Hereditary cystatin C amyloid angiopathy: monitoring the presence of the Leu-68 → Gln cystatin C variant in cerebrospinal fluids and monocyte cultures by MS

Bjarni ÁSGEIRSSON*[1], Sophie HAEBEL†, Leifur THORSTEINSSON‡, Erlendur HELGASON*, Kristbjörn Orri GUDMUNDSSON‡, Gunnar GUDMUNDSSON§ and Peter ROEPSTORFF†

*Department of Chemistry, Science Institute, University of Iceland, 107 Reykjavik, Iceland, †Department of Molecular Biology, Odense University, DK-5230 Odense M, Denmark, ‡Department of Medical Genetics, The Blood Bank, National University Hospital, 101 Reykjavik, Iceland, and §Department of Neurology, National University Hospital, 101 Reykjavik, Iceland

Hereditary cystatin C amyloid angiopathy (HCCAA) is an autosomal dominant condition in which the patients suffer at an early age from repeated cerebral haemorrhages. The development of HCCAA is directly linked to a Leu-68 → Gln (L68Q) mutation in the cystatin C protein sequence. The concentration of cystatin C in cerebrospinal fluid (CSF) of HCCAA patients is markedly diminished and cultivated monocytes from affected individuals accumulate cystatin C. The goal of this work was to characterize cystatin C isolated from CSF and monocyte cultures originating from healthy persons and HCCAA patients with respect to the L68Q mutation. Cystatin C was isolated by carboxymethylpapain affinity chromatography. Proteins from CSF and monocyte cultures that bound specifically to the carboxymethylated papain column were resolved by reverse-phase HPLC chromatography and tryptic peptides were subsequently analysed by matrix-assisted laser desorption ionization MS. No evidence for mutated cystatin C protein was found in CSF samples from healthy subjects or HCCAA patients, but approx. 60 % of the protein was found to be hydroxylated on Pro-3. No evidence was found for secretion of mutated cystatin C from HCCAA monocytes. However, we obtained evidence for the presence of mutated cystatin C in HCCAA monocytes. These results support the conclusion that the mutated cystatin C is retained in association with the monocytes and not secreted. An increased intracellular concentration would presumably promote the aggregation and denaturation of the mutated cystatin C, leading to the formation of amyloid fibrils and cell death.

INTRODUCTION

Hereditary cystatin C amyloid angiopathy (HCCAA) is an autosomal dominant disease characterized by the accumulation of amyloid fibrils in many tissues, including cerebral arteries [1]. Repeated cerebral haemorrhages eventually lead to paralysis, the development of dementia, and death at an early age [2]. Amyloid fibrils isolated from HCCAA-affected brains are composed of a mutant form of cystatin C, an extracellular inhibitor of cysteine proteinases [3]. The role of the cystatin C Leu-68 → Gln mutation (L68Q) in the mechanism of amyloid deposition in patients suffering from HCCAA (Icelandic type) is still unclear [4]. The concentration of cystatin C in the cerebrospinal fluid (CSF) of individuals carrying the mutation was found to be only one-third that of the normal reference group [5], suggesting that intracellular processing and eventual secretion might be at fault. In support of this contention is the finding that cultured monocytes from HCCAA patients secreted significantly less immunoreactive cystatin C than monocytes from healthy subjects. Furthermore immunostaining of cystatin C in fixed cells was in general stronger for monocytes grown from carriers of the mutated gene. Thus the intracellular accumulation of cystatin C L68Q due to blockage of the normal export mechanism might promote polymerization into amyloid fibrils, a process eventually leading to cell death and rupture, with local spreading of intracellular proteins [6,7]. More recent results obtained with a bacterial expression system for the L68Q cystatin C mutant showed that the mutant protein has a much greater tendency to dimerize and form aggregates than normal cystatin C [8]. It was therefore suggested that the change in physical characteristics of L68Q cystatin C might cause it to be trapped intracellularly and not secreted from the cells as efficiently as wild-type cystatin C [8] or perhaps not at all [7].

It has also been suggested that greater local cysteine proteinase activity, due to reduced levels of active cystatin C, might contribute to the destruction of vessel walls [7,9]. The mutant cystatin C has comparable inhibitory potency to that of wild-type cystatin C [8]. However, the observation that the dimerization of mutant cystatin C abolishes its inhibitory activity is compatible with a hypothesis placing uncontrolled proteinase activity in a central position as a causative agent for blood vessel rupture in HCCAA patients [7].

The cystatin C variant isolated from the brain-vessel amyloid deposits was also found to be truncated at the N-terminus. The first ten amino acid residues were missing [3,10]. Similar truncation could be produced with leucocyte elastase and purified cystatin C *in vitro* [11]. Because both cystatin C and leucocyte elastase are extracellular proteins it was suggested that such cleavage might be physiologically relevant in terms of regulating cysteine-proteinase inhibitory activity at sites of inflammation

Abbreviations used: CSF, cerebrospinal fluid; 4HCCA, α-cyano-4-hydroxycinnamic acid; HCCAA, hereditary cystatin C amyloid angiopathy; MALDI, matrix-assisted laser desorption ionization; TFA, trifluoroacetic acid.

