Diagnosis of Cerebral Amyloid Angiopathy by Enzyme-Linked Immunosorbent Assay of Cystatin C in Cerebrospinal Fluid

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An abnormally low level of cystatin C in the cerebrospinal fluid is a diagnostic marker for the hereditary form of brain hemorrhage associated with amyloidosis that was first identified in Iceland. We developed an assay for cystatin C to use in the diagnosis of patients with cerebral amyloid angiopathy and brain hemorrhage. This test consists of a sandwich enzyme-linked immunosorbent assay using monoclonal mouse anticystatin C and polyclonal rabbit anticystatin C antibodies. The cystatin C level was assayed in cerebrospinal fluid samples from 29 patients with brain hemorrhage and 45 control patients with other neurological diseases. Fifteen patients with brain hemorrhage showed low cystatin C levels (<70 ng/ml) in a clinical setting in which the positive and negative findings were compatible with a diagnosis of cerebral amyloid angiopathy. Immunohistological examination of brain tissue obtained by biopsy from two of the 15 patients confirmed the diagnosis of cerebral amyloid angiopathy and identified the deposition of cystatin C and β-protein. This enzyme-linked immunosorbent assay is simple to perform and may be useful for investigating patients suspected of having cerebral amyloid angiopathy with brain hemorrhage and the deposition of cystatin C. (Stroke 1991;22:860–866)

In 1972, Gudmundsson et al.1 first described families with hereditary cerebral hemorrhage with amyloidosis in Iceland (HCHWA-I). The affected individuals frequently developed brain hemorrhage while young and showed marked deposition of amyloid in the cerebral vessels. Ghiso et al.2 clearly showed that the amyloid protein is a variant of cystatin C (γ-trace), which is a cysteine proteinase inhibitor. In 1984, Grubb and coworkers.3,4 reported that the cystatin C concentration in the cerebrospinal fluid (CSF) of patients with HCHWA-I was abnormally low, and in 1988 we.5 reported the first case of HCHWA-I in Japan associated with deposition of cystatin C. In addition, we have described a similar nonfamilial disorder associated with the deposition of cystatin C and β-protein in the cerebral microvasculature.6 Maruyama et al.7 reported that β-protein and cystatin C immunoreactive substances are important in the pathogenesis of cerebral amyloid angiopathy (CAA) with brain hemorrhage. However, the diagnosis of CAA is difficult since histopathologic examination of a brain biopsy specimen or autopsy tissue is the only available method. We measured the cystatin C concentrations in the CSF of patients with brain hemorrhage by a new enzyme-linked immunosorbent assay (ELISA). A control study was performed in patients with various other neurological disorders. This simple and convenient ELISA for detecting cystatin C may be useful for the diagnosis of CAA associated with brain hemorrhage and low cystatin C levels.

Subjects and Methods

We studied 29 patients with brain hemorrhage (16 men and 13 women, ranging in age from 46 to 90 years) and 45 control patients with various other neurological diseases (29 men and 16 women ranging in age from 35 to 85 years). Brain hemorrhage was classified according to the location of the lesions shown by computed tomography. The 29 patients with brain hemorrhage had the following diagnoses: multiple brain hemmorhages in one; subcortical hemorrhage in 15, including two with accompanying thalamic hemorrhage; thalamic hemorrhage in four; putaminal hemorrhage in eight; and corona radiata hemorrhage in one. Most of these patients were
admitted to the Shimane Medical University Hospital, while some were seen at hospitals associated with Dokkyo Medical University, Jichi Medical University, Tottori University, and Tokyo Medical University. The CSF sample was obtained by conventional lumbar puncture during the chronic stage over the month following brain hemorrhage. A bloody or xanthochromic CSF specimen was rejected. We added benzamidion solution to the specimens as a preservative and stored them in a deep freezer at −70°C until use. The control patients had the following diagnoses: brain infarction in 27, Alzheimer’s disease in six, Parkinson’s disease in four, amyotrophic lateral sclerosis in five, and epilepsy in three.

