Immunohistochemical Characterization of Cerebrovascular Amyloid in 46 Autopsied Cases Using Antibodies to $\beta$ Protein and Cystatin C

Keiko Maruyama, MD, Shu-ichi Ikeda, MD, Tokuhiro Ishihara, MD, David Allsop, PhD, and Nobuo Yanagisawa, MD

Using immunohistochemical staining methods with antibodies to amyloid $\beta$ protein and human cystatin C, we examined cerebrovascular amyloid protein in the brains from 46 cases with cerebral amyloid angiopathy (seven with Alzheimer's disease, one with Down's syndrome, 18 with intracranial hemorrhage, 10 with cerebral infarction, and 10 elderly patients without any neurologic disorder). All cerebrovascular amyloid deposits in these 46 cases were consistently immunoreactive to anti-$\beta$ protein antibody. However, in nine cases some vascular walls with strong $\beta$ protein immunoreactivity also reacted less intensely with the anti-cystatin C antiserum. Of these nine cases, seven showed relatively heavy cerebrovascular amyloid deposition, and all seven had suffered a fatal subcortical hemorrhage presumably caused by cerebral amyloid angiopathy. Previous limited studies have suggested that the amyloid protein seen in elderly individuals with cerebral amyloid angiopathy is composed of $\beta$ protein. However, subcortical hemorrhage rarely occurs in such individuals. Our study shows that aged patients with different brain disorders commonly suffer from $\beta$ protein-type cerebral amyloid angiopathy, and we also suggest that the severity of $\beta$ protein-type cerebrovascular amyloid deposition is a fundamental factor in cerebral amyloid angiopathy-induced brain hemorrhage in the elderly. The nature of the cystatin C-immunoreactive substance in some of these vascular lesions is uncertain, but it might conceivably play an additional important role in the pathogenesis of brain hemorrhage in these cases. (Stroke 1990;21:397-403)

In cerebral amyloid angiopathy (CAA), amyloid is deposited in the walls of leptomeningeal and cortical blood vessels, mainly those of the cerebrum. CAA is seen in persons with various conditions including Alzheimer's disease and Icelandic-type (HCHWA-I) or Dutch-type (HCHWA-D) hereditary cerebral hemorrhage with amyloidosis and in nondemented elderly individuals. The incidence of CAA increases with age, and this disorder is also recognized as a cause of spontaneous cerebral hemorrhage in normotensive elderly persons. However, the pathogenesis of CAA has not been established, and the relation between CAA and cerebral hemorrhage is still incompletely understood.

Recent advances in the study of central nervous system amyloids have revealed the biochemical and immunohistochemical features of three different amyloid fibril proteins: $\beta$ protein, cystatin C ($\gamma$ trace), and protease-resistant prion protein (PrP 27–30 or PrP). CAA in the brains of patients with Alzheimer's disease or Down's syndrome consists of $\beta$ protein; this same protein has been identified in patients with HCHWA-D in patients with sporadic cerebral amyloid angiopathy with no significant number of neurofibrillary tangles or senile plaques (SCAA), and in asymptomatic elderly individuals. Amyloid protein isolated from the brains of patients with HCHWA-I is a variant of cystatin C. Amyloid plaques or CAA in the transmissible spongiform encephalopathies (Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, and kuru in humans or scrapie in animals) react with
antibodies to PrP. We characterized immunohistochemically the cerebrovascular amyloid deposits in the brains from 46 cases with different disorders using antibodies to β protein and human cystatin C, with special attention to the clinical manifestation of cerebral hemorrhage.

Subjects and Methods

Two to four blocks of neocortical brain tissue were obtained from 46 autopsied cases (Table 1), seven with Alzheimer's disease, one with adult Down's syndrome, 18 with intracranial hemorrhage (six with subcortical hemorrhage, six with putaminal and/or thalamic hemorrhage, four with subarachnoid hemorrhage caused by trauma or ruptured aneurysm, one with cerebellar hemorrhage, and one with pontine hemorrhage), 10 with cerebral infarction, and 10 elderly patients without any evidence of neurologic disorders. The age of these patients ranged from 37 to 94 (average 75) years.