[1] To whom correspondence should be addressed.

[11]. Our studies have shown that the cystatin C that is produced by HCCAA monocyte cell cultures is not N-terminally truncated, indicating that truncation occurs late in the pathway of amyloid fibril formation. Furthermore no sign of truncation could be detected in cystatin C from the CSF of HCCAA patients, either by isoelectric focusing or N-terminal sequence analysis [9,12]. However, these techniques did not have the potential to discriminate between the mutant and wild-type variants of cystatin C. Hence, in this study, we sought to detect the presence of the L68Q mutation directly by MS, in both cystatin C samples from CSF and cultured monocytes. Our results show that the mutant cystatin C is not secreted from HCCAA cells, indicating that intracellular accumulation is a prelude to amyloid fibrillar formation.

EXPERIMENTAL

Materials

Chemicals for the preparation of buffers and performance of SDS/PAGE, benzamidine, PMSF, papain (P3125), cysteine, α-cyano-4-hydroxycinnamic acid (4HCCA), n-octylglucoside and iodoacetic acid were obtained from Sigma. CNBr-Sepharose, Q-Sepharose and Sephadex G-25 (medium grade) were obtained from Pharmacia-LKB. Hybond-C nitrocellulose membranes for Western blotting were obtained from Amersham; 2,4,6-trihydroxyacetophenone was from Aldrich; sinapic acid was from Fluka; and modified sequencing-grade trypsin was from Promega. All other chemicals were of analytical grade.

Isolation and culture of monocytes

CSF and blood samples were collected from individuals carrying the mutated HCCAA cystatin C gene as confirmed by restriction fragment length polymorphism [13] and/or by PCR analysis [14]. Mononuclear cells were isolated by Isopaque/Ficoll gradient centrifugation and the monocytes by their adherence properties. Peripheral monocytes were then incubated for 7–10 days as described previously [6]. After collection of the supernatants from the culture plates, the cells were lysed with 0.05 % Triton X-100 and scraped off with a rubber 'policeman'. Benzamidinium chloride and PMSF were added to all samples at 2 mM to prevent proteolysis.

Quantification of cystatin C

Quantitative determination of cystatin C was performed by ELISA with standardized cystatin C [6,15].

Isolation of cystatin C

Methods were first developed with recombinant cystatin C that was a gift from Dr. Anders Grubb (University of Lund, Lund, Sweden). Preparation of carboxymethylpapain–Sepharose affinity resin was conducted as described originally [16] except that CNBr-Sepharose was purchased in activated form. A preparatory step with Q-Sepharose was generally employed, because the alkaline isoelectric point of cystatin C offered a rapid and efficient way to remove much of the contaminating proteins. Samples were diluted 1 : 1 with 0.25 M bicarbonate buffer, pH 9.0, and applied to Q-Sepharose resin pre-equilibrated with the same solution and contained in small disposable plastic columns (8 mm diameter). Cystatin C ran through the column without binding, leaving most other proteins behind. Fractions of appropriate size in relation to sample volume were collected and 10 μl of those fractions absorbing at 280 nm were spotted on nitrocellulose membranes and stained with anti-(cystatin C) antibody. Fractions containing cystatin C were applied to a carboxymethylpapain–Sepharose column (8 mm diameter). For small samples the resin volume was as little as 0.1 ml, whereas 0.5 ml of resin was used for samples with a volume larger than 10 ml. The sample was allowed to run through the column three times before the resin was washed extensively with saline (0.9 % NaCl). Elution procedures are detailed below.

Protein concentrations were determined either with the Coomassie Blue G250 dye-binding method or by measuring the absorbance at 280 nm. SDS/PAGE was performed in 16.5 % (w/v) polyacrylamide separation gels optimized for the resolution of low-molecular-mass peptides [17]; protein bands were stained with Coomassie Blue R250.

Microbore reverse-phase HPLC

Cystatin C was released from the carboxymethylpapain–Sepharose particles by washing with 10 % (v/v) formic acid, 45 % (v/v) ethanol and 45 % (v/v) distilled deionized water. The beads were washed twice with 0.1 ml aliquots, vortex-mixed and centrifuged. Combined supernatants (0.2 ml total) were separated by microbore reverse-phase HPLC on an Applied Biosystems 130A HPLC system equipped with a 2 mm C_4 column, with the following buffer system: A, 0.1 % (v/v) trifluoroacetic acid (TFA); B, 0.08 % (v/v) TFA in 90 % (v/v) acetonitrile.

