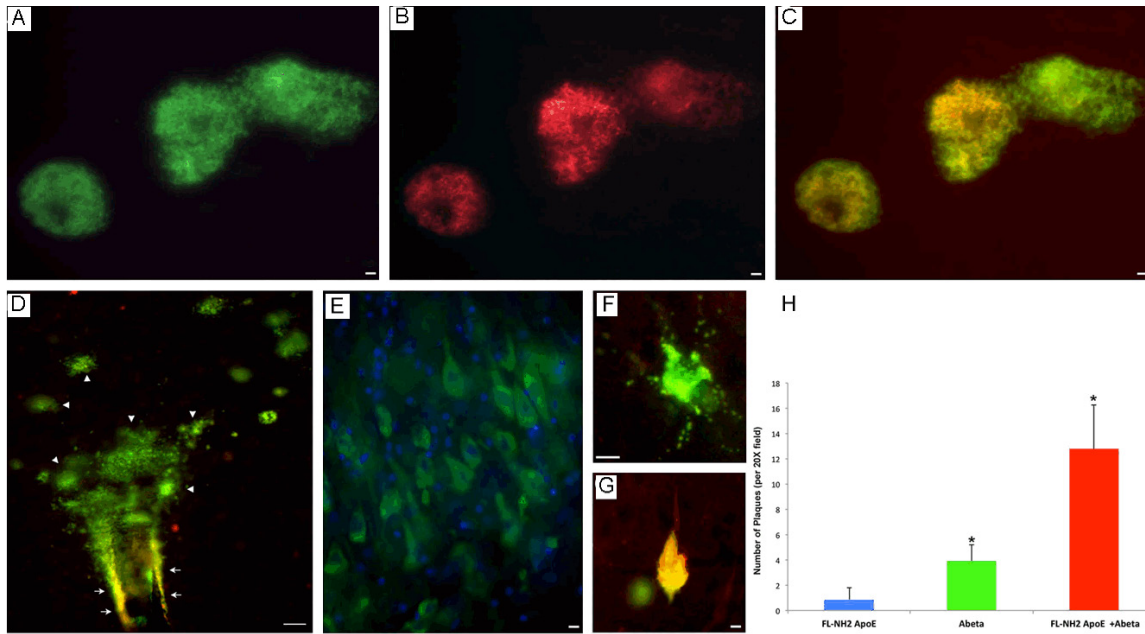
Figure 3. Distribution of beta-amyloid deposition in the vascular disease brain. (A-D) To confirm the presence of plaques, double-immunofluorescence studies were carried out using an anti-beta-amyloid antibody, 6E10 (green, A) together with a full-length amino-terminal antibody to apoE (red, B) with the overlap image shown in (C). The orange/yellow fluorescence in Panel C depicts where the two antibodies co-localize within plaques. (D) is identical to Panels A-C and depicts the labeling of apoE (arrows, yellow) predominantly within a blood vessel in which beta-amyloid plaques (arrowheads, green) are closely associated with in proximity. Representative immunofluorescence labeling in Panels A-D is shown utilizing Case #3, which had a confirmed APOE genotype of 4/4. (E-G) Besides plaques, application of 6E10 also indicated the presence of beta-amyloid within neurons with Hoechst nuclear staining (green, E, Case #4), degenerating glial cells (F, Case #1) and within NFTs (G, Case #1). Co-localization of 6E10 with full-length amino-terminal antibody was evident both within glial cells (F) and NFTs (G). (H) Quantification of plaques double-labeled by 6E10 and a full-length amino-terminal antibody to apoE. Data show the number of plaques labeled with the full-length apoE antibody alone (blue bar), 6E10 alone (green bar) or those plaques that were labeled with both antibodies (red bar). Plaques were identified in a 20X field within area CA1 by immunofluorescence overlap microscopy (n=3 fields for 4 different Pick cases) ±S.D., *$p<0.05$. Data indicated that roughly 76.5% of all identified plaques were labeled with both antibodies. All scale bars represent 10 μm except for Panel D, which represents 50 μm.

ed in entorhinal cortex islands. As a comparison to the size of NFTs found in VaD, PHF-1 labeling in a representative AD case is shown in **Figure 5B**. To validate these structures were indeed neuronal in nature, double-labeling experiments were carried out utilizing NeuN, a specific nuclear marker for neuronal cells. Co-localization between PHF-1 and NeuN was evident within large NFTs of the entorhinal cortex in the VaD brain (arrow, **Figure 5C-E**).

