Amyloid β-protein length and cerebral amyloid angiopathy-related haemorrhage

Mark O. McCarron,[1,2,CA] James A. R. Nicoll,[2] Janice Stewart,[2] Gregory M. Cole,[3] Fushen Yang,[3] James W. Ironside,[4] David M. A. Mann,[5] Seth Love[6] and David I. Graham[2]

Departments of [1]Neurology and [2]Neuropathology, Institute of Neurological Sciences, Southern General Hospital, Glasgow, G51 4TF, UK; [3]Department of Medicine, UCLA and Sepulveda VAMC, CA 91343, USA; [4]Neuropathology Laboratory, Department of Pathology, University of Edinburgh, Alexander Donald Building, Western General Hospital, Crewe Road, Edinburgh EH4 2XU; [5]Department of Pathological Sciences, University of Manchester, Stopford Building, Manchester, M13 9PT; [6]Department of Neuropathology, Frenchay Hospital, Bristol BS16 1LE, UK

[CA,2]Corresponding Author and Address

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The relationship between amyloid β-protein (Aβ) length and the apolipoprotein E (*APOE*) ε2 allele, which is over-represented in cerebral amyloid angiopathy-related haemorrhage (CAAH), has not previously been examined. Of 57 CAA patients studied, 37 had CAAH. All patients, particularly those with CAAH had more blood vessels immunoreactive for Aβ40 than Aβ42 in both the leptomeninges and cerebral cortex. CAAH patients had more Aβ40-immunoreactive blood vessels in the leptomeninges ($p < 0.001$) and cortex ($p = 0.027$) than had non-haemorrhage patients. Cortical blood vessels, the usual source of haemorrhage in CAAH, were more frequently Aβ42 immunoreactive in *APOE* ε2 carriers than in non-ε2 carriers ($p = 0.022$). The *APOE* ε2 allele may predispose to CAAH by increasing the seeding of cortical blood vessels by Aβ42. *NeuroReport* 11:937–940 © 2000 Lippincott Williams & Wilkins.

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INTRODUCTION

Processing of amyloid precursor protein by β- and γ-secretases is thought to generate amyloid β-protein (Aβ) with heterogeneous carboxyl termini. γ-secretase-mediated cleavage occurs predominantly at two sites, to produce Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42) [1]. Aβ42 is considerably more amyloidogenic and is the predominant form of Aβ in senile plaques in both demented [2] and non-demented individuals [3]. In Alzheimer's disease Aβ40-positive plaque frequency but not Aβ42 has been reported to increase with apolipoprotein E (*APOE* for gene; apoE for protein) ε4 allele dose [4]. In diffuse plaques Aβ deposition begins with Aβ42 [5].

Despite some initially conflicting data [6], vascular amyloid or cerebral amyloid angiopathy (CAA) appears to immunostain predominantly with antibodies to Aβ40 [7,8]. The amount of Aβ40 increases with the severity of CAA and the number of *APOE* ε4 alleles [9]. In hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D) Aβ40 is likewise the predominant vascular Aβ [10], although capillary Aβ42 deposits are thought to be present at an early stage in the development of this familial CAA [11].

Lobar haemorrhage is the main clinical manifestation of CAA, which otherwise usually remains clinically silent. Current evidence suggests that *APOE* genotype can influence the phenotype of CAA. While the ε4 allele predisposes to vascular Aβ deposition [12,13], it appears that the ε2 allele is associated with subsequent vessel rupture [14], probably by enhancing fibrinoid necrosis [15] or vessel wall cracking [16]. This study sought to characterize vascular Aβ in CAA with and without lobar haemorrhage and to examine the relationship between the *APOE* genotype and the length of Aβ in the cortical and leptomeningeal vasculature.

MATERIALS AND METHODS

Fifty-seven caucasian patients with pathological evidence of CAA were examined: 37 had a diagnosis of CAA-related haemorrhage and 20 had CAA without evidence of lobar haemorrhage. Among the CAA-related haemorrhage patients, 31 fulfilled the criteria for definite CAA-related haemorrhage in that a full postmortem examination demonstrated lobar, cortical or corticosubcortical cerebral haemorrhage with abundant Aβ in leptomeningeal and cortical blood vessels in the absence of other diagnostic lesions [13]. In six patients with lobar or superficial haemorrhage a biopsy at the time of haematoma evacuation demonstrated amyloid-laden blood vessels, making a diagnosis of probable CAA-related haemorrhage with

supporting pathological evidence [13]. Sections of formalin-fixed, paraffin-embedded tissue that comprised leptomeninges and cerebral cortex from the middle frontal gyrus or adjacent to the haematoma were assessed as described previously [15].

