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Short title: Diagnosis of cerebral amyloid angiopathy

Key Words: Cerebral amyloid angiopathy, Cystatin C, Enzyme-linked immunosorbent assay (ELISA), Cerebrospinal fluid, brain hemorrhage.

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Abstract

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 consisted of a sandwich enzyme immunosorbent assay using monoclonal mouse anti-cystatin C and polyclonal rabbit anti-cystatin C antibodies. The cystatin C level was assayed in cerebrospinal fluid samples from 29 patients with brain hemorrhage and in 45 control patients with other neurological diseases. Fifteen patients with brain hemorrhage showed low cystatin C levels (≦70 ng/ml), together 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 them confirmed the diagnosis of cerebral amyloid angiopathy and also identified the deposition of cystatin C and beta-protein. This enzyme-linked immunosorbent assay is simple to perform and may be potentially useful for investigating patients with suspected cerebral amyloid angiopathy with brain hemorrhage and the deposition of cystatin C.

Introduction

In 1972, Gudmundsson[1] first described families with hereditary cerebral hemorrhage with amyloidosis in Iceland (HCHWA-I). The affected individuals frequently developed brain hemorrhage while still young and showed marked deposition of amyloid in the cerebral vessels. Ghiso[2] has clearly shown that the amyloid protein is a variant of cystatin C (gamma-trace), which is a cysteine proteinase inhibitor. In 1984, Grubb[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 reported[5] the first case of HCHWA-I in Japan associated with deposition of cystatin C. In addition, we have described a similar non-familial disorder associated with the deposition of cystatin C and beta-protein in the cerebral microvasculature.[6] Maruyama[7] has reported that beta-protein and cystatin C immunoreactive substances were important in the pathogenesis of cerebral amyloid angiopathy (CAA) with brain hemorrhage. However, the diagnosis of CAA is difficult, since histopathological examination of a brain biopsy or autopsy tissue is the only available method. In this study, we measured the CSF cystatin C concentrations in 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 potentially useful for the diagnosis of CAA associated with brain

hemorrhage and cystatin C levels.

Materials and Methods

We studied 29 patients with brain hemorrhage (16 males and 13 females, ranging in age from 46 to 90 years) and 45 patients with various other neurological diseases (29 males and 16 females ranging in age from 35 to 85 years). Brain hemorrhage was classified according to the location of the lesions shown by computed tomography. These 29 patients with brain hemorrhage had the following diagnoses: multiple brain hemorrhage in 1 patient; subcortical hemorrhage in 15, including two with accompanying thalamic hemorrhage; thalamic hemorrhage in 4; putaminal hemorrhage in 8; and corona radiata hemorrhage in 1. Most of the patients were admitted to the Shimane Medical University Hospital, while some patients were seen at hospitals associated with Dokkyo Medical University, Jichi Medical University, Tottori University, and Tokyo Medical University. The control subjects had the following diagnoses: brain infarction (27), Alzheimer's disease (6), Parkinson's disease (4), amyotrophic lateral sclerosis (5), and epilepsy (3). Cerebrospinal fluid was obtained by conventional lumbar puncture during the chronic stage over a month period following brain hemorrhage. A bloody or xanthochromic CSF specimen was rejected We added benzamidium solution as a preservative to the specimens and stored them in a deep freezer (-70 ˚C) until use.

Quantitative determination of CSF cystatin C levels:

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 (Tosoh Corporation, Tokyo, Japan; column: ODS-120T) of this purified cystatin C showed a single sharp peak[8]. Cystatin C was diluted serially with 0.5% bovine serum albumin before use, and concentrations of this substance ranging from 0 to 1000 ng/ml were used as the standard antigen.

The mouse anti-cystatin C monoclonal antibody[9] and the rabbit anti-cystatin C polyclonal antibody[10] were kindly provided by Dr. Grubb (University of Lund, Malmö, Sweden). Goat peroxidase-labeled anti-rabbit IgG antibody was purchased from Cappelle Co.Ltd.(U.S.A.).

Enzyme-linked immunosorbent assay for cystatin C:

We employed the two-site sandwich method reported previously[8]. In brief, mouse anti-cystatin C monoclonal 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 (NUNC, Denmark; Immunoplate II 96-F) and allowed to stand at room temperature for 2 hr. to permit coating. After discarding the antibody, the wells were washed once with 0.05% TWEEN 20 phosphate buffered saline(PBS). Preliminary

determination of the optimal antibody dilution titer was done by the serial dilution antibody method. Each well was blocked by adding 250 µl of 0.5% egg albumin, let stand for 15 min., and then washed three times with TWEEN 20 PBS. Various concentrations of the cystatin C standard (0, 10, 50, 80, 100, 250, 500 or 1000 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 hr. at room temperature and then washed four times with Tween 20 PBS. A total of 50 µl of rabbit anti-cystatin C polyclonal antibody diluted with TWEEN 20 PBS (pH 7.9) was then added to each well and incubation was performed at room temperature for 2 hr. The residual antibody was then discarded and the wells were washed five times with 0.05% TWEEN 20 PBS. The optimal antibody dilution was also determined in a preliminary fashion by the serial dilution procedure. Next, 50 µl of goat peroxidase-labeled anti-rabbit IgG antibody (Cappelle, U.S.A.) was added and the wells were allowed to stand at room temperature for 2 hr. After the antibody was discarded, the wells were washed six times with 0.05% Teen 20 PBS.