Discussion

VaD is the seconding leading cause of dementia in the USA [1], yet the causes of this form of dementia remain largely nebulous. Clearly, risk factors that increase the potential for strokes or microbleeds including hypertension, hyperlipidemia, and artherosclerosis are also impor-

tant risk factors for VaD [6-9]. One of the major roles of apoE is to transport cholesterol in the CNS, and therefore, it has been hypothesized that loss of function of apoE may contribute to a heightened risk for VaD as it does for AD [17]. In this manner, it has been hypothesized that proteolytic cleavage of apoE may lead to loss of function and functionally link the inheritance of the *APOE4* allele to the heightened risk for AD [18]. Previous studies have indicated that apoE is more susceptible to proteolytic cleavage than the other isoforms of apoE [18]. However, attempts to link *APOE* polymorphism to enhanced VaD risk have proven to be elusive as conflicting reports have emerged (For example see, [22-25]). Compounding these results is the paucity of studies examining apoE pathology in the VaD brain. Indeed, we were unable to
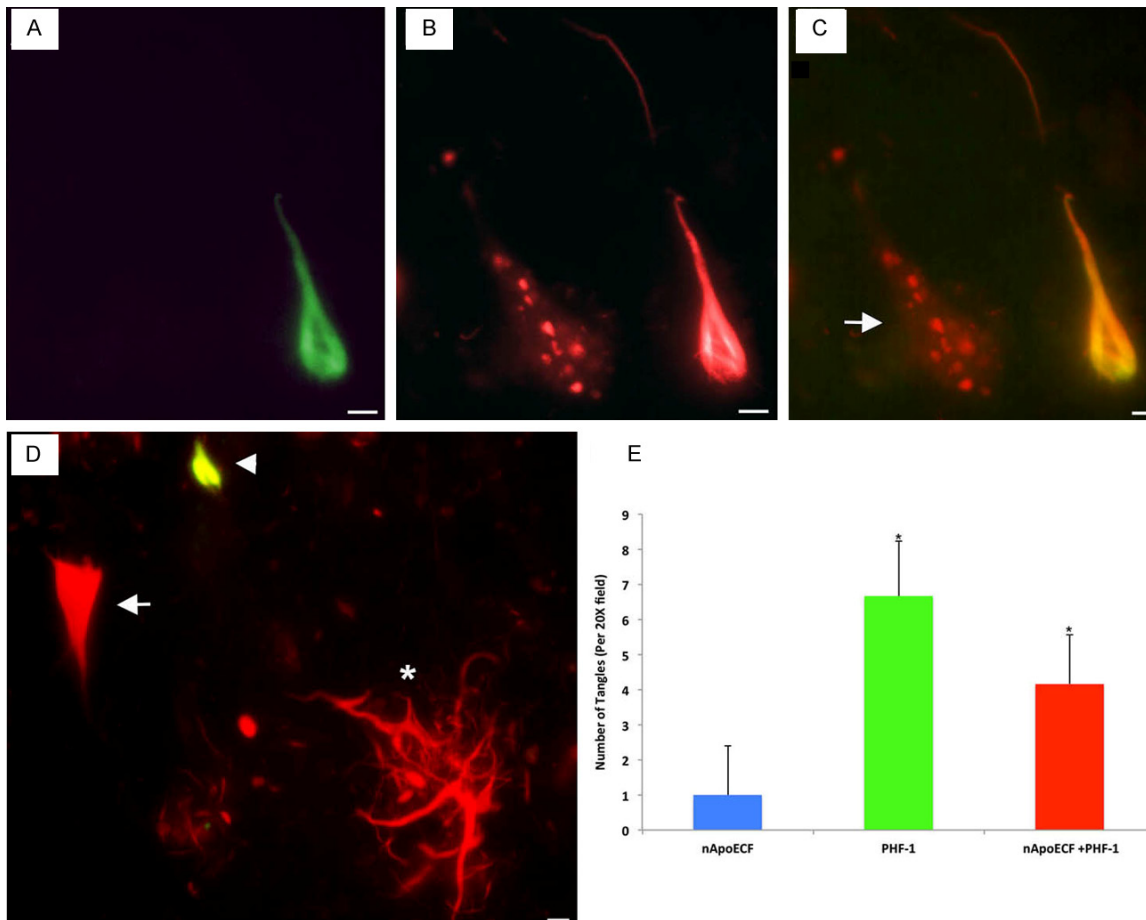
Figure 4. Co-localization of an amino-terminal fragment of apoE within NFTs of the vascular disease brain. (A-C) Representative immunofluorescence double-labeling in Case #1 identified as having an APOE genotype of 3/4. Utilizing the nApoECF antibody (green, Panel A) and PHF-1 (red, Panel B) revealed strong co-localization of the two antibodies within a NFT located in the entorhinal cortex (Panel C). Arrow in Panel C denotes a single-labeled tangle displaying vesicular punctate labeling with PHF-1 only. (D) Identical to Panel C illustrating co-localization of a single NFT (arrowhead) as well as the single-labeled, large NFT labeled with PHF-1 only. In addition, a neuritic plaque consisting of large PHF-1 labeled strands was evident within the field (asterisk, D). The representative staining shown in Panel D was accomplished using Case #6, which had a confirmed APOE genotype of 3/4. (E) Quantification of NFTs double-labeled by PHF-1 and nApoECF. Data show the number of NFTs labeled with nApoECF alone (blue bar), PHF-1 alone (green bar) or those NFTs that were labeled with both antibodies (red bar). NFTs were identified in a 20X field within hippocampal tissue sections by immunofluorescence overlap microscopy (n=3 fields using four different VaD cases) ±S.D., *p<0.05. Data indicated that roughly 38.4% of all identified NFTs were labeled with both antibodies. All scale bars represent 10 μm.