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and with alkaline Congo red. Cerebrovascular amyloid deposits were identified under a polarizing light microscope by their characteristic congophilia with apple-green birefringence. These sections were examined immunohistochemically using the streptavidin-biotin horseradish peroxidase method. The primary antibodies used in this study were a monoclonal antibody (4D12/2/6) raised against a synthetic peptide (AL1) consisting of residues 8–17 of amyloid β protein and an affinity-purified rabbit antiserum to the amino-terminal octapeptide of human cystatin C (AG8206). The tissue sections were immunostained with either 1:1,000–1:2,000-diluted 4D12/2/6 ascites fluid or with 1:1,000-diluted anti-cystatin C antiserum using the method described previously. The specificity of the immunostaining was confirmed by blocking the 1:1,000-diluted anti-β protein antibody with 1 mM synthetic peptide AL1 antigen or by preincubation of the cystatin C antiserum (1 ml diluted 1:1,000) with 25 µg human plasma cystatin C (Protogen AG, Weidenmattweg, Switzerland). The immunoreactive lesions in the vascular walls in each case were semiquantitatively divided into four grades of severity under low-power magnification: −, not observed; +, a few vessels involved, but seen only after extensive search; ++, more vessels involved, but some optic fields free of lesions; and ++++, many vessels involved.

Results

The results of Congo red and immunohistochemical staining are summarized in Table 1. Congo red staining revealed a variable degree of cerebrovascular amyloid deposits in both leptomeningeal and cortical blood vessels in all cases. Severe amyloid deposition within the vascular walls was seen in three cases with Alzheimer's disease (including one with subcortical hemorrhage) and in two cases with spontaneous subcortical hemorrhage. In these five cases some vessels with severe amyloid infiltration showed thickened vascular walls (mainly in the media) or a "double-barreled" formation (Figure 1). Congophilic angiopathy was in general less prominent in the cases with subarachnoid hemorrhage or cerebral infarction and in the aged individuals without neurologic com-

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Age (yr)</th>
<th>No.</th>
<th>Congo red</th>
<th>β protein</th>
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<tbody>
<tr>
<td>Alzheimer's disease</td>
<td>7</td>
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<td></td>
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<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Adult Down's syndrome</td>
<td>1</td>
<td>37</td>
<td>1</td>
<td>++</td>
<td>++</td>
</tr>
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<td>Intracranial hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated subcortical hemorrhage</td>
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<td>72–87</td>
<td>2</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>2</td>
<td>+++</td>
<td>+++</td>
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<td>6</td>
<td>60–86</td>
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<td>+</td>
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<td></td>
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<td>4</td>
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<td>4</td>
<td>68–93</td>
<td>3</td>
<td>+</td>
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<td></td>
<td>1</td>
<td>++</td>
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<tr>
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<td>54–74</td>
<td>1</td>
<td>+</td>
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<td></td>
<td>1</td>
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<tr>
<td>Cerebral infarction</td>
<td>10</td>
<td>65–85</td>
<td>5</td>
<td>+</td>
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<td></td>
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<td></td>
<td>5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Elderly individuals without central nervous system involvement</td>
<td>10</td>
<td>70–94</td>
<td>6</td>
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<td></td>
<td></td>
<td>4</td>
<td>+++</td>
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+, Few vessels involved; ++, more vessels involved, but some optic fields free of lesions; ++++, many vessels involved.

TABLE 1. Semiquantitative Estimation of Severity of Cerebrovascular Amyloid Deposits Using Congo Red Staining and Immunohistochemical Reaction to β Protein
FIGURE 1. Photomicrographs of typical vascular changes seen in heavily amyloid-laden leptomeningeal vessels. A and B are closely adjacent sections obtained from the same block from case 2 in Table 2, and an arrowhead indicates an identical vessel. A: Congo red-stained section viewed under polarized light. B: Immunostaining with anti-β protein antibody. All vessels in subarachnoid space are severely involved with β protein-type amyloid deposition, and some show degeneration of media with configuration of concentric vessels within a vessel ("double barreling"). Bars, 100 μm.