The coloring solution used was 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts (Nakarai, Kyoto, Japan) as a 15 mg/ml solution with 20 ml of 0.1 M citrate buffer (pH 4.0) and 10 ul of 30% H_2O_2. A volume of 200 µl was added to each well for color development. After 10 minutes the reaction was stopped by adding 50 ul of 0.5 M NaF and then after

a further 10 minutes the absorbance at 417 nm was read using a microplate photometer (Corona Electric, MTP-12, Ibaragi, Japan). A standard curve was devised showing the relationship between the known antigen concentrations and the absorbance (Fig. 1), 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 anti-cystatin C antibodies with amyloid light chain κ (ALκ)[11], amyloid A (AA)[11], or another proteinase inhibitor (trypsin inhibitor).[8]

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 (0-1000 ng/ml) to a fixed amount (50 ul) of control CSF.[8]

Histological study of cerebral amyloid angiopathy: During the acute stage, we obtained brain biopsies from four patients, two with subcortical hemorrhage and two with putaminal hemorrhage. Spinal fluid was obtained during the chronic stage. In one patient with putaminal hemorrhage, we obtained spinal fluid shortly before a second brain specimen was obtained at autopsy.

We evaluated these tissue specimens immunohistologically to detect cystatin C and beta-protein deposition 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 microscope by the apple-green birefringence in the involved vessels. Immunohistochemical study of the brain sections was performed using the avidin biotin complex method previously reported[6,12]. The primary antibodies used in this study were a polyclonal anti-cystatin C antibody,[3,4] provided by Dr. Grubb, and a monoclonal antibody raised against synthetic beta-protein,[13] presented by Dr. Allsop through Dr. Glenner. As the positive control for cystatin C, we used tissues from HCHWA-I patients with cerebral amyloid angiopathy. For negative controls, we used normal brain tissue and amyloid tissues from patients with various kinds of systemic amyloidosis. In the adsorption study, anti-cystatin C antibody was first adsorbed with the purified antigen (cystatin C, 1 mg : Ig G, 1 mg) and then was used as the primary antibody. The specificity of the anti-beta protein antibody was thoroughly examined in a previous study[13].

Results

To evaluate the specificity and accuracy of our new ELISA method, we conducted the following preliminary tests. First, the relationship between absorbance and cystatin C concentration was obtained for the standard antigen solutions. A linear relationship was found from 10 to 1000ng/ml (Fig.1). In this assay system, anti-cystatin C antibody reacted specifically with cystatin C and did not cross-react with ALκ, AA, or trypsin inhibitor[8]. In addition, the recovery test produced a rate of nearly 100%[8]. 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 tested. Values in control patients with brain infarction, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and epilepsy ranged between 100 ng/ml and 600 ng/ml. The mean + standard deviation (SD) was 231 ± 80.5 ng/ml. The mean ± 2SD (231-161=70 ng/ml) was taken as the cut-off level for CSF cystatin C. Among the patients with brain hemorrhage, fifteen had values below 70 ng/ml while 14 had higher values. The former group comprised 12 patients with subcortical hemorrhage, one with multiple relapsing hemorrhages, and two with thalamic hemorrhage who were virtually normotensive (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. A

diagnosis of CAA was confirmed in two of these patients by the histological evaluation of the 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 (Figs. 3A and 4A) and beta-protein (Figs. 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 ng/ml and 56 ng/ml, respectively). Table 2 summarizes the information on the 14 patients with brain hemorrhage and a CSF cystatin C level above 70 ng/ml. All were hypertensive. The diagnosis of CAA was ruled out in two of these patients (cases 16 and 21) by the Congo red staining and immunohistochemical study of brain tissue specimens.

Discussion

Cerebral amyloid angiopathy (CAA) has recently 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 present with multiple relapsing brain hemorrhages, especially subcortical hemorrhages.[14)-15)] The incidence of this disorder increases with age. Brain surgery is contraindicated in patients with brain hemorrhage associated with CAA, making early diagnosis highly desirable. At present, however, pathological diagnosis is the only definitive method. 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 reported[16)] that the amyloid found in HCHWA-I was a protein related to gamma-trace, a gastropancreatic neuroendocrine protein. Löfberg[17)] formulated the following hypothesis for the mechanism of the low levels of CSF cystatin C in HCHWA-I patients: cysteine proteinases are released from the walls of the small cerebral vessels of these patients, and consequently, cystatin C (a cysteine proteinase inhibitor) is consumed, leading to a decline in its CSF level. The inhibitor is then deposited in the form of amyloid with the structure of a cystatin C variant.

Löfberg's hypothesis is partially supported by a report by Davis [18)] that a patient who accidentally received an

intraspinal injection of chymopapain, a cysteine proteinase, developed multiple brain hemorrhages. His theory is also supported by Garvin,[19] who reported that infusion of cysteine proteinases into the CSF led to brain hemorrhage in an animal model.