Cystatin C was prepared from the urine of patients with chronic renal failure and purified at the New Drug Research Laboratory of Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan. Reverse-phase high-pressure liquid chromatography (ODS-120T column, Tosoh Corp., Tokyo, Japan) of this purified cystatin C showed a single sharp peak. Cystatin C was diluted serially with 0.5% bovine serum albumin before use, and concentrations ranging from 0 to 1,000 ng/ml were used as the standard antigen.

The monoclonal mouse anticystatin C antibody and the polyclonal rabbit anticystatin C antibody were kindly provided by Dr. Grubb, University of Lund, Malmö, Sweden. Peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) antibody was purchased from Cappelle Co., Ltd.

We employed the two-site sandwich method reported previously. In brief, monoclonal mouse anticystatin C antibody was diluted with 15 mM bicarbonate buffer (pH 9.6) to 1 μg/ml. A total of 50 μl of the diluted antibody was placed in each well of a microplate (Immunoplate II 96-F, NUNC, Denmark) and allowed to stand at room temperature for 2 hours to permit coating. After discarding the antibody, the wells were washed once with 0.5% bovine serum albumin and then washed three times with 0.05% Tween 20 PBS. Various concentrations of the cystatin C standard (0, 10, 50, 80, 100, 250, 500, or 1,000 ng/ml) or the CSF specimens to be tested were then added at a volume of 50 μl. The wells were subsequently left for 2 hours at room temperature and then washed four times with 0.05% Tween 20 PBS. A total of 50 μl of polyclonal rabbit anticystatin C antibody diluted with 0.05% Tween 20 PBS (pH 7.9) was then added to each well, and incubation was performed at room temperature for 2 hours. The residual antibody was then discarded, and the wells were washed five times with 0.05% Tween 20 PBS. Next, 50 μl of peroxidase-labeled goat anti-rabbit IgG antibody was added and the wells were allowed to stand at room temperature for 2 hours. After the antibody was discarded, the wells were washed six times with 0.05% Tween 20 PBS. The coloring solution used was 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Nakarai, Kyoto, Japan) as a 15 mg/ml solution with 20 ml of 0.1 M citrate buffer (pH 4.0) and 10 μl of 30% H₂O₂. A volume of 200 μl of each was added to each well for color development. After 10 minutes the reaction was stopped by adding 50 μl of 0.5 M NaF. After a further 10 minutes the absorbance at 417 nm was read using a microplate photometer (MTP-12, Corona Electric, Ibaragi, Japan). A standard curve was devised showing the relation between known antigen concentrations and absorbance, and we assayed the CSF cystatin C levels on the basis of this curve.

To evaluate the specificity of this system, we examined the cross-reactivity of the anticystatin C antibodies with amyloid light chain, amyloid A, or another proteinase inhibitor (trypsin inhibitor). To rule out interference with this assay system by CSF components, we tested cystatin C recovery by adding 50 μl of each concentration of cystatin C (0-1,000 ng/ml) to 50 μl of control CSF.

During the acute stage following brain hemorrhage, we obtained brain biopsy specimens from four patients, two with subcortical hemorrhage and two with putaminal hemorrhage. The CSF sample was obtained during the chronic stage. In one patient with putaminal hemorrhage, we obtained the CSF sample shortly before a second brain specimen was obtained at autopsy.

We evaluated these tissue specimens immunohistochemically to detect the deposition of cystatin C and β-protein in the cerebral vessels. The specimens were fixed with formalin, embedded in paraffin, and stained with hematoxylin and alkaline Congo red. Cerebrovascular amyloid deposits were identified under a polarizing light microscope by apple-green birefringence in the involved vessels. Immunohistochemical study of the brain sections was performed using the avidin/biotin

![Graph](http://stroke.ahajournals.org/)

**Figure 1.** Linear relation between absorbance and concentration from 10 to 1,000 ng/ml was demonstrated in standard curve of cystatin C enzyme-linked immunosorbent assay. Quantitative determination of cystatin C level was precise within this range.
complex method previously reported. The primary antibodies used in this study were a polyclonal anti-cystatin C antibody provided by Dr. Grubb and a monoclonal antibody raised against synthetic β-protein provided by Dr. Allsop through Dr. Glenner. As the positive control for cystatin C, we used tissues from HCHWA-I patients with CAA. For negative controls, we used normal brain tissue and amyloid tissues from patients with various kinds of systemic amyloidosis. In the adsorption study, anticystatin C antibody was first adsorbed with the purified antigen (1:1 wt:wt cystatin C:IgG) and then used as the primary antibody. The specificity of the anti-β-protein antibody had been thoroughly examined in a previous study.