Implications; in these cases patchy deposits of amyloid were commonly seen in the outer layer of the media and adventitia, but there were no significant vascular changes including aneurysmal formation, obliteration, or double barreling.

The cerebrovascular amyloid deposits in all cases examined were invariably immunoreactive to anti-β protein antibody. The severity, distribution, and morphologic appearance of these β protein–immunoreactive vascular lesions closely resembled that observed with Congo red. However, immunostaining with the anti-β protein antibody proved to be a significantly more sensitive technique, especially in demonstrating microvascular involvement (Table 1 and Figure 2, A).

In nine cases without any family history of stroke, some vessels with β protein immunoreactivity were also stained with anti-cystatin C antiserum (Table 2). Seven of these cases with dual immunoreactivity revealed recurrent serious subcortical hemorrhages probably caused by CAA, and among the remaining two cases, one suffered cerebral infarction but the other lacked any central nervous system symptoms. In the nine cases with dual immunoreactivity, all amyloid-laden vascular walls demonstrated by Congo red reacted intensely with anti-β protein antibody, frequently showing involvement of the entire vascular walls (Figure 2, A), while anti-cystatin C–immunoreactive lesions were restricted to only some of these vessels (Figure 2, B); the cystatin C–immunoreactive areas were observed mainly in the outer media to the adventitia of the larger cortical and leptomeningeal vessels, and the immunoreaction with anti-cystatin C antiserum was weaker than that with anti-β protein antibody (Figure 2 and Figure 3, A and B).

The vast majority of the remaining cases without subcortical hemorrhage showed mild or moderate anti-β protein–immunoreactive cerebrovascular lesions (Table 1). In addition to the cases with Alzheimer's disease, nondemented cases also had a variable number of senile plaques with a few congophilic neurofibrillary tangles in the cerebral cortex, and these senile plaque

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Severity of staining</th>
<th>β protein</th>
<th>Cystatin C</th>
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<tr>
<td>1</td>
<td>72</td>
<td>F</td>
<td>Subcortical hemorrhage</td>
<td>++ + + +</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>M</td>
<td>Subcortical hemorrhage</td>
<td>++ + + +</td>
<td>++</td>
<td>+</td>
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<tr>
<td>3</td>
<td>74</td>
<td>F</td>
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<td>4</td>
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<td>77</td>
<td>F</td>
<td>Subcortical hemorrhage</td>
<td>+ + +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>87</td>
<td>F</td>
<td>Subcortical hemorrhage</td>
<td>+ + + +</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>F</td>
<td>Alzheimer's disease with</td>
<td>++ + + + +</td>
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<td>+</td>
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<td>8</td>
<td>83</td>
<td>M</td>
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<td>++</td>
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</tr>
<tr>
<td>9</td>
<td>72</td>
<td>F</td>
<td>Bronchopneumonia</td>
<td>++</td>
<td>+</td>
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</tr>
</tbody>
</table>

No case with subcortical hemorrhage had hemorrhage in basal ganglia. F, female; M, male; +, few vessels involved; ++, more vessels involved, but some optic fields free of lesions; ++++, many vessels involved.
amyloid deposits were immunoreactive only to anti-β protein antibody (data not shown). No immunostaining was observed using either anti-β protein antibody blocked by preincubation with synthetic AL1 peptide or anti-cystatin C antiserum blocked by human plasma cystatin C (Figure 3, B and C).