In this study, we described an ELISA assay for cystatin C in the CSF. We confirmed the specificity of the antibodies used for cystatin C and the lack of interference by other substances in the CSF[8]. This ELISA is simpler and easier to perform than the RIA method reported by Grubb[3] and Löfberg.[17] Fifteen of our 29 patients with brain hemorrhage showed low CSF cystatin C levels. Based on analysis of all aspects of the clinical history and laboratory evaluations, we suspected them to have CAA associated with brain hemorrhage. In two of these 15 patients, immunohistopathological evaluation of brain tissue specimens was possible and confirmed the diagnosis of CAA by demonstrating immunoreactive cystatin C and beta-protein in the amyloid deposits. We have previously reported that age-related beta-protein deposition may precede the deposition of cystatin C in CAA associated with brain hemorrhage[6]. Recently, Vinters[20] reported that A4(beta-) peptide may colocalize with gamma-trace (cystatin C) in the cerebral microvessels. In the two cases in which we have brain tissue available, the deposition of cystatin C and beta-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.

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References

1) Gudmundsson G, Hallgrimsson J, Jonasson TA, Bjarnasson O: Hereditary cerebral hemorrhage with amyloidosis. Brain 1972; 95: 387-404

2) Ghiso J, Jensson O, Frangione B: Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C). Proc Natl Acad Sci U.S.A. 1986; 83: 2974-2978

3) Grubb A. Jensson O, Gudmundsson G, Arnason A, Löfberg H, Malm J: Abnormal metabolism of gamma-trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. New Engl J Med 1984; 311: 1547-1549

4) Grubb A, Löfberg H: Human gamma-trace. Structure, function and clinical use of concentration measurements. Scand J Clin Lab Invest 1985 ;45 :7-13

5) Fujihara S, Shimode K, Kobayashi S, Tsunematsu T, Kagawa R: Possibly familial cerebral amyloid angiopathy in Japan, Immunohistochemical identification of gamma-trace. Clin Neurol 1988; 28: 453-458

6) Fujihara S, Shimode K, Nakamura M, Kobayashi S, Tsunematsu T: cerebral amyloid angiopathy with the deposition of cystatin C (gamma-trace) and beta-protein. Progress in Clinical and Biological Research 1989; 317: 939-944

7) Maruyama K, Ikeda S, Ishihara T, Allsop D, Yanagisawa N: Immunohistochemical characterization of cerebrovascular amyloid

in 46 autopsied cases using antibodies to beta-protein and cystatin C. Stroke 1990; 21: 397-403

8) Shimode K, Fujihara S, Nakamura M, Kobayashi S, Tsunematsu T: Diagnosis of cerebral amyloid angiopathy with cerebral hemorrhage by enzyme-linked immunosorbent assay (ELISA) of cystatin C in the cerebrospinal fluid. Clin Neurol 1990; 30: 288-293

9) Olafsson I, Löfberg H, Abrahamson M, Grubb A: Production, characterization and use of monoclonal antibodies against the major extracellular human cysteine proteinase inhibitors, cystatin C and kininogen. Scand J Clin Lab Invest 1988; 48: 573-582

10) Löfberg H, Grubb AO, Brun A: Human brain cortical neurons contain gamma-trace. Rapid isolation, immunohistochemical and physiochemical characterization of human gamma-trace. Biomed Res 1981; 2: 298-306

11) Fujihara S, Balow JE, Costa JC, Glenner GG: Identification and classification of amyloid in formalin-fixed, paraffin-embedded tissue sections by the unlabeled immunoperoxidase method. Lab Invest 1980; 43: 358-365

12) Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase techniques:a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577-580

13) Allsop D, Landon M, Kidd M, Lowe JS, Reynolds GP, Gardner A: Monoclonal antibodies raised against a subsequence of senile

plaque core protein react with plaque cores, plaque periphery and cerebrovascular amyloid in Alzheimer's disease. Neurosci Let 1986; 68: 252-256

14) Tomonaga M: Cerebral amyloid angiopathy in the elderly. J Am Geriatr Soc 1981; 29: 151-157

15) Vinters HV: Cerebral amyloid angiopathy; a critical review. Stroke 1987; 18: 311-324

16) Cohen DH, Feiner H Jensson O, Frangione B: Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis(HCHWA) is related to the gastroentero-pancreatic neuroendocrine protein,gamma-trace. J Exp Med 1983; 158: 623-628

17) Löfberg H, Grubb AO, Nilsson EK, Jensson O, Gudmundsson G, Blondal H, Arnason A, Thorsteinsson L: Immunohistochemical characterization of the amyloid deposits and quantitation of pertinent cerebrospinal fluid proteins in hereditary cerebral hemorrhage with amyloidosis. Stroke 1987; 18: 431-440

18) Davis RJ, North RB, Campbell JN, Suss RA: Multiple cerebral hemorrhages following chymopapain chemonucleolysis. J Neurosurg 1984; 61: 169-171

19) Garvin PJ, Jennings RB, Smith L, Gesler RM: Chymopapain. A pharmacologic and toxicologic evaluation in experimental animals. Clin Orthop 1965; 41: 204-223

20) Vinters HV, Secor DL, Pardridge WM, Gray F: Immunohistochemical study of cerebral amyloid angiopathy. Widespread Alzheimer A4 peptide in cerebral microvessel walls

colocalizes with gamma trace in patients with
leukoencephalopathy. Ann Neurol 1990; 28: 34-42

Table 1. Fifteen patients with brain hemorrhage and low CSF cystatin C levels (CAA was confirmed histologically in 2 patients and 13 had suspected CAA).
A low cystatin C level was defined as ≦ 70 ng/ml.
Immunohistochemical examination of brain tissue from patients 1 and 2 showed CAA with cystatin C and beta-protein deposition.

