Immunohistochemical Characterization of the Amyloid Deposits and Quantitation of Pertinent Cerebrospinal Fluid Proteins in Hereditary Cerebral Hemorrhage With Amyloidosis

Helge Lofberg, Anders O. Grubb, Elise K. Nilsson, Olafur Jennis, Gunnar Gudmundsson, Hannes Blondal, Alfred Arnason, and Leifur Thorsteinsson

Cystatin C, a protein inhibitor of lysosomal cysteine proteinases, was demonstrated by immunohistochemical techniques to be present in the birefringent amyloid deposits of the small arteries in the cerebrum, cerebellum, and leptomeninges of 10 Icelandic individuals with hereditary cerebral hemorrhage with amyloidosis. Specimens from other organs were investigated in one of the patients, and amyloid angiopathy characterized by an immunoreactivity of cystatin C was found in a submandibular lymph node. No immunoreactivity of amyloid fibril protein AA, κ or λ immunoglobulin light chain, or prealbumin was observed. Significantly low cerebrospinal fluid concentrations of cystatin C were found in all 9 investigated individuals with hereditary cerebral hemorrhage with amyloidosis. The concentrations of β₂-microglobulin, albumin, and IgG in the cerebrospinal fluid were within normal limits. Isoelectric focusing showed that cystatin C from the cerebrospinal fluid of 9 patients with hereditary cerebral hemorrhage with amyloidosis had an isoelectric point identical to that of normal individuals. This investigation demonstrates that hereditary cerebral hemorrhage with amyloidosis may be diagnosed by two laboratory methods: immunohistochemical investigation of cystatin C in brain tissue specimens and quantitation of cystatin C in cerebrospinal fluid.

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A HEREDITARY type of amyloidosis localized to small arteries in the central nervous system has been described in Icelandic individuals dying from cerebral hemorrhage. The disorder is inherited in an autosomal dominant mode and is clinically manifested by recurrent multifocal intracerebral hemorrhages in young adults. The amyloid substance deposited in the cerebral vessel walls of these patients has recently been characterized and found to be related to a protein called cystatin C. An abnormally low cerebrospinal fluid (CSF) concentration of cystatin C has been reported to be characteristic for individuals suffering from this type of intracerebral hemorrhage and for those asymptomatic family members who carry the trait of amyloid angiopathy and who later in life will develop manifest hemorrhages. Cystatin C, also called γ-trace, is an inhibitor of lysosomal cysteine proteinases and earlier had been demonstrated in human body fluids and in normal and neoplastic neuroendocrine cells in monkeys and humans.

The present investigation was undertaken to elucidate the nature and tissue distribution of the amyloid deposits in hereditary cerebral hemorrhage with amyloidosis and to compare the diagnostic potential of immunohistochemical procedures with that of quantitative analysis of CSF proteins.

Subjects and Methods

Report of a Case

A 26-year-old Icelandic woman (BE) was admitted to the Neurological Department, University Hospital in Reykjavik, with a left-sided hemiparesis and hyperreflexia. On examination she had no hypertension, and the total protein concentration and cell content of her CSF were within normal limits. Carotid and vertebral angiography were normal. During the next 10 years she had at least 5 new cerebrovascular insults, with signs from both right and left hemispheres. On one occasion the CSF was blood-stained. She partially recovered after each episode, but a striking feature was a progressive mental deterioration. Computed tomography (CT) showed intracerebral hemorrhages. Finally she died shortly after her last insult.

Postmortem examination showed 3 fresh hemorrhages in the white matter of her left cerebral hemisphere (Figure 1). Remnants of old hemorrhages were...
Coronal slices of the left cerebral hemisphere of a deceased 36-year-old Icelandic woman (BE) suffering from hereditary cerebral hemorrhage with amyloidosis. Three separate fresh hemorrhages were seen; one was localized in the lower part of the left frontal lobe, another large one in the left frontoparietal region, and a third in the upper part of the left parieto-occipital lobe. Remnants of old hemorrhages were found in the basal ganglia and subcortical white matter. (The frontal pole is seen in the upper left of A and the occipital pole in the lower right of B.) × 2 minification.
Human Tissue and Fixation