Discussion

CAA has been noted as an important cause of cerebral hemorrhage in normotensive aged persons1,2,5; this type of cerebral hemorrhage usually occurs in the cerebral hemispheres as a large subcortical hematoma, frequently resulting in death.3,4 At present, three different types of cerebrovascular amyloid protein are identified3,26; β protein, cystatin C, and PrP. PrP-immunoreactive amyloid protein has been found only in the transmissible spongiform encephalopathies,13 and persons with these disorders rarely show CAA.21 Several biochemical10,14,27 and immunohistochemical15,28,29 studies have already established that β protein is the main component of the amyloid fibrils of both senile plaques and cerebrovascular amyloidosis seen in persons with Alzheimer's disease and adults with Down's syndrome. Moreover, it has been shown that the vascular amyloid protein in patients with SCAA and in asymptomatic elderly persons is also composed of β protein.16–20 Therefore, it has been suggested that the appearance of β protein-type CAA is closely related to the aging process. However, there has been no extensive study investigating cerebrovascular amyloid protein in a large number of CAA-affected patients with diverse cerebral pathologic conditions. In our study of 46 elderly cases with different cerebral disorders and CAA, cerebrovascular amyloid protein in all cases reacted with the monoclonal antibody to β protein. This observation is consistent with the previous limited studies mentioned above and provides strong evidence that β protein-type CAA is the common form seen in aged persons.

In nine of these 24 cases, cystatin C-immunoreactive lesions were also observed in some portion of the
cerebral vessels that showed an intense immunoreactivity to β protein. Cystatin C inhibits lysosomal cysteine proteinases,30 and a variant of cystatin C12 with a single substitution of glutamine for leucine at position 68 was originally reported to be the main component of amyloid in HCHWA-I. This disorder is transmitted as an autosomal dominant trait, and almost all patients affected by cystatin C CAA develop a massive brain hemorrhage and die before the age of 50 years.31-32 The location of the cerebral hemorrhages in patients with HCHWA-I32 is closely similar to that seen in aged patients with nonfamilial CAA. However, the frequency of cerebral hemorrhages in the latter patients is reported to range from 2% to 10%,8,9,33 and the reason for this low incidence has not been explained. Interestingly, in our series only the patients showing coexistence of β protein and cystatin C immunoreactivity in their diseased cerebral vessels suffered fatal subcortical hemorrhages, possibly attributable to CAA. The degree of cerebrovascular amyloid deposition in these patients seemed to be relatively greater than that in cases without cerebral hemorrhages.

The pathologic mechanism of cerebral bleeding caused by CAA has been claimed to be as follows:4,34 amyloid deposition weakens the vascular walls by destroying their normal structure, and when these structures are severely involved with heavy amyloid deposition, miliary aneurysmal formation or double barreling occurs, sometimes associated with bleeding. On the basis of our pathologic and immunohistochemical findings, we consider that the severity of (possibly β protein-type) amyloid deposition in the cerebrovascular walls might be a principal cause of subcortical cerebral hemorrhage seen in the elderly.

It is uncertain whether the cystatin C-immunoreactive lesions contain genuine cystatin C amyloid fibrils or whether they contain unpolymerized cystatin C (or cross-reactive substances) adsorbed onto or trapped within the bundles of β protein amyloid fibrils. Similar immunohistochemical findings were reported in two cases with HCHWA-D35 and in seven Japanese cases, including two probable familial ones.36 Cysteine proteinases are usually localized to lysosomes but are also secreted into the extracellular space under certain conditions,37 and it has been suggested that proteolytic enzymes may have a potential pathologic role in cerebral hemorrhage,38,39 possibly by decreasing the resistance of the vascular walls. It is possible that the accumulation of β protein amyloid fibrils alters the homeostasis of proteinases and their inhibitors, increasing the accumulation of these proteins in the cerebrovascular walls, and this
process might contribute to the development of brain hemorrhage. Further studies (including chemical analysis of the vascular amyloid proteins in cases with dual immunoreactivity) are necessary to determine the exact chemical nature of the immunoreactive substances.

With regard to the specificity of our immunostaining, the monoclonal antibody to β protein (4D12/2/6) does not react with the cerebrovascular amyloid deposits in patients with HCHWA-I, 20 and in our study the anti-cystatin C antiserum did not react with senile plaque amyloid seen in the cases with Alzheimer's disease. Accordingly, the 4D12/2/6 and AG8206 antibodies show no immunohistochemical cross-reactivity to cystatin C and β protein amyloid, respectively. Furthermore, all immunohistochemical reactions in this study were completely blocked by inhibited controls, involving preincubation of antibodies with their cognate peptides.

Acknowledgments

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KEY WORDS • amyloid • cerebral hemorrhage
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