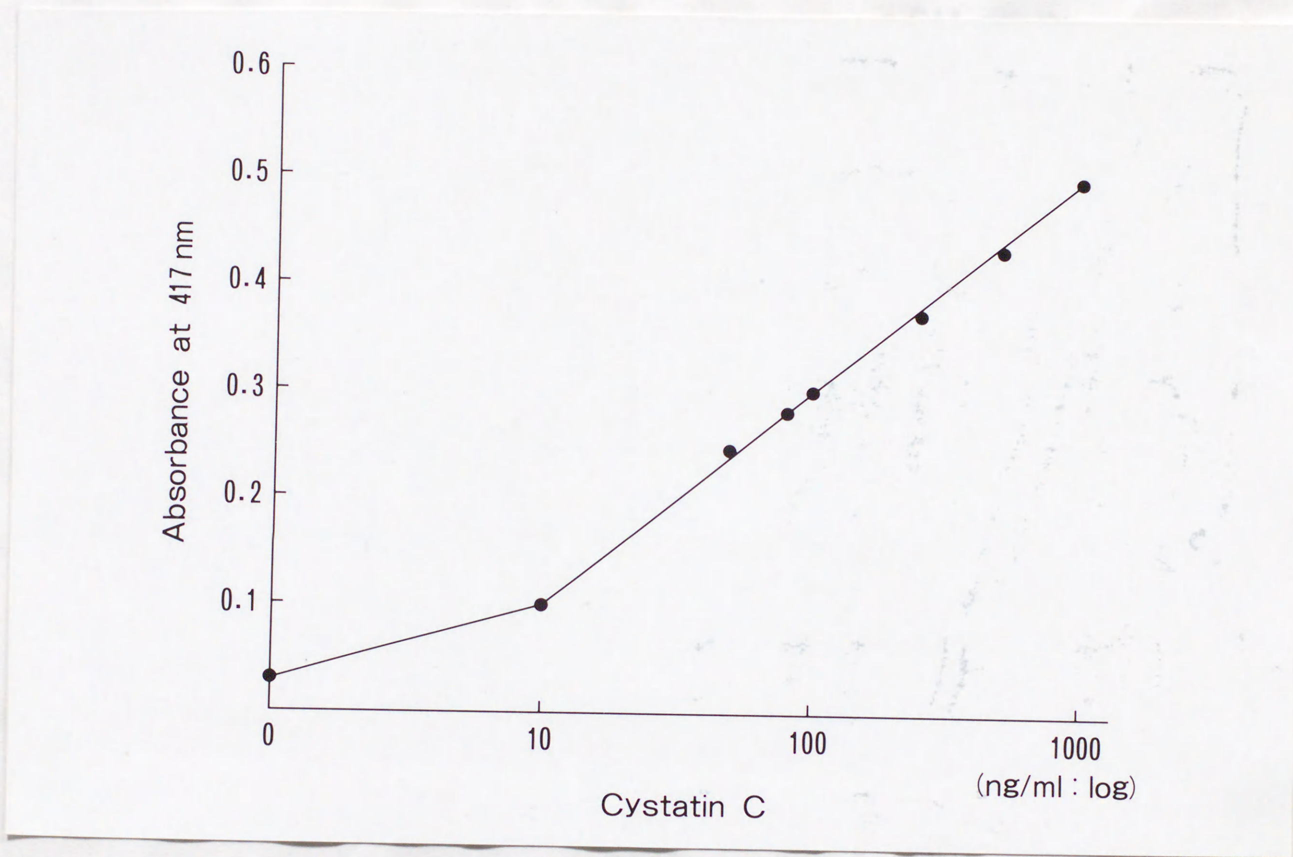
Case	Age	Sex	Site of hemorrhage	Hypertension	Cystatin C level in CSF(ng/ml)
1	65	F	Subcortical(temporal)	-	56
2	76	M	Subcortical(frontal)	-	60
3	63	M	Subcortical(parietal)	-	24
4	81	F	Subcortical(frontal)	-	16
5	72	M	Subcortical(frontal) and thalamic	-	70
6	53	F	Subcortical(occipital)	-	48
7	72	M	Subcortical(frontal)	-	70
8	46	M	Subcortical(parietal)	+	48
9	62	M	Subcortical(occipital) and thalamic	-	68
10	86	M	Subcortical(parietal)	-	68
11	64	F	Subcortical(parietal)	-	55
12	90	M	Subcortical(occipital)	-	46
13	75	F	Multiple lesions	-	32
14	84	F	Thalamus	-	23
15	58	F	Thalamus	-	58

Table 2. Fourteen patients with hypertensive brain hemorrhage and normal CSF cystatin C levels. CAA was ruled out at autopsy or operation in 2 patients.

A normal CSF level of cystatin C was defined as above 70 ng/ml. The brain tissues of patients 16 and 21 were negative for CAA by both Congo red staining or by immunohistochemical examination.

