Vascular Smooth Muscle Actin Is Reduced in Alzheimer Disease Brain: A Quantitative Analysis

JOHN F. ERVIN, BA, CATHERINE PANNELL, MARI SZYMANSKI, RN-C, KATHLEEN WELSH-BOHMER, PHD, DONALD E. SCHMЕCHЕL, MD, AND CHRISTINE M. HULETTE, MD

Abstract. We analyzed smooth muscle actin (SMA) immunoreactivity in brain blood vessels of 10 ApoE 4,4 Alzheimer disease (AD) patients and 10 ApoE 3,3 AD patients matched for age, sex, and duration of dementia. We also examined 10 cognitively and neuropathologically normal controls matched for age and sex. Vascular SMA immunoreactivity in the arachnoid, grey matter, and white matter was quantified by image analysis. There was less SMA immunoreactivity in blood vessels of all AD patients when compared to cognitively and neuropathologically normal controls (p < 0.001). In addition, arachnoidal vessels of ApoE 4,4 AD patients had less SMA immunoreactivity than ApoE 3,3 AD patients (p < 0.05). There is decreased vascular SMA density in arachnoid, grey matter, and white matter blood vessels in patients with AD when compared to age matched, cognitively and neuropathologically normal controls. The severity of the loss of SMA within the AD group may depend on ApoE type.

Key Words: Alzheimer disease; ApoE; Blood vessels; Image analysis; Smooth muscle actin.

INTRODUCTION

ApoE functions in lipid transport and maintenance of membrane integrity (1). It has been shown to play a role in nerve regeneration (2). In humans, ApoE exists in 3 allelic forms ε2, ε3, and ε4. Inheritance of the allele ApoE-ε4 increases the risk and lowers the age of onset for familial and sporadic Alzheimer disease (AD) (1, 3–5). In AD patients, ApoE-ε4 is associated with increased frequency and severity of amyloid angiopathy (6, 7). It is also associated with impaired recovery from head injury and stroke (8–11), which are risk factors for dementia, and also with vascular dementia (12–14). In addition to putative roles in the pathogenesis of central nervous system disease, the lipid transport molecule, ApoE, has been implicated in the development of systemic atherosclerosis (15–18). Thus, ApoE seems to be the common denominator in many pathological processes. It plays an important role in the pathogenesis of atherosclerosis, both systemic and cerebrovascular, and in 2 forms of dementia: vascular dementia and AD. ApoE thus plays an important role in 2 common pathologies associated with late-life dementia. Furthermore, some authors have suggested that microvascular disease is the primary pathological event in the development of Alzheimer-type dementia (19, 20).

AD is characterized pathologically by the formation of senile neuritic plaques that contain βA4 amyloid. In vitro studies have shown that ApoE-ε4 has a higher avidity for βA4 amyloid than ApoE-ε3 (21). Individuals with the ApoE 4/4 genotype have a higher burden of βA4 amyloid in senile neuritic plaques and in vessel walls than individuals with the ApoE 3/3 genotype (6, 22). Previous work had suggested that vascular pathology in AD may also vary as a function of ApoE type (23). The present study was undertaken to extend and strengthen these early observations of vascular pathology in AD. We sought to analyze the severity of microvascular disease by immunostaining with smooth muscle actin, an important component of arteries, arterioles, and large veins, by examining cortical brain tissue from normal controls and 2 groups of AD patients.

MATERIALS AND METHODS

Controls and demented subjects were enrolled in the autopsy program of the Joseph and Kathleen Price Bryan Alzheimer Disease Research Center according to standard protocols approved by the Duke University Medical Center Institutional Review Board (IRB) (24). Autopsies were performed according to institutional guidelines. Brains were subsequently examined neuropathologically and diagnosed as AD or normal according to NIA-Reagan Institute criteria (25). Brains were banked according to approved protocols. ApoE genotyping was performed according to established methods (4). Results of the analysis of 30 cases (i.e. 20 AD cases and 10 controls) are reported here.