Primary antibodies to Aβ40 (polyclonal 34-40, 1:1000) and Aβ42 (monoclonal 7A3 raised to Aβ37-42 and which recognises Aβ42, 1:12,000) were used. The antibodies were previously characterised using dot blot and preadsorption [7,17] and in direct and sandwich ELISA assays (Cole *et al.*, unpublished). The presence of amyloid-laden blood vessels was confirmed with an alkaline Congo red stain [18].

Adjacent tissue sections were used for immunohistochemistry. After the sections had been dewaxed, endogenous peroxidase was blocked by treating the sections with 3% hydrogen peroxide for 30 min. Sections were then treated with 80% formic acid for 8 min and blocked for 1 h in 20% goat serum. Sections were incubated overnight with the primary antibody in blocking serum. Immunostaining was demonstrated by the avidin–biotin technique and visualized using 3,3′-diaminobenzidine as the chromogen. The sections were dehydrated, counterstained with hematoxylin and mounted.

For each antibody, leptomeningeal and cortical blood vessels (arteries, arterioles and venules) were scored separately, blind to *APOE* genotype, by a semiquantitative procedure as described previously [15]. Briefly, zero signifies that no blood vessels were stained; +, fewer than one-third of the blood vessels were immunostained; ++, between one- and two-thirds of the blood vessels were positively stained; +++, more than two-thirds of the blood vessels were stained. The χ^2 test with Yate's correction was used for statistical analyses and where the number of alleles was too small, Fisher's exact test was used.

RESULTS

The study group consisted of 57 patients, 38 women and 19 men, of mean age 71 years. In all of these cases Congo red staining revealed apple-green birefringent blood vessels when these were viewed under crossed polarizing filters, confirming the presence of vascular amyloid. As reported previously [15], the 37 patients in the haemorrhage group had an excess of *APOE* ε2 alleles (ε2 0.24, ε3 0.57 and ε4 0.19), while the patients with CAA without haemorrhage had an excess of the ε4 allele (ε2 0.08, ε3 0.58 and ε4 0.35).

Comparison of the number of cases with different proportions of vascular deposition of each Aβ species (dichotomizing between score 0/+ and ++/+++) demonstrated that CAA-related haemorrhage patients had more Aβ40 than Aβ42 blood vessel immunoreactivity in both the leptomeninges ($p < 0.001$) and cortex ($p < 0.001$, Table 1). There was a similar, although non-significant finding in the CAA patients without haemorrhage. In addition, more CAA-related haemorrhage patients had greater Aβ40-positive scores in the leptomeninges ($p < 0.001$) and in the cortex ($p = 0.027$) than the non-haemorrhage patients. More patients in the haemorrhage group also had the higher density Aβ42-immunoreactive scores in the leptomeninges and cortex than had the non-haemorrhage group, but the differences were not statistically significant (Table 1).

When analysed with respect to the patients' possession of *APOE* ε2 or *APOE* ε4 alleles, cortical vascular Aβ immunostaining revealed genotype differences. Although the absolute numbers of cases were small, ε2 carriers more frequently had greater than one-third of their cortical blood vessels staining for Aβ42 than had non-ε2 carriers (Table 2, $p = 0.022$). However, *APOE* ε4 carriers had no more Aβ40 or Aβ42 immunoreactive blood vessels in the leptomeninges and cortex than had non-ε4 carriers (Table 3).

DISCUSSION

Our previous study demonstrated that a greater proportion of leptomeningeal and cortical blood vessels are affected with amyloid angiopathy (using an antibody that recognizes all species of Aβ) [15] in patients with CAA-related haemorrhage than in patients with CAA but without haemorrhage. The present study shows that this is also the case for Aβ40 (with a similar but non-significant excess of Aβ42-positive blood vessels). Both Aβ species were present in a greater proportion of leptomeningeal than cortical blood vessels. However, primary subarachnoid haemorrhage is

Table 1. Cases of cerebral amyloid angiopathy with a high density of Aβ40- or Aβ42-immunoreactive blood vessels (score ++ or +++) in the leptomeninges or cortex. The cases have been subdivided according to the presence or absence of associated lobar haemorrhage.

	Haemorrhage ($n = 37$)		No haemorrhage ($n = 20$)		p
	n	%	*n*	%	
Leptomeningeal Aβ40*	36	97	11	55	<0.001
Leptomeningeal Aβ42	20	54	6	30	0.14
Cortical vascular Aβ40†	22	59	5	25	0.027
Cortical vascular Aβ42	8	22	1	5	0.14

p value column represents comparison between haemorrhage and non-haemorrhage groups.
*Comparison of the proportion of leptomeningeal blood vessels containing Aβ40 with the proportion containing Aβ42 in haemorrhage cases yielded $p < 0.001$ and in non-haemorrhage cases this was not significant, $p = 0.11$.
†Comparison of the proportion of cortical blood vessels containing Aβ40 with the proportion containing Aβ42 in haemorrhage cases yielded $p < 0.001$ and in non-haemorrhage cases did not reach significance, $p = 0.18$.