Tryptic peptide mapping of cystatin C

Tryptic digestion was performed in 50 mM NH_4HCO_3 (pH 7.8)/5 mM n-octylglucoside for 1–3 h at 37 °C. Enzyme-to-substrate ratios were estimated as approx. 2–10 % by weight. The commonly used protocols for trypsin digestion failed (3–5 % enzyme-to-substrate ratio in 50 mM NH_4HCO_3, pH 7.8) because no peptides were observed by matrix-assisted laser desorption ionization (MALDI) MS. However, the addition of 5 mM n-octylglucoside to the normal digestion buffer (50 mM NH_4HCO_3, pH 7.8) resulted in good cleavage for reduced and alkylated cystatin as well as native protein.

On-target digestions were performed by depositing 0.5 μl of sample dissolved in 10 mM n-octylglucoside on the target, followed by 1 μl of 50 mM NH_4HCO_3, pH 7.8, and 0.5 μl of 0.01 mg/ml trypsin. The target was then kept for 1 h at 37 °C in a box containing water to lower the evaporation rate.

MS analysis

MALDI MS was performed on a Bruker Reflex instrument (Bruker-Franzen Analytic, Bremen, Germany) with the 'Laser-One' software (M. Mann and P. Mortensen, EMBL Heidelberg, Heidelberg, Germany) for data acquisition and processing. The spectra were calibrated on the matrix dimer or trimer peaks at m/z 379.093 or m/z 568.139.

In general, 4HCCA was used as matrix and sample preparation performed according to the 'sandwich' method. A thin layer of matrix was prepared by fast evaporation of 1 μl of a saturated solution of 4HCCA in 99 % (v/v) acetone/1 % (v/v) water, followed by deposition of 0.5 μl of 2 % (v/v) TFA, 0.5 μl of sample and 0.2 μl of a 4HCCA solution [15 g/l in 70 % (v/v) acetonitrile]. The samples subjected to on-target digestion were prepared by adding 0.5 μl of 2 % (v/v) TFA to the dry peptide mixture followed by 0.5 μl of 4HCCA solution [15 g/l in 70 % (v/v) acetonitrile]. Samples prepared with 4HCCA were washed by depositing a drop of 0.1 % (v/v) TFA on the dry sample and then removing the liquid carefully by touching the drop with a piece of tissue. The spectrum in Figure 3 was obtained with 2,4,6-

trihydroxyacetophenone (saturated solution in methanol) by preparing a thin layer of matrix and depositing the sample on it.

RESULTS

Cystatin from CSF

Cystatin C is a minor component of most biological fluids. The characterization of sequence heterogeneity in samples containing very small amounts of cystatin C required isolation methods that were both specific and minimized the number of handling steps. Affinity chromatography on immobilized carboxymethylpapain has previously been used for the isolation of cystatins [16,18]. Figure 1(A) shows the results of two experiments with carboxymethylpapain affinity columns to isolate CSF cystatin C from HCCAA patients. Very few proteins bound to the affinity resin, with a major component running in the expected area for the molecular mass of cystatins. Figure 1(B) shows the cystatin C amino acid sequence and tryptic peptides that can be expected from the reduced protein.

Cystatin C from CSF samples obtained either from healthy individuals or HCCAA patients was isolated by affinity chromatography on carboxymethylpapain, further purified after elution by reverse-phase HPLC and analysed by MALDI MS. The intact molecular mass spectra obtained for two representative

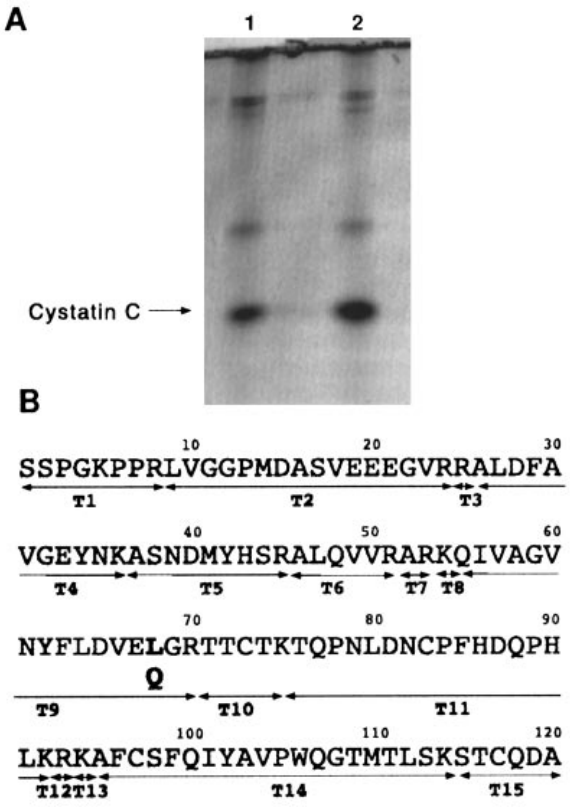

Figure 1 Efficiency of immobilized carboxymethylated papain in retaining cystatin C and tryptic peptides of the cystatin C sequence

(**A**) SDS/PAGE of proteins from CSF that bound to inactive carboxymethylpapain immobilized on Sepharose beads. The following samples were analysed: lane 1, CSF from HCCAA patients M71 and MG (pool of 1.85 ml); lane 2, CSF from HCCAA patients M83 and PH (pool of 1.45 ml). The arrowhead marks the position of the marker corresponding to cystatin C. (**B**) Tryptic peptides of the cystatin C sequence. The site of the Leu-68 → Gln mutation is indicated in bold. The expected tryptic peptides are indicated by arrows (T1 to T15).