Autopsy specimens were obtained 3 hours post mortem from the cerebrum, cerebellum, brainstem, dura mater, pituitary gland, submandibular gland, thyroid gland, pancreas, adrenal gland, kidney, liver, lung, spleen, myocardium, breast, eye, vagus nerve, obturator nerve, external iliac artery, and external iliac vein of BE. The tissues were frozen without fixation or fixed in a solution of 10% formalin, in Bouin’s fluid13 (0.04 M picric acid, 3.2 M paraformaldehyde, 0.83 M acetic acid), in cold 96% ethanol,18 or in B-5 fixative19 (0.22 M mercuric chloride, 0.15 M sodium acetate, 1.3 M paraformaldehyde) or freeze-dried and fixed in diethylpyrocarbonate vapor20 or in paraformaldehyde vapor at 60°C for 3 hours.

Specimens were also taken at autopsy from the brain of 5 men (22, 26, 30, 33, and 38 years old) and 4 women (29, 30, 33, and 37 years old) who had died from cerebral hemorrhages. They belonged to 6 Icelandic families, and all of them had close relatives who earlier had died from hereditary cerebral hemorrhage with amyloidosis. The tissues from the patients were fixed in a solution of 10% formalin.

**Human Body Fluids**

**Hereditary Cerebral Hemorrhage with Amyloidosis.** Samples of CSF and blood plasma were collected from 9 Icelandic individuals of both sexes, 26–38 years old, suffering from recurrent cerebral hemorrhages (BE was included in this patient group). All of them underwent neuroradiologic investigations including CT. Three of the patients died from their disorder, and postmortem examination of their brains showed congophilic amyloid deposition in the cerebral vessel walls. The 6 other living patients had parents, brothers, or sisters who previously had died from apoplectic stroke. The brains of these deceased relatives had shown amyloid angiopathy at histopathologic examination.

**Healthy Reference Population.** CSF and plasma samples were also analyzed from 44 adults with minor neurologic complaints but with normal CSF cell count, normal CSF–plasma gradients of albumin and IgG, and normal electrophoretic pattern of the CSF. No diagnoses could be established in this group although careful clinical and laboratory investigations were performed.

**Patients with Cerebrovascular Disorders without Known Amyloidosis.** CSF and plasma samples were collected from 28 individuals (mean age 60 years, range 33–90 years). Seven of them had intracerebral hemorrhages, 13 had cerebral infarctions, and 8 had reversible ischemic neurologic deficits or transitory ischemic attacks. The hemorrhages and infarctions were diagnosed by neuroradiologic investigations or at autopsy.

**Patients with Multiple Sclerosis.** CSF and plasma samples were investigated from 22 patients suffering from multiple sclerosis. The criteria of Schumacher et al21 were used to establish the diagnosis.

**Patients with Protruded Intervertebral Disks.** CSF and plasma samples were collected from 10 patients with intervertebral disk protrusion. The diagnosis was based on clinical examination and myelography and confirmed by operation in 8 cases. No derangements of the central nervous system of these patients could be demonstrated.

**Preservation of the Samples**

The CSF samples were obtained by lumbar puncture in tubes containing the serine protease inhibitor benzamidine chloride. Its final concentration in the samples invariably exceeded 1 mM. The samples were immediately frozen at −20°C. The plasma samples were anticoagulated with ethylenediaminetetraacetate and stored at −20°C, but no protease inhibitor was added to these samples as no degradation of cystatin C in plasma has been observed.
Primary Antisera

A polyclonal rabbit antiserum against human cystatin C, affinity chromatography purified sequence-specific antibodies against the amino-terminal octapeptide of human cystatin C, purified mouse monoclonal antibodies against human cystatin C, polyclonal rabbit antiseria against amyloid fibril protein AA, human amyloid P component, β2-microglobulin, albumin, and IgG Fc fragments were produced at the laboratories. Rabbit antibodies against κ (034610A) and λ (034033) immunoglobulin light chains were obtained from Ortho Diagnostic Systems, Raritan, N.J. A rabbit antiserum against prealbumin was obtained from Dakopatts a/s, Copenhagen, Denmark.

Polypeptides

Human cystatin C, human amyloid P component, human κ and λ light chains, and human prealbumin were isolated at the laboratories.