Results

To evaluate the specificity and accuracy of our new ELISA method, we conducted the following preliminary tests. First, the relation between absorbance and cystatin C concentration was obtained for the standard antigen solutions. A linear relation was found from 10 to 1,000 ng/ml (Figure 1). In this assay system, the anticystatin C antibody reacted specifically with cystatin C and did not cross-react with amyloid light chains, amyloid A, or trypsin inhibitor. In addition, the recovery test produced a rate of nearly 100%. Thus, it was confirmed that no component in the CSF other than cystatin C influenced the assay results.

Figure 2 shows the CSF cystatin C levels in the patients and controls. Levels in the controls ranged from 100 to 600 ng/ml. The mean ± standard deviation (SD) was 231 ± 80.5 ng/ml. Two SD below the mean (231−161=70 ng/ml) was taken as the cutoff level for CSF cystatin C. Among the patients with brain hemorrhage, 15 had cystatin C levels below 70 ng/ml while 14 had higher levels. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage. The latter group comprised 19 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with subcortical hemorrhage and thalamic hemorrhage.
with thalamic hemorrhage who were virtually non-motensive (Table 1). On the basis of their clinical features, we suspected that these 15 patients had CAA (Table 1). They had no risk factors for brain hemorrhage, such as hypertension, leukemia, or thrombocytopenia, except for case 8. The diagnosis of CAA was confirmed in two of these patients by the histological evaluation of brain biopsy specimens. One of the two patients had a right temporal hemorrhage (case 1), and the other had a right frontal hemorrhage (case 2).

Immunohistochemical study of brain tissue showed that CAA was accompanied by the deposition of cystatin C (Figures 3A and 4A) and β-protein (Figures 3B and 4B) in these two patients. In the adsorption test, positive immunoreactivity for amyloid in the microvessels was almost completely abolished by the adsorption of antibodies with their corresponding purified antigens. Both patients had a low CSF cystatin C level (60 and 56 ng/ml).

Table 2 summarizes the information on the 14 patients with brain hemorrhage and a normal cystatin C level in the CSF (Table 1). They had no risk factors for brain hemorrhage, such as hypertension, leukemia, or thrombocytopenia, except for case 8. The diagnosis of CAA was confirmed in two of these patients by the histological evaluation of brain biopsy specimens. One of the two patients had a right temporal hemorrhage (case 1), and the other had a right frontal hemorrhage (case 2).

**Discussion**

Recently CAA has attracted attention as a possible cause of brain hemorrhage. Patients with CAA show amyloid deposition that is limited to the small cerebral vessels and frequently suffer multiple relapsing brain hemorrhages, especially subcortical hemorrhages.14,15 The incidence of CAA increases with age. Brain surgery is contraindicated in patients with brain hemorrhage associated with CAA, making early diagnosis highly desirable. At present, however, examination of pathology specimens is the only definitive method of diagnosis. Members of an Icelandic family showed multiple brain hemorrhages at an early age, and later autopsy studies revealed remarkable deposition of amyloid in the cerebral vessels (HCHWA-I). Cohen et al16 reported that the amyloid found in HCHWA-I was a protein related to γ-trace, a gastropancreatic neuroendocrine protein. Löfberg et al17 formulated a hypothesis for the mechanism of low CSF levels of cystatin C in HCHWA-I patients: cysteine proteinases are released from the walls of the small cerebral vessels and consequently cystatin C (a cysteine proteinase inhibitor) is consumed, leading to a decline in its level in the CSF. The inhibitor is then deposited in the form of amyloid with the structure of a cystatin C variant.

The hypothesis of Löfberg et al17 is partially supported by a report by Davis et al18 of a patient who accidentally received an intraspinal injection of chymopapain, a cysteine proteinase, and developed multiple brain hemorrhages. The theory is also supported by the work of Garvin et al,19 who reported that the infusion of cysteine proteinases into the CSF led to brain hemorrhage in an animal model.