Table 2. Cases of cerebral amyloid angiopathy with a high density of Aβ40- or Aβ42-immunoreactive blood vessels (score ++ or +++) in the leptomeninges or cortex. The cases have been subdivided according to their apolipoprotein E ε2 status.

	ε2 carriers ($n = 18$)		Non-ε2 carriers ($n = 39$)		p
	n	%	*n*	%	
Leptomeningeal Aβ40	17	94	30	77	0.14
Leptomeningeal Aβ42	8	44	18	46	0.87
Cortical vascular Aβ40	9	50	18	46	0.99
Cortical vascular Aβ42	6	33	3	8	0.022

Table 3. Cases of cerebral amyloid angiopathy with a high density of Aβ40- or Aβ42-immunoreactive blood vessels (score ++ or +++) in the leptomeninges or cortex. The cases have been subdivided according to their apolipoprotein E ε4 status.

	ε4 carriers ($n = 22$)		Non-ε4 carriers ($n = 35$)		p
	n	%	*n*	%	
Leptomeningeal Aβ40	16	73	31	89	0.16
Leptomeningeal Aβ42	8	36	18	51	0.4
Cortical vascular Aβ40	7	32	20	57	0.11
Cortical vascular Aβ42	2	9	7	20	0.46

unusual in CAA [19]; most haemorrhages are intracerebral and are thought to arise from rupture of Aβ-laden cortical blood vessels [20]. Secondary subarachnoid haemorrhage is common because of the close proximity of these lobar haemorrhages to the subarachnoid space.

Previous studies of asymptomatic CAA patients have shown that the Aβ40 species is more abundant in the cerebral vasculature than the Aβ42 species, and in this study we confirm that this is particularly the case in CAA-related haemorrhage, both in leptomeningeal and cortical blood vessels. Alonzo and colleagues found in CAA patients without haemorrhage that Aβ42 was always present in a blood vessel which contained Aβ40 and *vice versa* [9]. However, in the current study some blood vessels could be clearly identified which contained Aβ40 but not Aβ42 in adjacent sections. The reason for these discrepant findings is not clear but may reflect different immunostaining methods or primary antibodies. This did not appear to be a specific feature of cases with haemorrhage.

Evidence has accumulated implicating the *APOE* ε4 allele in Aβ deposition in the cerebral vasculature [12,13]. Seeding of Aβ deposits at the capillary level has been shown to be initially by Aβ42 in patients with an APP693 mutation, which causes the Dutch familial form of CAA-related haemorrhage, HCHWA-D. Seeding by Aβ42 is also thought to be the initial event in sporadic CAA as well as in Alzheimer's disease [21]. The *APOE* ε4 allele has not been shown to be associated with this initial step in Alzheimer's disease but rather is thought to facilitate Aβ40 deposition on to Aβ42-seeded plaques [4]. In Alzheimer's disease, in which there is a very low ε2 frequency, it is unclear what promotes the initial seeding of Aβ42. The high *APOE* ε2 frequency in CAA-related haemorrhage and the fact that only 50% of patients with CAA-related haemorrhage have neuropathological evidence of Alzheimer's disease [22] suggest that there are differences in the mechanism of Aβ deposition in these two conditions.

Progression of CAA has been attributed to continued vascular deposition of Aβ40, the amount of which increases with *APOE* ε4 allele dose [9]. However, previous studies implicated the *APOE* ε2 allele rather than ε4 as a risk factor for haemorrhage in CAA [14,23], an effect possibly mediated by the promotion of CAA-associated vasculopathic complications, specifically fibrinoid necrosis [15] or vessel wall splitting [16]. The finding in the current study that proportionately more ε2 than non-ε2 carriers had large numbers of cortical blood vessels with deposits of Aβ42 suggests a further mechanism to explain the association of ε2 with CAA-related haemorrhage.