Immunoochemical Techniques

Cystatin C was localized by the avidin-biotin-peroxidase complex and the two-step immunofluorescence techniques. The concentrations of cystatin C and β2-microglobulin were determined by enzyme-amplified single radial immunodiffusion and those of albumin and IgG by electroimmunoassay and unmodified single radial immunodiffusion, respectively.

Isoelectric focusing was performed in polyacrylamide gel slabs with pH gradients from 7 to 11 established by use of commercial ampholytes (Ampholines, LKB, Bromma, Sweden) and with a final voltage of 140 V/cm. Immediately after focusing, all proteins were transferred to and immobilized on sheets of nitrocellulose. Proteins reacting with various antisera were then visualized by use of peroxidase-labelled secondary antibodies and the enzyme substrate 3-amino-9-ethylcarbazole.

Antiserum Control Procedures

The specificity of the antibodies against cystatin C was investigated by absorption experiments; 100 μl of the solutions of the primary antiseria diluted according to their working dilutions were incubated overnight with 2.5 μg cystatin C, 5 μg κ light chain, 5 μg λ light chain, 22 μg amyloid P component, and 10 μg prealbumin.

Statistical Analysis

The Wilcoxon rank sum test was used to evaluate differences between groups in two-tailed comparisons.

Results

Immunostaining

Immunohistochemical investigation using polyclonal, sequence-specific, and monoclonal antibodies showed an immunoreactivity of cystatin C in the walls of small arteries in the cerebrum, cerebellum, brainstem, and leptomeninges of a 36-year-old woman (BE) who died from intracerebral hemorrhage (Figure 3). The vessel walls also demonstrated immunoreactivity of the amyloid P component. The immunoreactive substance was stained by alkaline Congo red and showed a yellow-green birefringence under polarized light (Figure 3). In addition, cystatin C was demonstrated in the walls of small-sized arteries located in the medullary sinus of a submandibular lymph node of the patient (Figure 4). These lymph node arteries were also characterized by a yellow-green polarization color after Congo red staining. The amyloid deposits showed no immunoreactivity of amyloid fibril protein AA, κ or λ light chain, or prealbumin when the tissues were unfixed or fixed in a solution of 10% formalin, in Bouin’s fluid, cold ethanol, B-5 fixative, diethylpyrocarbonate vapor, or paraformaldehyde vapor.

No congophilic birefringent material and no vascular deposits reacting with the antiserum against cystatin C were found in the dura mater, pia mater, submandibululary gland, thyroid gland, pancreas, adrenal gland, kidney, liver, lung, spleen, myocardium, breast, eye, vagus nerve, obturator nerve, external iliac artery, or external iliac vein of BE.

In 9 other Icelandic individuals who had died from hereditary cerebral hemorrhage an immunoreactivity of cystatin C and a birefringence characteristic for amyloid deposits were demonstrated in the walls of the small arteries in the cerebral and cerebellar parenchyma and in the leptomeninges.

Fixation

A distinct immunostaining of cystatin C was seen in the amyloid deposits of the cerebral vessels when unfixed tissues or paraformaldehyde-vapor-fixed tissues of BE were investigated by polyclonal, sequence-specific, and monoclonal antibodies. Vapor-fixation in diethylpyrocarbonate and fixation in Bouin’s fluid, in cold ethanol, or in B-5 fixative resulted in an immunostaining of cystatin C of medium-grade intensity. Fixation in a solution of 10% formalin did not result in any staining when the monoclonal antibodies were used and in a weak immunostaining when the polyclonal and sequence-specific antibodies against cystatin C were used.

Specificity of the Immunostaining

The specificity of the antibodies was tested by absorption experiments. No decrease in immunoreactivity was observed when the polyclonal, sequence-specific, or monoclonal antibodies against cystatin C were absorbed by the amyloid P component, κ or λ light chains, or prealbumin.