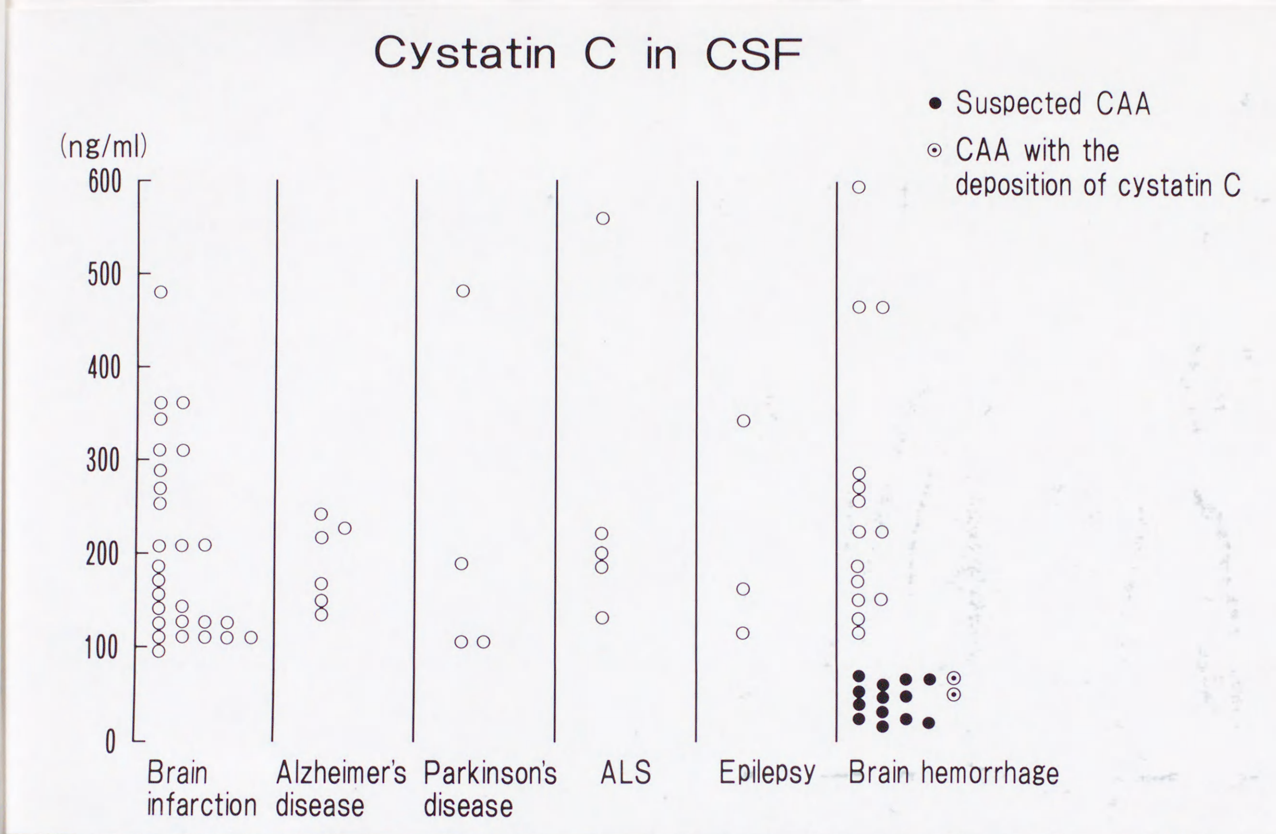
Case	Age	Sex	Site of hemorrhage	Hypertension	Cystatin C level in CSF(ng/ml)
16	75	M	Putamen	+	260
17	56	F	Putamen	+	105
18	73	F	Putamen	+	230
19	53	M	Putamen	+	600
20	88	M	Putamen	+	470
21	53	M	Putamen	+	100
22	74	F	Putamen	+	180
23	70	F	Putamen	+	150
24	49	M	Thalamus	+	225
25	72	M	Thalamus	+	470
26	68	F	Corona radiata	+	280
27	72	M	Subcortical(parietal)	+	145
28	76	F	Subcortical(frontal)	+	175
29	52	M	Subcortical(frontal)	+	290

Figure 1.



A linear relationship between 10 and 1000 ng/ml was demonstrated in the standard curve of the cystatin C ELISA. Quantitative determination of the cystatin C level was precise within this range.

Figure 2.



CSF levels of cystatin C. In the control group, including patients with brain infarction, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and epilepsy, the cystatin C level ranged between 100 and 600 ng/ml. Among the 29 patients with brain hemorrhage, 15 had levels below 70 ng/ml while 14 had levels above 70 ng/ml Two patients in the group with low level were confirmed to have CAA and the deposition of cystatin C was observed by the study of brain tissue specimens.

Figure 3A.