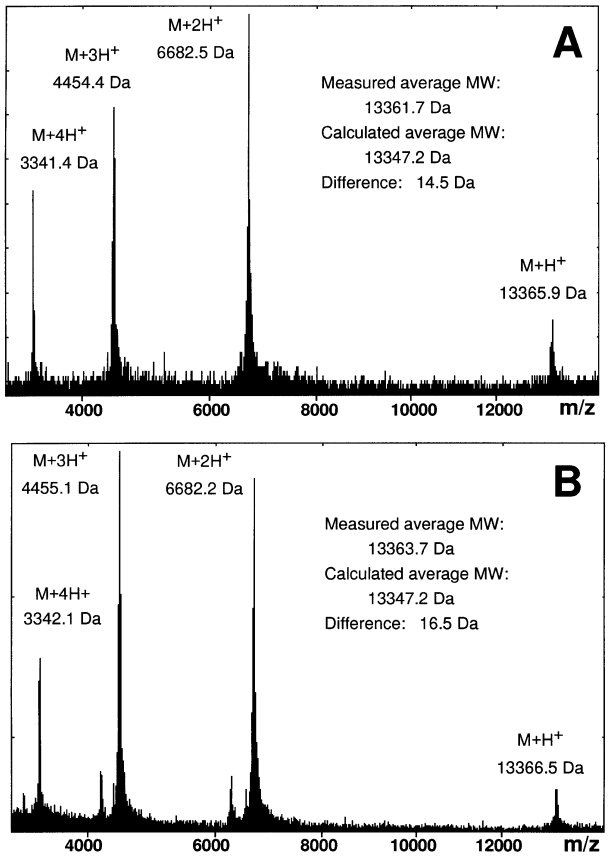

Figure 2 MALDI MS analysis of intact cystatin C isolated from CSF originating from normal subjects and an HCCAA individual

(**A**) CSF from healthy persons was collected in the presence of serine proteinase inhibitors for direct analysis. (**B**) CSF from an HCCAA patient. The average molecular masses determined from singly to triply charged peaks are listed and compared with the theoretical molecular mass.

samples are shown in Figure 2. The spectrum in Figure 2(A) is that of cystatin C isolated from pooled CSF of healthy individuals; that in Figure 2(B) was obtained with cystatin C from HCCAA patients. No sign of N-terminal truncation was found, judging by the total measured average mass. However, in Figure 2(B) the more highly charged ions were accompanied by small peaks arising from a peptide with measured average mass of 12542.3 Da. This mass coincides with the calculated mass for cystatin C sequence starting with residue Leu-9 (12540.3 Da), indicating that proteolytic breakdown during collection or storage of some of the HCCAA samples had occurred. Similarly, cystatin C isolated from urine has been found with N-terminal sequences starting at residues Gly-4, Lys-5, Arg-8, Leu-9 or Val-10 owing to endogenous proteolytic activity [19,20].

When comparing the molecular masses measured in Figure 2 with the calculated ones, a very similar mass shift was observed for both normal and HCCAA cystatin C, increasing the calculated masses by 14.5 and 16.5 Da respectively. Although this mass difference is close to that expected from the L68Q mutation, it was equally likely that the same post-translational modification, unrelated to the mutation, was present in both proteins. To explore this further, peptide mapping was performed.

Figure 3 shows the MALDI mass spectrometric tryptic peptide map obtained from reduced and alkylated CSF cystatin C from

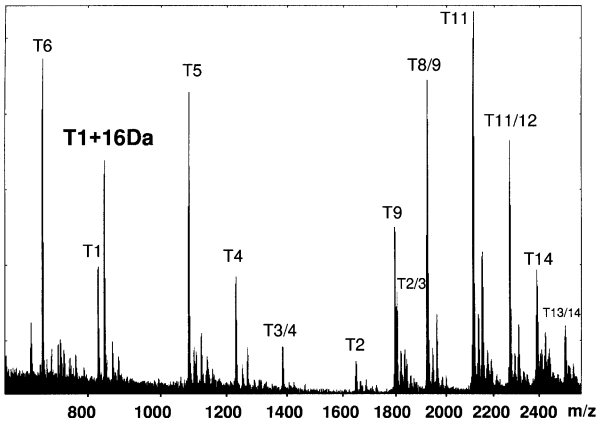

Figure 3 Tryptic peptide mapping of human cystatin C from normal CSF samples with MALDI MS

Cystatin C was isolated from CSF samples by using carboxymethylpapain–Sepharose affinity beads and digested with trypsin after reduction/alkylation and desalting on a microbore C_4 reverse-phase HPLC column. The matrix was 2,4,6-trihydroxyacetophenone. The sequence coverage resulting from the detected peptides is 89%. The observation of a peak at the mass of T1 + 16 Da locates a modification to peptide T1.