The cases selected from the Kathleen Price Bryan Brain Bank were matched for age and gender. The AD groups were also matched for duration of dementia and Braak stage (26). None of the cases studied here exhibited significant cerebrovascular atherosclerosis in the vessels of the circle of Willis or in the arachnoidal blood vessels. These carefully matched cases were selected in order to study variations between 3 major groups: ApoE 4,4 AD, ApoE 3,3 AD, and Normal. There were 5 male and 5 female ApoE 4,4 AD subjects with an average age of 76. There were 5 male and 5 female ApoE 3,3 AD subjects with an average age of 75. There were 10 cognitively
Incubated for 1 hour at 37°C body to smooth muscle actin (SMA; clone 1A4; DAKO, Carpinteria, CA) was used. The antibody was diluted 1:100 and incubated for 1 hour at 37°C. A biotinylated horse anti-mouse IgG secondary antibody (Vector Labs, Burlingame, CA) 1:300 was incubated for 20 min at 37°C. Horseradish peroxidase labeled streptavidin (DAKO) 1:500 was incubated for 20 min at 37°C. The sections were developed with 0.5 mg/mL diaminobenzidine DAB (Sigma, St. Louis, MO) solution for 3 min.

Within each cortical section, 3 anatomical regions (arachnoid, grey matter, and white matter) were examined. Five arteries from each region were selected for detailed image analysis. Images of the vessels were captured using a Pixera Penguin 600CL camera attached to a Nikon Optiphot-2 microscope. The images were saved using ViewFinder 3.0 software and analyzed using Metamorph 4.12 image analysis software from Universal Imaging (Downington, PA). Images were captured and analyzed randomly. The technician was blinded as to diagnosis. Two images of each vessel were taken. The first image allowed visualization of the entire structure and the second image was captured after adjusting the exposure time to allow specific visualization of the tunica media of the artery.

The amount of positive staining in the tunica media of the artery wall was quantified using Metamorph 4.12. The first image that was captured was used to determine the pixel area of the vessel wall. The vessel was divided into 2 regions using a drawing tool. Region 1 represented the entire tunica media including the lumen and Region 2 represented the lumen only. The pixel area of the vessel wall was determined by subtracting the pixel area of the lumen from the total pixel area. Using the second image of the artery that was captured, the pixel area of the positively stained portion of tunica media was measured. By creating a region around the image and a threshold for dark objects, the pixel area of the positively stained area was determined. Dividing the pixel area of the positively stained portion by the pixel area of the tunica media, a percentage of staining was established. This was completed for 5 vessels in each of the 3 anatomical layers for both the middle frontal and inferior parietal cortical regions in each case.

The percentage of vessel staining was analyzed statistically using a Microsoft Excel spreadsheet. Graphs were created to demonstrate trends between different groups of subjects. A Student t-test was used to determine significance between the different groups. Comparisons with p values ≤0.05 were considered to be significantly different.

### RESULTS

Visual examination with light microscopy revealed that SMA-positive staining was seen only in the tunica media. The differences in intensity of SMA staining between groups was obvious on visual inspection (Fig. 1). The positively stained vessels in the arachnoid were of large caliber and arteries had thick walls and a relatively small lumen. Occasional veins in the arachnoid exhibited some SMA immunoreactivity; however, veins were not included in this analysis. Arteries in the grey matter were of intermediate caliber, and arteries in the white matter were the smallest in diameter. (Data not shown) SMA immunoreactivity in the vasculature was quantified by image analysis. Comparison of SMA densities between the different patient groups highlighted a pattern in vascular SMA density that was similar for the middle frontal and

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normal control subjects with an average age of 71.3 years at death (range 60 to 80 years). These subjects had undergone cognitive testing within 1 year of death as previously described (27, 28). All control subjects were neuropathologically normal. There was no large vessel atherosclerosis. There were no neuritic plaques and no histological evidence of vascular amyloid as demonstrated by immunostain for β amyloid peptide. Mild neurofibrillary change was seen only in the entorhinal cortex, corresponding to Braak Stage I (26). Details of the study population are shown in Table 1.