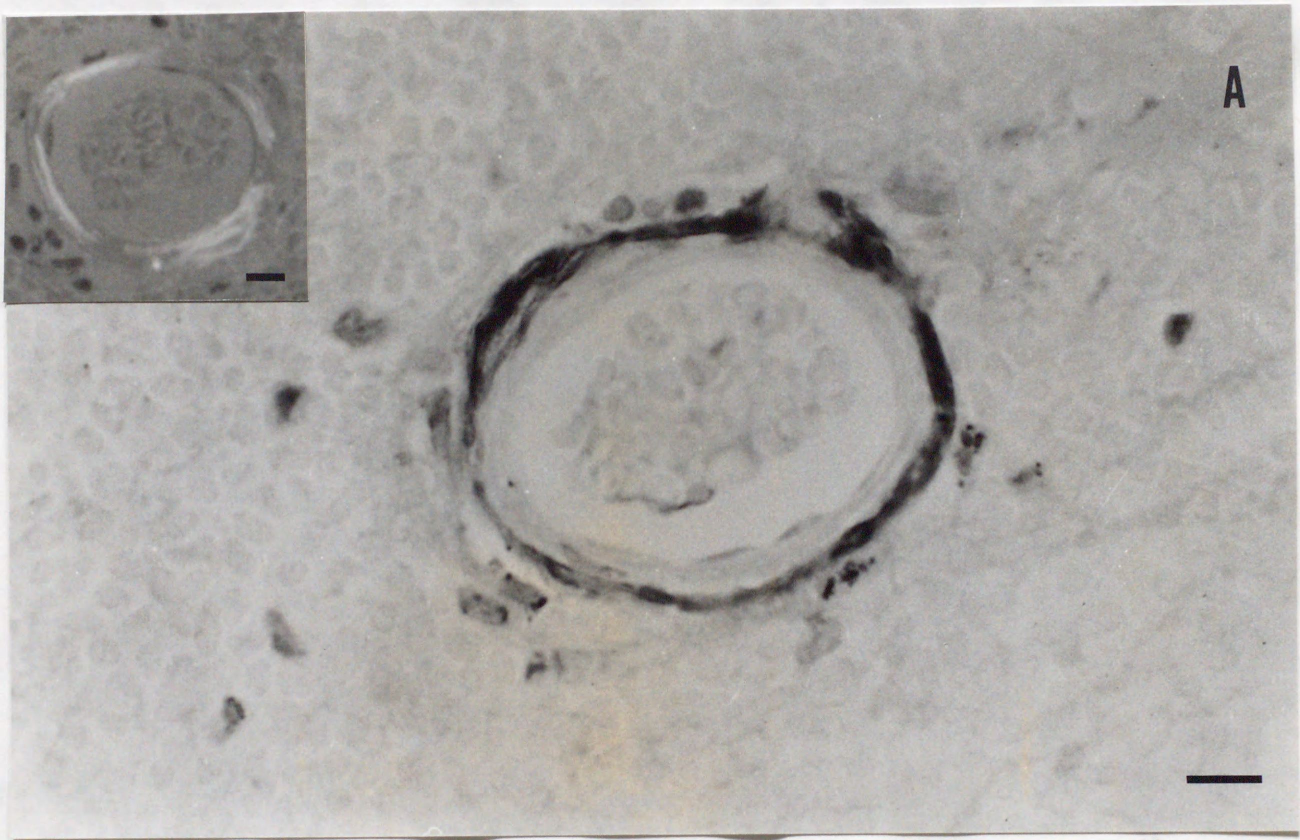
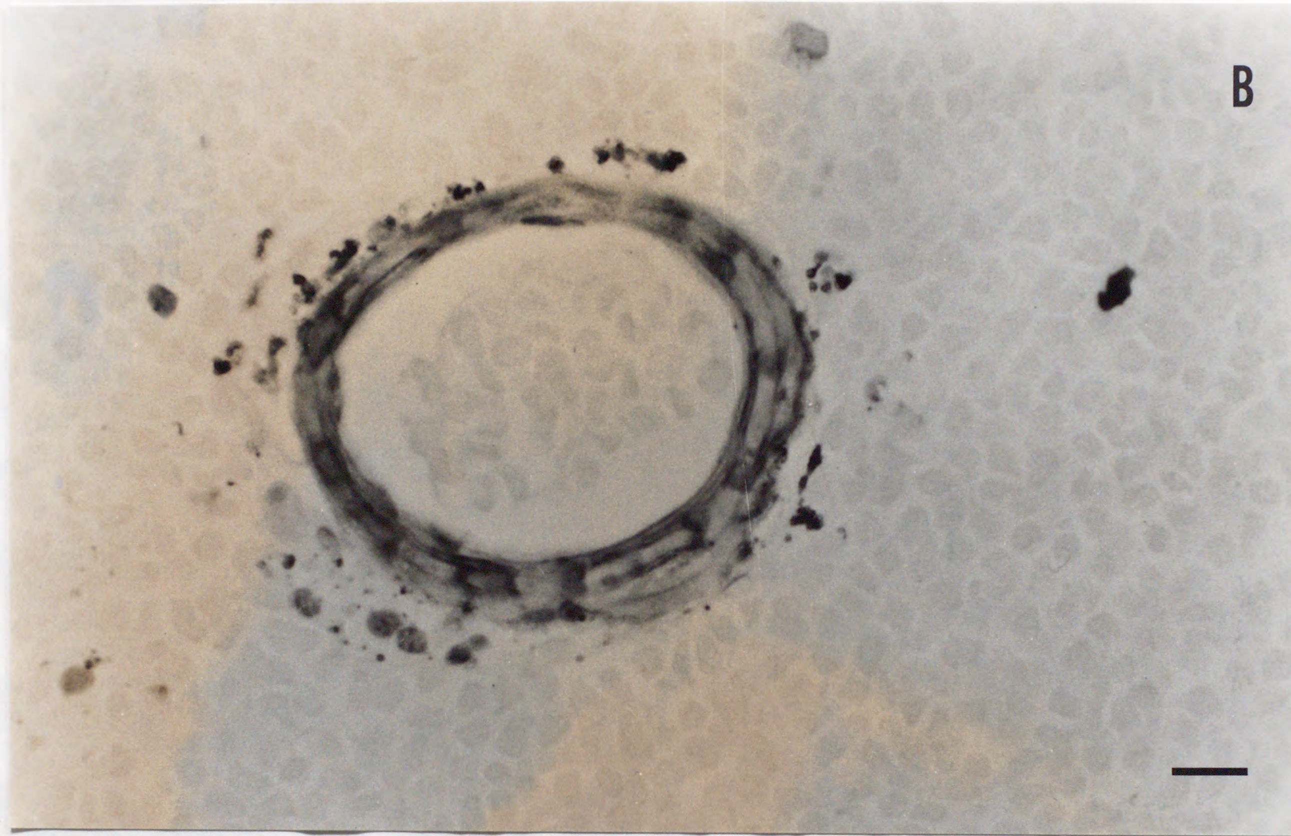