We describe an ELISA procedure for cystatin C in the CSF. We confirmed the specificity for cystatin C of the antibodies used and the lack of interference by other substances in the CSF.20 This ELISA is simpler and easier to perform than the radioimmunoassay reported by Grubb et al1 and Löfberg et al.17 Fifteen of our 29 patients with brain hemorrhage showed low CSF cystatin C levels. Based on analysis of all aspects

**Table 2. Characteristics of 14 Patients With Hypertensive Brain Hemorrhage and Normal Cystatin C Levels in Cerebrospinal Fluid**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Site of hemorrhage</th>
<th>Hypertension</th>
<th>Cystatin C (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>75</td>
<td>M</td>
<td>Putamen</td>
<td>+</td>
<td>250</td>
</tr>
<tr>
<td>17</td>
<td>56</td>
<td>F</td>
<td>Putamen</td>
<td>+</td>
<td>105</td>
</tr>
<tr>
<td>18</td>
<td>73</td>
<td>F</td>
<td>Putamen</td>
<td>+</td>
<td>230</td>
</tr>
<tr>
<td>19</td>
<td>53</td>
<td>M</td>
<td>Putamen</td>
<td>+</td>
<td>600</td>
</tr>
<tr>
<td>20</td>
<td>88</td>
<td>M</td>
<td>Putamen</td>
<td>+</td>
<td>470</td>
</tr>
<tr>
<td>21</td>
<td>53</td>
<td>M</td>
<td>Putamen</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>22</td>
<td>74</td>
<td>F</td>
<td>Putamen</td>
<td>+</td>
<td>180</td>
</tr>
<tr>
<td>23</td>
<td>70</td>
<td>F</td>
<td>Putamen</td>
<td>+</td>
<td>150</td>
</tr>
<tr>
<td>24</td>
<td>49</td>
<td>M</td>
<td>Thalamus</td>
<td>+</td>
<td>225</td>
</tr>
<tr>
<td>25</td>
<td>72</td>
<td>M</td>
<td>Thalamus</td>
<td>+</td>
<td>470</td>
</tr>
<tr>
<td>26</td>
<td>68</td>
<td>F</td>
<td>Corona radiata</td>
<td>+</td>
<td>280</td>
</tr>
<tr>
<td>27</td>
<td>72</td>
<td>M</td>
<td>Subcortical (parietal)</td>
<td>+</td>
<td>145</td>
</tr>
<tr>
<td>28</td>
<td>76</td>
<td>F</td>
<td>Subcortical (frontal)</td>
<td>+</td>
<td>175</td>
</tr>
<tr>
<td>29</td>
<td>52</td>
<td>M</td>
<td>Subcortical (frontal)</td>
<td>+</td>
<td>290</td>
</tr>
</tbody>
</table>

Normal cystatin C level defined as >70 ng/ml. M, male; F, female. Brain tissues of cases 16 and 21 were negative for cerebral amyloid angiopathy by both Congo red staining and immunohistochemical examination.

**Figure 4.** Photomicrographs of case 2 (Table 1) showing both cystatin C (A) and β-protein (B) immunoreactivity in cerebral vessels. A and B are closely adjacent sections from same block. Amyloid deposition associated with cystatin C and β-protein deposits is seen in small cerebral vessels. Bars=50 μm.
of the clinical history and laboratory evaluations, we suspected these patients of having CAA associated with brain hemorrhage. In two of these 15 patients, immunohistopathologic evaluation of brain tissue specimens confirmed the diagnosis of CAA by demonstrating immunoreactive cystatin C and β-protein in the amyloid deposits. We have previously reported that age-related β-protein deposition may precede the deposition of cystatin C in patients having CAA associated with brain hemorrhage. Recently, Vinters et al reported that A4 (β-) peptide may colocalize with γ-trace (cystatin C) in the cerebral microvessels. In the two patients for whom we had brain tissue specimens, the deposition of cystatin C and β-protein in the small vessels appears to have been mediated by the same mechanism. The new ELISA method described here is simple to perform and provides a convenient tool for assaying cystatin C levels in the CSF. Such data might aid in the laboratory diagnosis of CAA, although further studies are necessary to confirm this.

Acknowledgments

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Key Words • amyloid • cerebral hemorrhage • cerebrospinal fluid • cystatins
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