uncover a single study of apoE pathology in the VaD brain after an exhaustive literature search. Therefore, the primary purpose of this study was to examine apoE immunoreactive pathology in VaD utilizing a custom, in house antibody to the amino-terminal fragment of apoE as well as full-length antibodies.

Screening seven pathologically confirmed cases of VaD (**Table 1**) with our nApoECF antibody revealed three consistent staining features: 1) punctate labeling within blood vessels;

2) labeling of late-stage tangles primarily in layer II of the entorhinal cortex; 3) staining of reactive astrocytes. The staining within blood vessels was the most consistent finding utilizing the nApoECF antibody (**Table 2**) and suggests that cleavage of apoE within blood vessels could disrupt normal cholesterol transport and therefore, contribute to the cerebral vascular disease observed in this disease. Staining within reactive astrocytes may be indicative of the strong inflammatory component that has been associated with VaD [26]. In addition, a
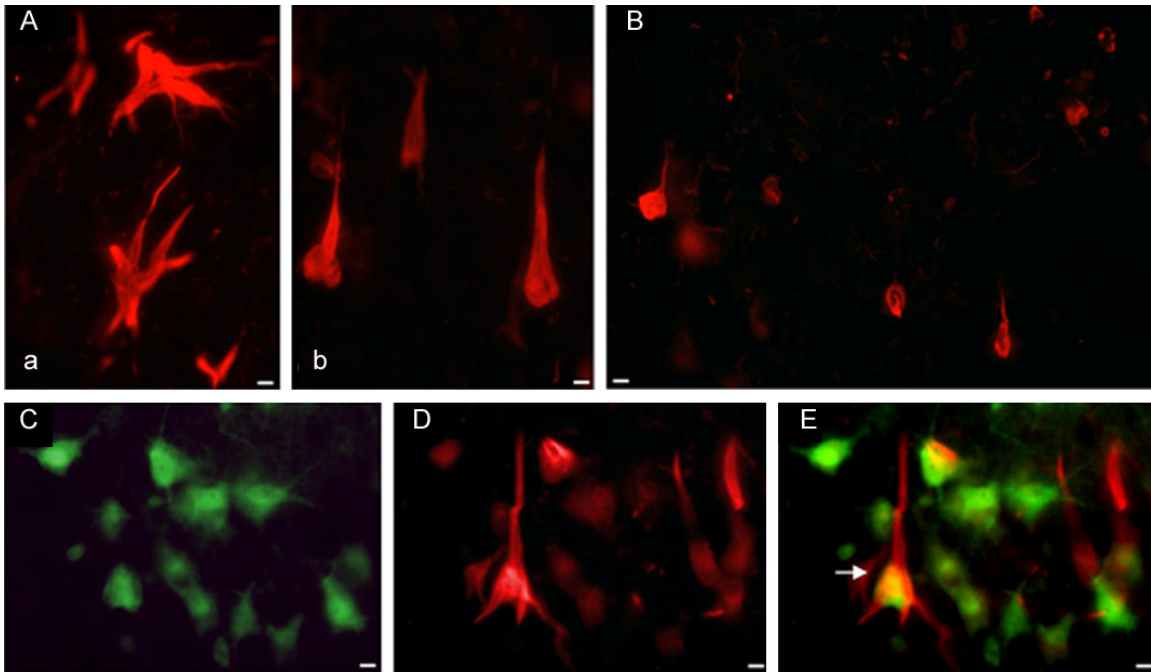
Figure 5. Presence of large, stellate neurofibrillary tangles in layer II of the entorhinal cortex in vascular dementia. (A, a and b) Representative single-label staining from VaD Case #1, showing staining in very large tangle-like structures in layer II of the entorhinal cortex. (B) As a comparison, the relative size of NFTs from an AD subject are shown following staining with PHF-1. Note the large size differential between the two types of tangles (Panel A vs. Panel B). (C-E) Double-label immunofluorescence experiments in VaD Case #1 utilizing the neuronal marker, NeuN, (green, C) together with PHF-1 (red, D), with the overlap image (arrow, E). All scale bars represent 10 μm.