Figure 3B.



Photomicrographs of Congo red staining and immunostaining with anti-cystatin C antibody and beta-protein of cerebral vessels from brain tissue preparations obtained during surgery in case 1 (Table 1). A and B are closely adjacent sections obtained from the same tissue block. A: Immunostaining with anti-cystatin C antibody. B: Immunostaining with anti-beta-protein antibody. Insert: Congo red staining viewed under polarized light. Amyloid deposition associated with cystatin C and beta-protein deposits is seen in the cerebral vessels. Bars = 20 um.

Figure 4A.

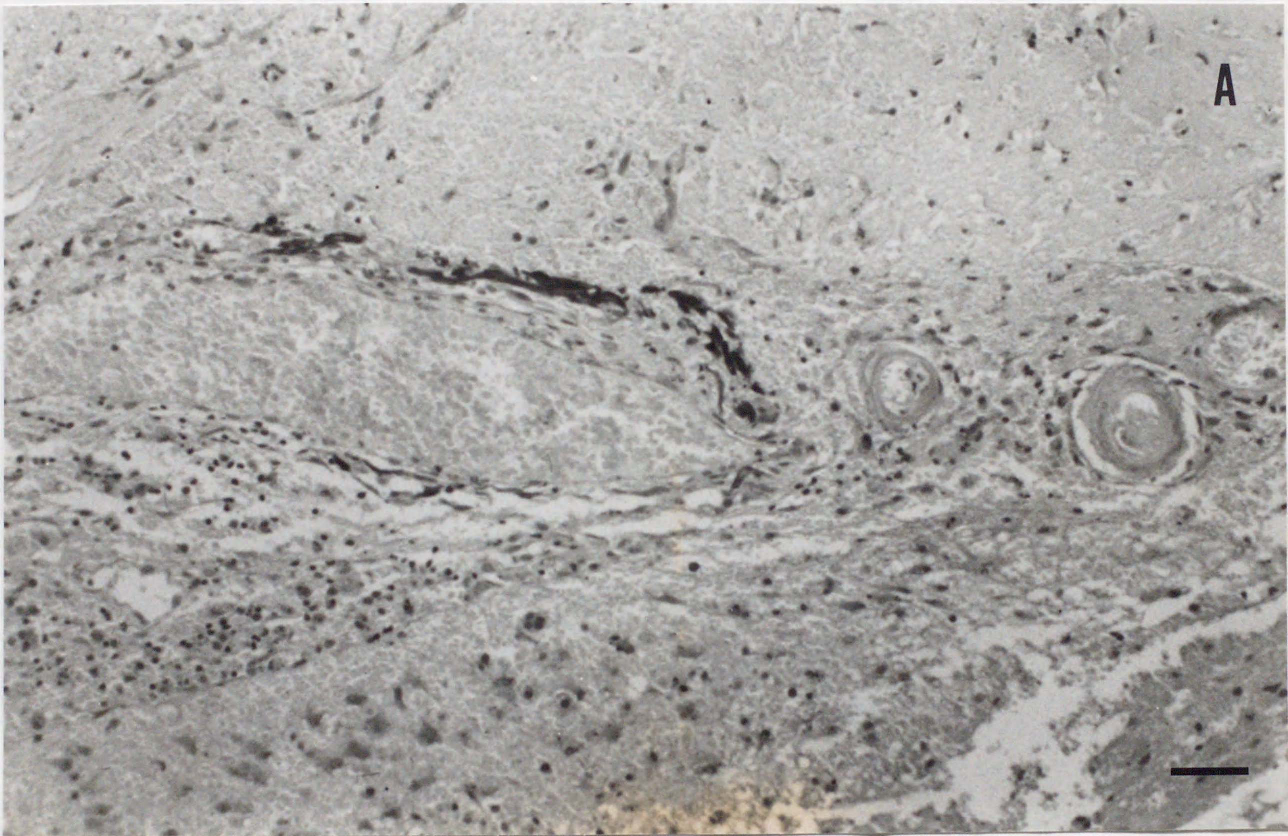
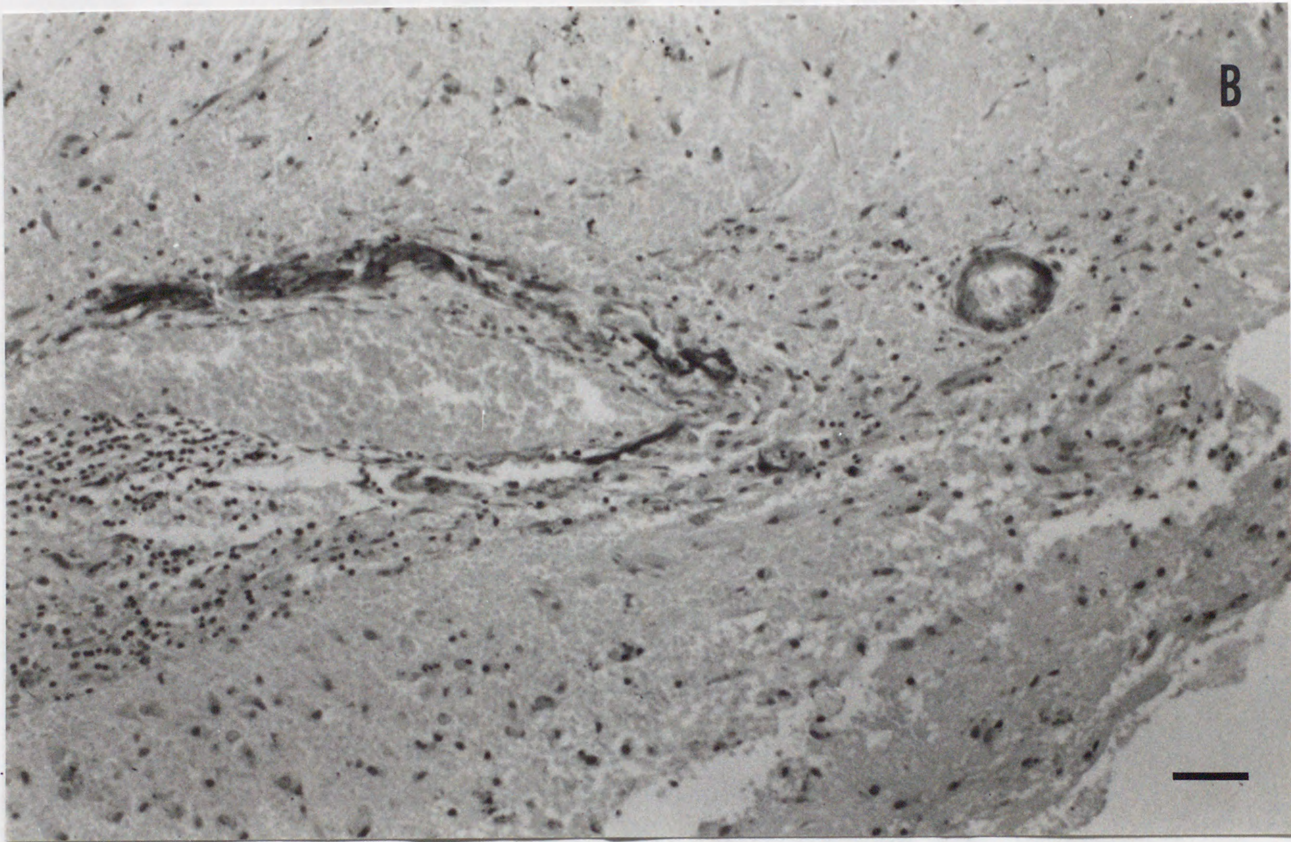