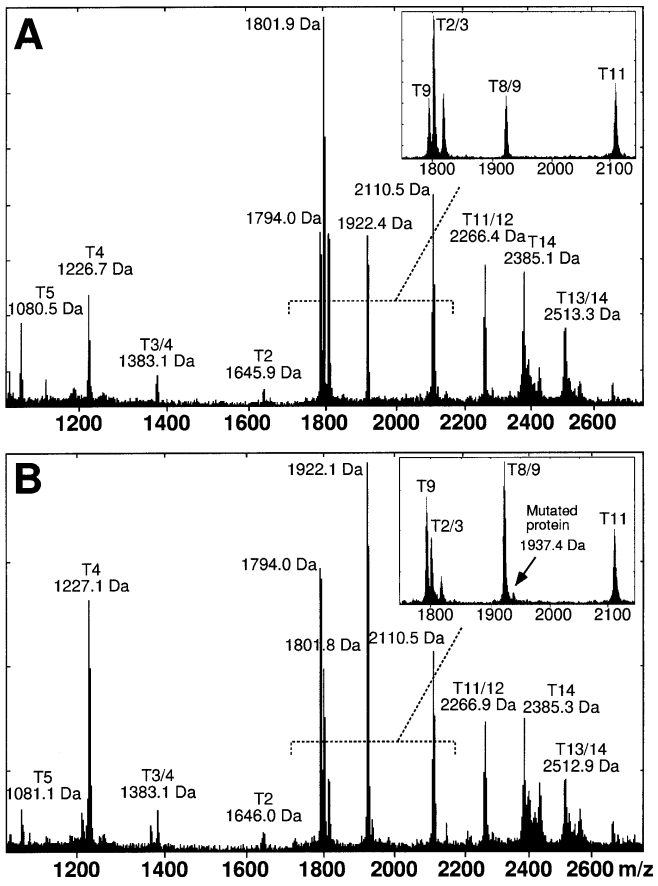

Figure 4 Analysis of cystatin C tryptic peptides originating from the CSF of HCCAA patients by MALDI MS

Cystatin C in CSF from HCCAA individuals was purified on carboxymethylpapain–Sepharose beads. The peptide masses were analysed after tryptic digestion. The matrix used was 4HCCA. (**A**) Spectrum showing no signs of mutated cystatin C in the CSF. (**B**) This sample was the only one that indicated the presence of very small amounts of mutated protein in CSF. Three other independent analyses were negative.

a healthy person. For the reduced protein, eight of the fifteen expected tryptic peptides plus a number of incompletely digested fragments (labels as in Figure 1B) were observed, which represents a sequence coverage of 89%. A sequence coverage of 100% could be obtained if the peptides resulting from the digestion of unreduced protein were also considered (results not shown). The observation of an additional peak at the mass of T1 + 16 Da indicated that Pro-3 was hydroxylated and explained the mass shift observed for the intact CSF cystatin C samples (Figure 2). Pro-3 hydroxylation was subsequently confirmed by N-terminal sequencing (results not shown). The proline-to-hydroxyproline ratio was found to be approx. 1:1.5 (approx. 60% hydroxylated protein), which agrees well with the original sequencing results for cystatin C from urine of patients with renal failure [21]. Although the hydroxylation at Pro-3 was not noted in N-terminal sequences previously determined for CSF cystatin C samples from an HCCAA individual or normal subjects [9], the present study confirmed that CSF cystatin C is in fact hydroxylated to a similar level as urinary cystatin C.

Figure 4 shows the mass spectrum obtained after the tryptic digestion of CSF cystatin C from an HCCAA-affected individual. The search for the L68Q mutation in the tryptic peptide map was favoured by very strong signals for peptide T9 and the incompletely digested fragment T8 + T9, both carrying residue 68. In most of the CSF samples analysed from HCCAA patients (a total of three), no signal corresponding to a mutated peptide was detected (Figure 4A). However, one HCCAA sample out of four apparently contained an extremely small amount of the mutated form. As seen in Figure 4(B), the evidence for this is a very small signal associated with the T8 + T9 peak and might represent 1–2% of the total cystatin C. Peak T9 overlaps with peptide T2 + T3, thus masking the possible presence of corresponding satellite peak. These results indicated that the mutated form of cystatin C was practically absent from the CSF.