The results of this study, although based on a small number of patients, suggest that the *APOE* ε2 allele increases the frequency of cortical vascular deposition of Aβ42. This appears somewhat paradoxical in view of the protective effect of the ε2 allele in Alzheimer's disease. However, Weller and colleagues have suggested that Aβ generated in the cerebral cortex can drain via periarterial interstitial pathways, probably complexed with apoE, and may become deposited in blood vessel walls [24]. It could be argued that the apoE2 isoform more effectively clears Aβ from the cerebral cortex into periarterial interstitial fluid drainage pathways, where the Aβ is deposited in the cortical vasculature. A previous report of CAA-related haemorrhage with very few plaques in a patient with a presenilin-2 mutation and an *APOE* ε2/ε3 genotype is consistent with our suggestion that the ε2 allele directs more Aβ42 vascular deposition [25]. The number of blood vessels with Aβ42 may be at least as important as the total amyloid vascular load in increasing the risk of cerebral haemorrhage, possibly through the development of fibrinoid necrosis [15]. This would explain the observation that

patients possessing ε2 are more likely to have multiple CAA-related haemorrhages and have haemorrhages at a younger age than individuals without ε2 [14]. The role of *APOE* ε4 in CAA-related haemorrhage may be to promote the deposition of Aβ40 in blood vessels already seeded with Aβ42. So in ε4 carriers, although fewer cortical blood vessels contain Aβ42, the severity of CAA that results from the subsequent deposition of increasing amounts of Aβ40 in those blood vessels may eventually lead to their rupture and haemorrhage.

CONCLUSION

This study suggests a potential mechanism to explain how *APOE* ε2 may contribute to CAA-related haemorrhage by influencing the composition of cortical vascular Aβ. We have demonstrated an association between *APOE* ε2 and an increased number of Aβ42-immunoreactive cortical blood vessels, whereas previous work has shown that the ε4 allele leads to the progressive deposition of Aβ40 on seeded blood vessels [9]. The different influences of *APOE* ε2 and *APOE* ε4 along this pathogenic pathway suggest how individuals with the *APOE* ε2/ε4 genotype may be susceptible to CAA-related haemorrhage at a particularly young age.

REFERENCES

1. Selkoe DJ. *Nature 399* **(suppl.)**, A23–A31 (1999).
2. Roher AE, Lowenson JD, Clarke S *et al. J Biol Chem* **268**, 3072–3083 (1993).
3. Fukumoto H, Asami-Odaka A, Suzuki N *et al. Am J Pathol* **148**, 259–265 (1996).
4. Gearing M, Mori H and Mirra SS. *Ann Neurol* **39**, 395–399 (1996).
5. Iwatsubo T, Odaka A, Suzuki N *et al. Neuron* **13**, 45–53 (1994).
6. Roher AE, Lowenson JD, Clarke S *et al. Proc Natl Acad Sci USA* **90**, 10836–10840 (1993).
7. Mak K, Yang F, Vinters HV *et al. Brain Res* **667**, 138–142 (1994).
8. Savage MJ, Kawooya JK, Pinsker LR *et al. Amyloid Int J Exp Clin Invest* **2**, 234–240 (1995).
9. Alonzo NC, Hyman BT, Rebeck GW and Greenberg SM. *J Neuropathol Exp Neurol* **57**, 353–359 (1998).
10. Mann DM, Iwatsubo T, Ihara Y *et al. Am J Pathol* **148**, 1257–1266 (1996).
11. Natte R, Yamaguchi H, Maat-Schieman MLC *et al. Alzheimer's Reports* **2 (suppl 1)**, S22 (1999).
12. Schmechel DE, Saunders AM, Strittmatter WJ *et al. Proc Natl Acad Sci USA* **90**, 9649–9653 (1993).
13. Greenberg SM, Rebeck GW, Vonsattel JP *et al. Ann Neurol* **38**, 254–259 (1995).
14. Nicoll JA, Burnett C, Love S *et al. Ann Neurol* **41**, 716–721 (1997).
15. McCarron MO, Nicoll JAR, Stewart J *et al. J Neuropathol Exp Neurol* **58**, 711–718 (1999).
16. Greenberg SM, Vonsattel JG, Segal AZ *et al. Neurology* **50**, 961–965 (1998).
17. Tekirian TL, Cole GM, Russell MJ *et al. Neurobiol Aging* **17**, 249–257 (1996).
18. Francis RJ. Amyloid. In: Bancroft JD, Stevens A, eds. *Theory and Practice of Histological Techniques*, 3rd edn. Edinburgh: Churchill Livingstone, 1990: 155–175.
19. Ohshima T, Endo T, Nukui H *et al. Stroke* **21**, 480–483 (1990).
20. Yamada M, Itoh Y, Otomo E *et al. J Neurol Neurosurg Psychiatry* **56**, 543–547 (1993).
21. Jarrett JT, Berger EP and Lansbury PT, Jr. *Biochemistry* **32**, 4693–4697 (1993).
22. Vinters HV. *Stroke* **18**, 311–324 (1987).
23. Greenberg SM, Briggs ME, Hyman BT *et al. Stroke* **27**, 1333–1337 (1996).
24. Weller RO, Massey A, Newman TA *et al. Am J Pathol* **153**, 725–733 (1998).
25. Nochlin D, Bird TD, Nemens EJ *et al. Ann Neurol* **43**, 131–135 (1998).