Figure 4B.



Photomicrographs of case 2 (Table 1) showing both cystatin C and beta-protein immunoreactivity in cerebral vessels. A and B are closely adjacent sections from the same block. A: Immunostaining with anti-cystatin C antibody. B: Immunostaining with anti-beta-protein antibody. Amyloid deposition associated with cystatin C and beta-protein deposits is seen in the small cerebral vessels. Bars = 50 um.

inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

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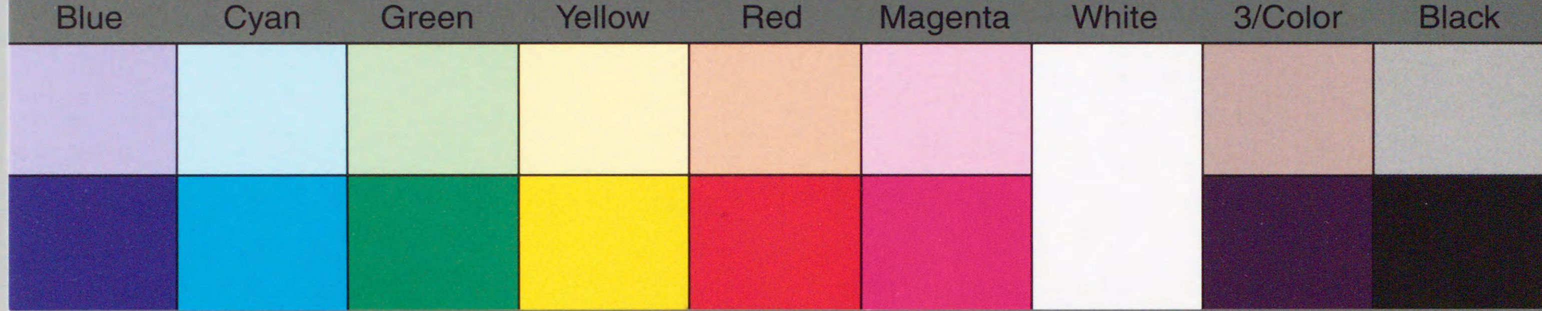
Blue Cyan Green Yellow Red Magenta White 3/Color Black



Kodak Gray Scale

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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19