Cystatin from monocyte cultures

Monocyte culture samples, both culture media and lysed cells, were passed through a shallow layer of Sephadex G-25 to collect any insoluble aggregated material if present, followed by a Q-Sepharose anion-exchange column that retained most contaminating proteins. The cystatin C in flow-through fractions was then captured by carboxymethylpapain immobilized on Sepharose beads. All the Sephadex G-25 and carboxymethylated papain resins were washed with elution solvent; the eluted material was checked for the presence of cystatin C by reverse-phase HPLC followed by trypsin mapping using MALDI MS.

Figure 5 shows examples of reverse-phase HPLC separations. Trypsin digestions of all samples from the fractions indicated in Figure 5 with arrows were performed on the target (see the Materials and methods section) to minimize sample losses. Protein material eluted from immobilized carboxymethylated papain was found to contain cystatin C except for the HCCAA cell sample, in which the cystatin C was recovered from the Sephadex G-25 instead of the papain resin. The average molecular mass of the intact cystatin C isolated from normal cell culture medium (Figure 5A, fraction 3) was determined as 13344 Da (results not shown), which is close to the sequence mass of the unhydroxylated intact cystatin C (13347 Da). An overlap of protein peaks could be recognized in the HPLC traces of Figure 5 that predictably gave rise to some unexpected peptides in the tryptic maps, including those for cystatin B.

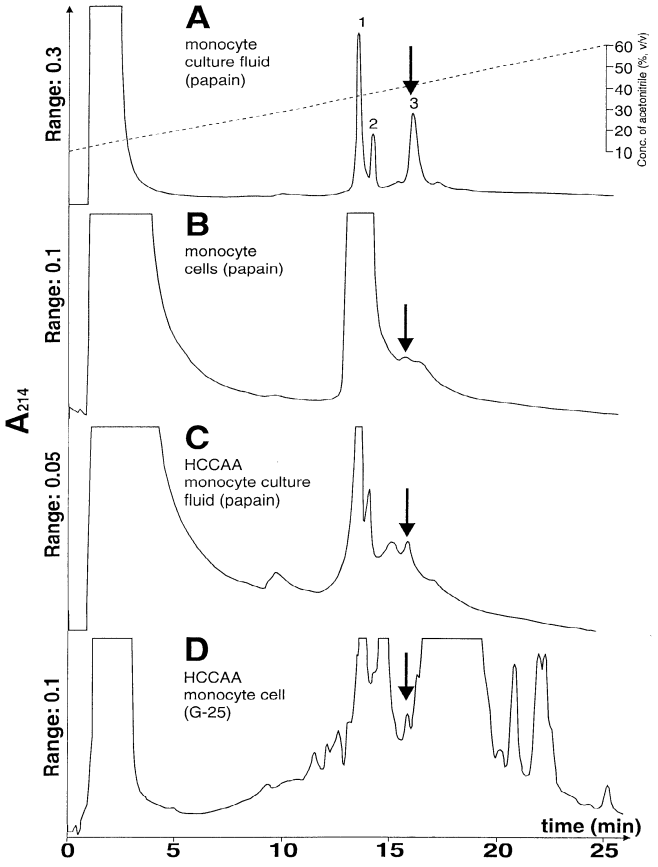

Figure 5 Reverse-phase HPLC separation of proteins from monocyte cultures that bound to carboxymethylated papain immobilized on Sepharose beads

The material applied to the reverse-phase HPLC column was recovered from carboxymethylpapain–Sepharose beads [or Sephadex G25 precolumn in (**D**)] by washing with 45% (v/v) ethanol/45% (v/v) water/10% (v/v) formic acid. The microbore C_4 reverse-phase HPLC column was eluted with an acetonitrile/TFA gradient. Absorbance was monitored at 214 nm. (**A**) Normal monocyte culture medium; (**B**) lysed monocytes; (**C**) HCCAA monocyte culture medium; (**D**) lysed monocytes from HCCAA patients.