Paraffin-embedded tissue sections from the middle frontal cortex and the inferior parietal cortex of each subject were immunostained with SMA. Sections were cut at 8 µm, deparaffinized in xylene, and hydrated through graded ethanol. Endogenous peroxidase activity was blocked with 2% H2O2 in methanol. Antigen retrieval was performed by boiling the sections in citrate buffer pH 6.0 for 30 min. A monoclonal antibody to smooth muscle actin (SMA; clone 1A4; DAKO, Carpinteria, CA) was used. The antibody was diluted 1:100 and incubated for 1 hour at 37°C. A biotinylated horse anti-mouse IgG secondary antibody (Vector Labs, Burlingame, CA) 1:300 was incubated for 20 min at 37°C. Horseradish peroxidase labeled streptavidin (DAKO) 1:500 was incubated for 20 min at 37°C. The sections were developed with 0.5 mg/mL diaminobenzidine DAB (Sigma, St. Louis, MO) solution for 3 min.
Fig. 1. SMA immunostain of arachnoid blood vessels. **Left panel:** ApoE 4,4 AD. **Right panel:** ApoE 3,3 AD. The intima is at the left in each panel.

**TABLE 2**

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<th>Statistical Differences in SMA Staining Intensity of the Blood Vessels from 3 Cortical Regions</th>
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Within each block, the group and area compared is listed first, with the p value listed below.

inferior parietal lobes when each area was examined alone (data not shown). Data obtained from vessels in the middle frontal and inferior parietal lobes were combined. SMA densities in both males and females were similar when the sexes were examined alone (data not shown). Therefore, data obtained from males and females were also combined. Details of the statistical analysis of the differences between the SMA staining intensity of the blood vessels from 3 anatomical regions and between patient groups are shown in Table 2.

Examination of SMA immunoreactivity within the normal control group demonstrated that there was more vascular SMA in the arachnoid arteries than in the grey matter or in the white matter arteries. There was also more SMA immunoreactivity in the grey matter arteries than in the white matter arteries. These comparisons were significant at the p < 0.001 level.

Analysis of the difference in SMA between the combined AD groups and the cognitively and pathologically normal control group showed that there was much more staining of SMA in the vessels from all regions in the control group than in the AD group. The difference between arachnoid vessel staining in the normal group when compared to the AD groups was highly significant (p < 0.001). The difference between grey matter vessel staining in the normal group when compared to the AD groups was also highly significant (p < 0.001). The difference between white matter vessel staining in the normal group when compared to the AD groups was highly significant (p < 0.001; Fig. 2).
Comparison of vascular SMA staining between the ApoE 3,3 AD or the ApoE 4,4 group and the cognitively and pathologically normal control group also showed much more staining of SMA in the vessels from all regions in the control group than in either AD group. The difference between arachnoid vessel staining in the normal group when compared to the ApoE 4,4 or the ApoE 3,3 AD group was very highly significant ($p < 0.0001$). The difference between grey matter vessel staining in the normal group when compared to the ApoE 4,4 or the ApoE 3,3 AD group was highly significant. Comparison of the ApoE 4,4 AD group with normal group was significant at $p < 0.0001$. Comparison of the ApoE 3,3 AD group with the normal group was only slightly less significant at $p < 0.001$. The difference between white matter vessel staining in the normal group when compared to the ApoE 4,4 or ApoE 3,3 AD group was also very highly significant ($p < 0.0001$).

There was no difference in staining between the arachnoid and the grey matter arteries in either the ApoE 4,4 or ApoE 3,3 AD groups. The comparison of arachnoid vessel staining with white matter vessel staining was different for the ApoE 3,3 AD group ($p < 0.001$) and for the control group ($p < 0.001$), but not for the ApoE 4,4 group. The comparison of arachnoid vessel staining with white matter vessel staining for the ApoE 4,4 AD group was, however, on the cusp of significance ($p = 0.06$). SMA staining in the arachnoid vessels when compared to the grey matter vessels was different only in the control group ($p < 0.001$).