Concentration of Proteins in Body Fluids

The concentration of cystatin C (mean 2.5 mg/l, range 1.7–3.5 mg/l) in the CSF of 9 patients with hereditary cerebral hemorrhage with amyloidosis (including BE) was far below the cystatin C concentrations of the patients with other cerebrovascular disorders, multiple sclerosis, prolapsed intervertebral disks, and the healthy reference population (Table 1). The differences between the patient group with amyloid angiopathy and the 4 reference groups were all highly significant (p < 0.01), and the mean CSF concentration of cystatin C in the amyloid angiopathy group was
Table 1. Concentrations of Cystatin C, β₂-Microglobulin, Albumin, and IgG in Cerebrospinal Fluid (CSF) and Plasma Samples From Patients With Hereditary Cerebral Hemorrhage With Amyloidosis and From 4 Reference Groups

<table>
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<tr>
<th></th>
<th>Healthy reference population</th>
<th>Prolapsed intervertebral disks</th>
<th>Multiple sclerosis</th>
<th>Cerebrovascular disorders without known amyloidosis</th>
<th>Hereditary cerebral hemorrhage with amyloidosis</th>
<th>Hereditary cerebral hemorrhage with amyloidosis</th>
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<td>CSF cystatin C (mg/l)</td>
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<tr>
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<td>1.1–4.2</td>
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<td>Plasma β₂-microglobulin (mg/l)</td>
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<td>p</td>
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All p values refer to comparisons with the group of patients with hereditary cerebral hemorrhage with amyloidosis.

only about a third of the mean values of the reference groups.

The levels of β₂-microglobulin (a protein of similar molecular size as cystatin C), albumin, and IgG were also analyzed to allow a comparison with the levels of cystatin C. In contrast to the cystatin C concentration, the CSF concentrations of β₂-microglobulin, albumin, or IgG did not differ between the groups (Table 1). No consistent differences in the blood plasma concentrations of cystatin C or β₂-microglobulin among the 5 groups were observed (Table 1).

Isoelectric Focusing

Isoelectric focusing of CSF and plasma samples followed by immunoblotting with the use of a polyclonal rabbit antiserum against human cystatin C revealed that the isoelectric point of cystatin C from 9 patients with hereditary cerebral hemorrhage with amyloidosis was identical to that of cystatin C from healthy individuals. Immunoreactive molecules with the same isoelectric points could also be demonstrated with monoclonal antibodies against native human cystatin C and with an antiserum against the amino-terminal octapeptide of cystatin C.

Discussion

The clinical diagnosis of cerebral amyloid angiopathy may be considered in cases with a history of recurrent multifocal intracerebral hemorrhages. The most reliable method to establish the definite diag-
FIGURE 3. Examination of leptomeningeal small arteries outside the cerebellum of BE. A: Demonstration of the amyloid deposits by Congo red staining. B: Birefringence of the congophilic amyloid fibrils in polarized light (yellow-green color). C: Immunoreactivity of cystatin C in the amyloid deposits of the leptomeningeal vessel walls (brown color). A polyclonal antiserum against human cystatin C was used in the avidin-biotin-peroxidase complex technique. D: Immunofluorescence of cystatin C (yellow color) using sequencesspecific antibodies against the amino-terminal octapeptide of human cystatin C. × 320 magnification.
FIGURE 4. Characterization of the amyloid deposits in the walls of small arteries in a submandibular lymph node of BE (the same patient as in Figure 2). A: Alkaline Congo red staining. × 800 magnification. B: Immunofluorescence staining of cystatin C (yellow color) with use of a polyclonal antiserum against cystatin C. × 320 magnification.
nosis of amyloidosis is to demonstrate birefringent amyloid material in the small arteries of tissue specimens obtained at brain biopsy or autopsy. Only a few percent of all intracerebral hemorrhages have been reported to be due to amyloidosis.

Recurrent intracerebral hemorrhages in young individuals have previously been reported to be strongly associated with familial forms of cerebrovascular amyloidosis in Iceland and Holland. In the Icelandic individuals the congophilic substance has been localized to the walls of small vessels in the central nervous system. The amyloid protein has been isolated from the vascular deposits and found to be a variant of the neuroendocrine protein cystatin C. This protein, which also is called γ-trace, has a known primary structure and a molecular weight of 13,260. It is a potent inhibitor of the lysosomal cysteine proteinases cathepsin B, H, and L, and has been suggested to play a regulatory role in the degradation of proteins and postribosomal processing of peptides. Cystatin C occurs in all investigated human extracellular fluids. The concentration of cystatin C in CSF is particularly high, and in normal adults the level in CSF is 5.5 times higher than that in blood plasma. Cystatin C is present intracellularly in brain cortical neurons, in the luteinizing-hormone-containing cells, and in certain ACTH-containing cells of the adenohypophysis, in the calcitonin-containing C-cells of the thyroid gland, the glucagon-containing A-cells of the pancreatic islets, and in the adrenal medulla. Cystatin C is also found in nonhyposecreting pituitary adenomas.