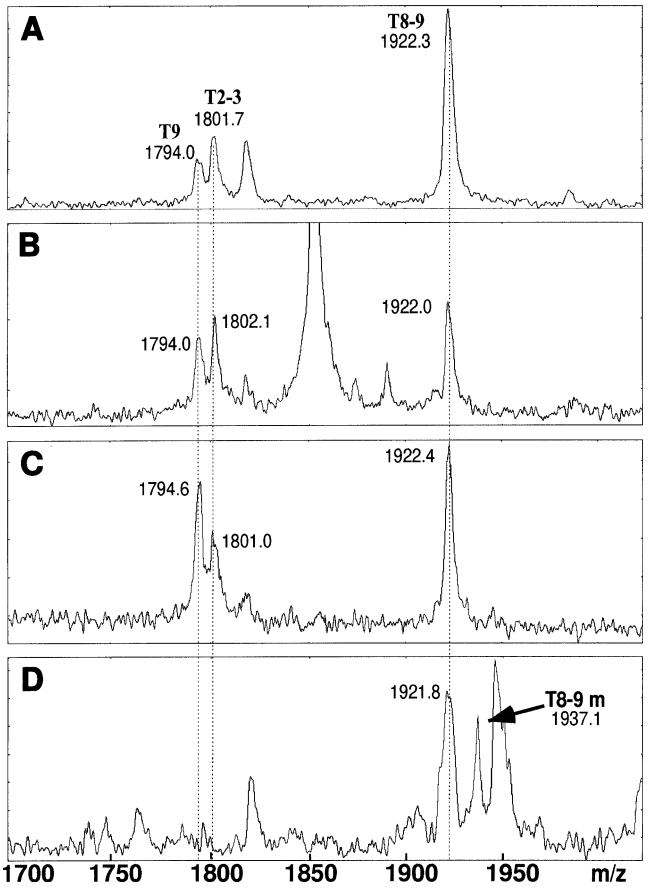

Figure 6 Tryptic peptide maps of cystatin C from monocytes acquired by MALDI MS

Monocytes from HCCAA patients and healthy individuals were cultured before the isolation of cystatin C by papain-affinity chromatography and reverse-phase HPLC as described in the legend to Figure 5. (**A**) Normal monocyte culture medium, fraction 3; (**B**) normal lysed monocytes, fraction 2; (**C**) HCCAA monocyte culture medium, fraction 4; (**D**) HCCAA lysed monocytes, protein recovered from Sephadex G25 beads.

Figure 6 shows the mass spectra obtained after tryptic digestion of the four samples shown in Figure 5 (arrows). Only the mass range between 1700 and 2000 Da is represented, where peptides T9, T8 + T9 and the potentially mutated equivalents (with 15 Da higher mass) are expected. The peptide map obtained with fraction 3 from the reverse-phase HPLC separation of normal cell medium (Figure 5A) showed that only approx. 10–20% of the cystatin C isolated was indeed hydroxylated (m/z area not shown in Figure 6A), and indicated that the protein secreted from monocytes was hydroxylated to a much lesser extent than CSF or urinary cystatin C.

The mass spectra in Figure 6 showed no evidence for the presence of the mutated cystatin C variant in extracellular fractions recovered from the culture supernatants (Figures 6A and 6C) nor in normal monocytes (Figure 6B). However, direct evidence for the exclusive association of the L68Q cystatin C mutant with HCCAA monocytes was obtained (Figure 6D). In the spectrum in Figure 6(D) a peak at mass 1937.1 Da is observed in addition to that of the T8 + T9 peptide at 1921.8 Da. The former value is in excellent agreement with the calculated molecular mass (1937.4 Da) of the mutated tryptic fragment T8 + T9.

ELISA measurements were performed before and after the carboxymethylpapain affinity step, to gain information about the efficiency of the cystatin binding to the resin. As summarized in Table 1, retention by the papain–Sepharose was more than 70% for the HCCAA monocyte culture supernatant (6.4 ng/ml before and 1.8 ng/ml after the column) and well in excess of 80% for cystatin C found in the lysed HCCAA cells (6.4 ng/ml before and less than 1 ng/ml after the column). If the mutated form of cystatin C were present, it would constitute one-half of the immunoreactive material. Because our analysis covers a significantly higher proportion of the total cystatin C from the original samples than 50%, the results obtained here should be qualitatively the same as if all the immunoreactive material had been analysed. ELISA results also showed that a somewhat higher proportion (31–47%) of the total immunoreactive cystatin C in normal samples did not bind to the immobilized carboxymethylpapain. Saturation of the carboxymethylpapain resin with cystatin was not the reason for this phenomenon because no additional cystatin C was bound when the first effluent was passed through a second column containing fresh carboxymethylpapain–Sepharose. The most obvious explanation for this lack of binding affinity would be that many of the native cystatin

Table 1 Quantification of cystatin C in samples from monocyte cell cultures

Immunoassay by ELISA was performed after a Sephadex G25 (0.8 cm × 3 cm) chromatography step to test for possible retention of aggregates and then after each of two consecutive carboxymethylpapain–Sepharose (0.8 cm × 0.8 cm) steps to check for saturation of the binding capacity of the affinity resin. Results are means of three determinations for each sample. Abbreviation: CMP, carboxymethylpapain.

	Cystatin C (ng/ml)				
Source	Initial	After Sephadex G25	After first CMP column	After second CMP column	Recovered for further analysis (%)
Normal lysed cells	13.3	10.0	4.9	4.9	63
Normal supernatant	17.0	13.0	5.4	5.4	68
HCCAA lysed cells	8.4	6.4	< 1.0	< 1.0	88
HCCAA supernatant	6.4	6.4	1.8	1.8	72

Table 2 Quantification of cystatin C in samples from CSF

Immunoassay by ELISA was performed after an initial Sephadex G25 chromatography step to test for possible retention of aggregates and then after two consecutive carboxymethylpapain–Sepharose steps to check for saturation of the binding capacity of the affinity resin. Results are means of triplicate determinations. The Sephadex G25 step was by-passed for HCCAA CSF. Abbreviation: CMP, carboxymethylpapain.