Both AD groups had less vascular SMA immunoreactivity in the arachnoid than in the grey matter. This pattern was the inverse of the normal pattern where there was more SMA immunoreactivity in the arachnoid vessels than in the grey matter vessels. Comparison of grey matter to white matter vessel staining within each group was significant. The comparison of staining in grey matter and white matter was significant for the ApoE 4,4, group ($p = 0.01$). Comparison of staining in grey matter and white matter was more highly significant for the ApoE 3,3 group ($p < 0.001$).

The difference between arachnoid vessel staining between ApoE 4,4 and ApoE 3,3 AD patients was significant ($p < 0.05$). The difference between grey matter vessel staining between ApoE 4,4 and ApoE 3,3 AD patients was marginally significant ($p = 0.05$). The difference in white matter vessel staining between ApoE 4,4 and ApoE 3,3 AD patients was not different (Fig. 3).

DISCUSSION

Comparison of SMA densities in the arteries between the different patient groups highlight a pattern in vascular SMA density that was similar for the middle frontal and inferior parietal lobes when each area was examined alone. However, vascular SMA density did vary with the anatomical region, with diagnosis, and with ApoE genotype. Within the normal control group there was more SMA immunoreactivity in arteries of the arachnoid than in arteries of the grey matter. The grey matter arteries in turn exhibited more immunoreactivity than the white matter arteries. This may be due to normal anatomical variation. Arteries in the arachnoid are of larger caliber than arteries of the grey matter, which are in turn larger in diameter than arteries in the white matter.

Intriguingly, this normal anatomical variation was abolished in both of the AD groups. In both AD groups there was less vascular SMA immunoreactivity in the arachnoid than in the grey matter. However, the normal relationship of greater immunoreactivity in the grey matter than in the white matter was preserved in both AD groups, but the difference between the grey matter and the white matter was less marked in both of the AD groups than in the control group.

Comparison of the SMA immunoreactivity in the arachnoid vessels, cortical vessels, and the white matter vessels between the combined AD groups and the controls was highly statistically significant. There was a great difference in arterial SMA immunoreactivity in the AD group vessels from each area when compared to the normal control group. This diminishment of SMA immunoreactivity in AD was accentuated in ApoE 4,4 AD subjects. The ApoE 4,4 AD group not only exhibited less SMA density in all areas than the control group, but they also exhibited less SMA immunoreactivity in the arachnoid and in the grey matter than the ApoE 3,3 AD group.
Unfortunately, the control group consisted entirely of ApoE 3,3 individuals. We deliberately selected controls who were age and sex matched with the AD groups. We also wanted to include only cognitively normal individuals who had no evidence of possible AD upon post-mortem examination. Although anecdotal reports of normal elderly with no histopathological evidence of possible AD by CERAD criteria and at least 1 ApoE 4 allele exist, these cases are very rare. In the Kathleen Price Bryan Bank we have available a total of 134 neuropathologically normal brains. Of these, 77 are CERAD 1a. Ten of these CERAD 1a elderly controls are ApoE 3,4 and only 1 is ApoE 4,4. The brains from 57 elderly controls have a few or moderate neuritic plaques and are, therefore, CERAD 1b. Nine of these CERAD 1b controls are ApoE 3,4 and only 1 is ApoE 4,4. Since there were only 2 normal ApoE 4,4 cases identified in the bank, no statistically valid comparison could be made.

The arachnoid arteries show the highest density of SMA. This may be due to increased numbers of smooth muscle cells present in the media of the vessel wall in this location. A simple explanation would be that this disparity is due to the increased size of arachnoid vessels. These large arteries are required to carry blood under high pressure and may require a greater actin density for normal function. This same explanation would hold for the relative size of vessels in the grey matter as compared to the white matter. However, some distinct physiological difference or function between the arachnoid vessels and the cortical vessels cannot be excluded.

We have demonstrated loss in arterial SMA immunoreactivity in AD brains when compared to cognitively and neuropathologically normal controls. Changes in cerebrovascular density and the presence of vascular abnormalities are common in normal aging (30) and in Alzheimer disease (19). Such alterations are thought to be related to brain metabolism, neuronal loss, degenerative changes and environmental, genetic, and toxic factors. In AD, the vasculature of the leptomeninges and cortex is often altered by amyloid deposition. The resultant condition, cerebral amyloid angiopathy (CAA), is a prominent feature of AD (31). The severity of vascular amyloid deposition varies with the presence of the allele ApoE-e4 (6, 22). ApoE-e4 has also been shown to have a high avidity for βA4 amyloid using in vitro models (21).