The present postmortem examination of the Icelandic woman BE demonstrated multiple intracerebral hemorrhages of various ages (Figure 1) and a generalized amyloid angiopathy throughout the brain. No changes typical for Alzheimer's disease were observed. The immunohistochemical investigation of tissue specimens from 10 Icelandic individuals with hereditary cerebral amyloidosis showed immunoreactivity of cystatin C in amyloid deposits in the walls of small cerebral, cerebellar, pontine, and leptomeningeal arteries (Figure 3). No immunoreactivity against other known amyloidogenic proteins (amyloid fibril protein AA, κ and λ light immunoglobulin chains, or prealbumin) was detected. As in other types of amyloidosis and consistent with an earlier study the amyloid P component was demonstrated to occur in the amyloid deposits.

Only vessel walls of the central nervous system have been reported earlier to be affected by amyloidosis in individuals with hereditary cerebral hemorrhage with amyloidosis. However, in the present investigation unexpected amyloid deposits of cystatin C were found in small arteries of a submandibular lymph node (Figure 4). This finding showed that minor amyloid deposits may also be formed outside the central nervous system in the Icelandic type of cerebral amyloid angiopathy.

In contrast to an earlier report that has shown that freeze-drying and vapor-fixation in diethylpyrocarbonate is the fixation method of choice for immunohistochemical demonstration of native cystatin C in the cytoplasm of neuroendocrine cells, the present experiments showed that most fixation methods are useful when cystatin C occurs as an amyloid fibrilar protein with β-pleated sheet conformation. Even routine fixation with a solution of 10% formalin resulted in a weak immunostaining of the protein.

It has previously been demonstrated that the diagnosis of hereditary cerebral hemorrhage with amyloidosis is strongly supported by an abnormally low CSF concentration of cystatin C. The present investigation shows that the CSF concentrations of β₂-microglobulin, albumin, and IgG are virtually normal and cannot be used to identify patients with this type of cerebral amyloid angiopathy (Table 1). The general CSF protein turnover in such patients therefore does not seem to be abnormal in contrast to the turnover of cystatin C.

The isoelectric point of cystatin C in CSF and blood plasma samples from patients with hereditary cerebral hemorrhage with amyloidosis was found to be identical to that of cystatin C from healthy individuals. This observation indicates a normal structure of cystatin C in these patients and excludes the possibility that the low concentrations were due to a proteolytic degradation of the cystatin C molecules in pertinent CSF samples.

In humans the cysteine proteinase chymopapain has been used for enzymatic digestion of the nucleus pulposus in patients with prolapsed intervertebral disks. Enzyme erroneously injected into the subarachnoid space has recently been reported to cause multiple cerebral hemorrhages in these patients. In animal experiments injection of a cysteine proteinase into the CSF has also resulted in cerebral hemorrhage. These observations indicate a possible pathophysiologic role of cysteine proteinases in cerebral hemorrhage. We propose the hypothesis that patients with hereditary cerebral hemorrhage with amyloidosis might have a raised release of cysteine proteinases in the walls of their small arteries. Cystatin C, a physiologic inhibitor of cysteine proteinases in the extracellular fluids, might be consumed and thereby be accumulated as amyloid fibrils in the vessel walls. The increased metabolism of cystatin C might explain its low concentration in the CSF.

The present and earlier investigations show that at a clinical suspicion of the diagnosis of hereditary cerebral hemorrhage with amyloidosis, two laboratory tests can be used to corroborate or refute the diagnosis. One test, immunohistochemical search for amyloid deposits of cystatin C, probably has a nearly perfect specificity, but if used ante mortem it requires a hazardous brain biopsy. The other test, measurement of the CSF concentration of cystatin C, has a somewhat lower specificity and analytic errors due to proteolysis might occur, but it requires only a small amount of CSF from the patient.

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KEY WORDS • cerebral hemorrhage • amyloidosis • immunohistochemistry • cerebrospinal fluid proteins • protease inhibitors
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