	Cystatin C (μg/ml)				
Source	Initial	After Sephadex G25	After first CMP column	After second CMP column	Recovered for further analysis (%)
Normal CSF	3.58	3.13	1.35	1.10	69
HCCAA CSF	2.5* (range 1.7–3.5)	–	0.02	0.02	99

* Published values.

C molecules were already interacting specifically with other proteins. Table 2 shows ELISA results for CSF samples, in which recovery of cystatin C was at least 70 %. Generally, 4–40 pmol of cystatin C was recovered for further analysis by trypsin digestion and MS.

DISCUSSION

The cause of Icelandic HCCAA is linked to the presence of amyloid fibrils in blood vessels found in the central nervous system [12,22,23]. These amyloid fibrils are composed of a mutated and truncated variant of the proteinase inhibitor cystatin C that eventually accumulates sufficiently to cause haemorrhage and death. The present study has provided the first evidence to support previous suggestions that the mutated form of cystatin C is not present in extracellular fluids but remains associated with the cells, either intracellularly or possibly imbedded in the outer membrane.

Suggestions of how the mutation might affect normal cystatin C function have focused on the increased tendency of L68Q cystatin C to aggregate spontaneously at physiological temperatures [8]. Because dimeric L68Q cystatin C is ineffective as a proteinase inhibitor the extracellular inhibitory potential would decrease, leading to an enhancement of localized proteolytic activity.

Our previous results showed that cultured monocytes from individuals carrying the cystatin C mutation secreted immunoreactive cystatin C less efficiently than monocytes from healthy controls. This was coincident with an increased accumulation of intracellular cystatin C in monocyte cultures from the HCCAA patients [6]. The finding that cystatin C concentrations in CSF of HCCAA patients were only one-third of normal values [5] also gave a strong indication that the mutated cystatin C was poorly secreted and accumulated in cells because of abnormal metabolism. Analysis of cystatin mRNA levels in monocytes cultivated from HCCAA individuals have shown that transcription from the normal and mutated alleles proceeds at equal rates [7]. On the evidence presented here, it is the L68Q cystatin C that accumulates inside cells, eventually leading to the formation of aggregated fibrillar material.

In addition to carrying the Leu-68 to Gln-68 mutation, the main cystatin C protein component of amyloid fibrils was also found to be truncated with ten of the N-terminal residues missing [3,10]. It is still unclear what role, if any, this truncation has in promoting amyloid formation, and at what stage the cleavage takes place in the process [3,9]. Cystatin C from HCCAA individuals has not been found to be N-terminally truncated by employing either isoelectric focusing and Edman sequencing of CSF cystatin C [9], Western blotting and immunostaining after SDS/PAGE of monocyte cystatin C [6] or analysis by MS (present study). N-terminally truncated variants were not generally detected in fresh CSF or monocyte samples, but some cystatin C devoid of residues up to and including Arg-8 was recovered by carboxymethylpapain binding in a few CSF samples subjected to long-term storage. Although an important interaction between human cystatin C and carboxymethylpapain is provided by the sequence lying N-terminally to Gly-11 [11], the inhibitory mechanism does not normally involve cleavage of the inhibitor [24], and human cystatin C is still inhibitory towards papain with a K_i in the nanomolar range after removal of the first ten residues by leucocyte elastase [11,25]. The absence of truncated cystatin C in our samples provides evidence to suggest that truncation occurs at a late stage in the formation of the amyloid fibrils. The truncation at Gly-11 as observed in cystatin C amyloid fibrils [3,10] might therefore require the denaturation of cystatin C to precede fibrillar formation [13].

In the present study we have presented direct evidence, with the use of cell cultures, that the mutated form of cystatin C involved in amyloid fibril formation remains associated with the cells and is apparently not secreted into the culture medium. Furthermore the expression of mRNA coding for the amyloidogenic cystatin C is not affected by the pathogenic mutation [7]. Therefore our results support the previous suggestion that the decreased secretion of cystatin C and the increased intracellular concentration of cystatin C observed in monocytes isolated from HCCAA patients [6] is the direct consequence of altered properties of L68Q mutated protein molecules. It can be further suggested that the intracellular accumulation of aggregated cystatin C finally leads to rupture of the cells and that amyloid fibril formation eventually causes the destruction of the smooth-muscle cells and their replacement by L68Q protein in the media of brain arteries. Overall this would consequently result in inadequate concentrations of cystatin C to down-regulate the cysteine proteinases released from inflammatory cells that are believed to accumulate at sites of vascular damage [7]. The hypothesis that the aggressive form of vascular damage observed in HCCAA is due to this combination of effects resulting in uncontrolled and excessive tissue proteolysis [7,9] is supported by our present results.

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