Vascular βA4 deposition in AD and the normal aging brain is found in both the large and small arteries and arterioles of the leptomeninges and cortex (31). A relationship between smooth muscle cell deterioration and βA4 has been seen, but the precise pathophysiological mechanisms involved are still controversial. Some
authors believe that smooth muscle cells release βA4 amyloid and other extracellular components as they are injured and begin to degenerate. It has been hypothesized that this protein deposition is a physiologic response or repair mechanism, which may unfortunately result in cellular suicide (13, 14, 32, 33). Other studies suggest that the deposition of βA4 amyloid comes first, and as it increases it damages the smooth muscle cells, which then exhibit degenerative changes (34). The latter implies that βA4 has a toxic role. This idea is supported by studies implicating βA4 amyloid as a neurotoxin (20, 35).

Furthermore, detailed analysis of potential drainage routes for β amyloid have demonstrated that β amyloid in the cortex, associated with plaques, is in direct continuity with β amyloid in the capillaries. This suggests that there is a direct drainage route from the brain into the perivascular spaces of the capillaries. However, there is a distinctly different pattern seen for arterial β amyloid. Arterial β amyloid is not in direct continuity with cortical β amyloid. This would suggest that functional changes in ageing arteries, such as loss of SMA, may result in failure to adequately eliminate β amyloid (38).

Increasing knowledge about CAA and its prominence in AD has ignited interest in the vasculature and the role it might play in the pathogenesis of AD. Vascular degeneration is a consequence of CAA that has been qualitatively assessed (14). These studies have shown that vessels affected by CAA are thickened with βA4 amyloid and show a partial or complete loss of SMA staining. The neuropathological findings reported here are based upon brains from AD patients and controls who were very closely matched for a variety of clinical and pathological criteria, including age, sex, duration of dementia and Braak stage. All brains lacked any significant atherosclerosis in the vessels at the base. They also lacked any evidence of microvascular disease such as lacunes or microinfarcts; thus, the only difference between the AD groups was ApoE type. ApoE 3,3 and ApoE 4,4 AD cases were evaluated diagnostically according to CERAD criteria NIA Reagan Institute Guidelines. Therefore, all AD cases exhibited frequent neuritic plaques and sufficient neurofibrillary tangles to meet consensus guidelines for intermediate or high likelihood AD. The vasculature of these 2 AD groups, however, is slightly different. ApoE 4,4 AD cases had less smooth actin density in the vessels of the arachnoid layer compared to ApoE 3,3 AD. This observation implies more smooth muscle cell deterioration in arachnoid arteries ApoE 4,4 AD than in ApoE 3,3 AD.

Findings from studies of Apolipoprotein E knockout mouse models of AD would support this observation. The mouse normally expresses only 1 type of ApoE. Some investigators believe that the ApoE knockout mouse thus mimics the human ApoE 4,4 homozygous condition. ApoE knockout mice have been studied extensively but lack any histopathological evidence of the senile plaques and neurofibrillary tangles that are the hallmarks of human AD. The mice do, however, exhibit an impaired blood-brain barrier (36, 37).

We have observed significantly decreased SMA in the cerebral blood vessels of patients with AD compared to cognitively and neuropathologically normal elderly controls. In addition, ApoE 4,4 AD patients had less vascular smooth muscle action immunoreactivity in arachnoid arteries than ApoE 3,3 AD patients. This observation suggests vascular disease is important in the pathogenesis of AD and that ApoE4 may enhance the degeneration of smooth muscle cells within the cerebral vasculature. This may result in an impaired blood brain barrier or inadequate drainage of β amyloid. Our findings would also suggest that different pathogenetic mechanisms may play varying roles in different genetic forms of Alzheimer disease.

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REFERENCES


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