previous study has documented the presence of extensive gliosis in the VaD brain supporting our current findings [27]. The finding of nApoECF within NFTs was not surprising, as this antibody strongly labels NFTs in the AD brain [19]. The localization of the nApoECF within NFTs was confirmed following co-localization experiments with PHF-1. In general, we observed three to four times less tangles in VaD as compared to AD. Thus, in a previous study we documented the presence of approximately 20-25 PHF-1-labeled neurons per 20X field [19] as compared to the current study whereby only 6-7 PHF-1-labeled neurons were documented (**Figure 4E**). Another interesting feature of PHF-1 labeling were the large size of neurons stained within clusters of layer II in the entorhinal cortex. These cells most likely are stellate neurons representing an early stage in tangle pathology as has been previously described in Down's syndrome [28]. The extent of co-localization between our nApoECF antibody and PHF-1 was only 38%. This value was much lower for the co-localization of the same two antibodies in AD, which we have previously reported to be 60%. The overall low percent co-localization would suggest that the cleavage of apoE and tangle pathology is poorly correlated as supported by a calculated Pearson coefficient of 0.27.

To verify the localization of our nApoECF antibody within NFTs, co-localization experiments were carried out using full-length antibodies to either the N- or C-terminal regions of apoE. As expected, we confirmed the co-localization of our in house antibody with the full-length N-terminal antibody but not the C-terminal antibody to apoE. Application of the full-length N-terminal apoE antibody to VaD cases also revealed strong immunolabeling within extracellular plaques that co-localized with an anti-beta-amyloid antibody, 6E10. An interesting feature of the nApoECF antibody is that it does not label extracellular plaques in the AD brain [19], nor did it label plaques observed in VaD. These data suggest that extracellular plaques consist primarily of full-length apoE, moreover, the presence of cleaved apoE within NFTs supports the hypothesis that the cleavage event of

apoE occurs intracellularly or within blood vessels, but not in the extracellular compartment.

A final observation regarding apoE in the VaD brain that was shown in the present study was the staining of the nApoECF antibody in cases that had known genotypes of 3/3. Human apoE is polymorphic with three major isoforms, apoE2, apoE3, and apoE4, which differ by single amino acid substitutions involving cysteine-arginine replacements at positions 112 and 158 [16]. Our in house nApoECF antibody recognizes the N-terminal fragment of apoE in 3/3 and 3/4, and 4/4 genotypes [19]. In VaD, we observed widespread staining of the nApoECF in cases expressing all three different genotypes (**Table 2**). However, there appeared to be a general trend for less apoE-immunoreactive pathology in VaD cases with known genotypes of 3/3 (**Table 2**). This is consistent with previous observations that apoE4 is highly susceptible to proteolysis compared to the other major isoforms of apoE [29].

In conclusion, we have reported for the first time the presence of cleaved apoE in the VaD brain. The localization of an amino-terminal fragment of apoE was determined to largely be confined within NFTs, blood vessels, and reactive astrocytes. ApoE immunoreactive pathology was documented in all seven VaD cases examined including those cases with a confirmed *APOE* genotype of 3/3. The presence of cleaved apoE within the hippocampus of VaD cases provides for pathological data supporting the potential link between *APOE* polymorphism and enhanced risk for VaD. In addition, the potential loss of function of apoE following cleavage may contribute to disease progression in a disorder whereby cerebral vascular risk factors play such an important role.

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Disclosure of conflict of interest

None.

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