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Instability of the Amyloidogenic Cystatin C Variant of Hereditary Cerebral Hemorrhage with Amyloidosis, Icelandic Type*

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A cystatin C variant with L68Q substitution and a truncation of 10 NH_2-terminal residues is the major constituent of the amyloid deposited in the cerebral vasculature of patients with the Icelandic form of hereditary cerebral hemorrhage with amyloidosis (HCHWA-I). Variant and wild type cystatin C production, processing, secretion, and clearance were studied in human cell lines stably overexpressing the cystatin C genes. Immunoblot and mass spectrometry analyses demonstrated monomeric cystatin C in cell homogenates and culture media. While cystatin C formed concentration-dependent dimers, the HCHWA-I variant dimerized at lower concentrations than the wild type protein. Amino-terminal sequence analysis revealed that the variant and normal proteins produced and secreted are the full-length cystatin C.

Pulse-chase experiments demonstrated similar levels of normal and variant cystatin C production and secretion. However, the secreted variant cystatin C exhibited an increased susceptibility to a serine protease in conditioned media and in human cerebrospinal fluid, explaining its depletion from the cerebrospinal fluid of HCHWA-I patients. Thus, the amino acid substitution may induce unstable cystatin C with intact inhibitory activity and predisposition to self-aggregation and amyloid fibril formation.

Hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I)[1] (1, 2), also called hereditary cystatin C amyloid angiopathy (3), is an autosomal dominant form of cerebral amyloid angiopathy (CAA). The amyloid deposits mainly in the cerebral and spinal arteries and arterioles and leads to recurrent hemorrhagic strokes causing serious brain damage and eventually death before the age of 40 years (1, 2). Some amyloid deposition is also present in other tissues, such as skin and lymph nodes (4). Immunohistochemical studies and sequence analysis of amyloid extracted from brain vessels of affected patients have revealed it is a variant of cystatin C (5, 6).

Cystatin C, or γ-trace, is a basic protein composed of 120 amino acid residues (7). It is a cysteine proteinase inhibitor, which inhibits the activities of papain, ficin, and the human cathepsins B, H, and L *in vitro* (8, 9). Cystatin C is found in all body fluids and tissues examined and is 5.5 times more concentrated in cerebrospinal fluid (CSF) than in plasma (10–13).

The amyloid protein isolated from leptomeninges of HCHWA-I patients is truncated cystatin C, which starts at Gly^{11} of the normal protein (5, 6, 14) (see Fig. 1 for the sequence of normal and variant cystatin C). Truncated forms of cystatin C starting at Leu^9 and Val^{10} are present in normal human urine in addition to the full-length protein (15). Removal of the NH_2-terminal decapeptide of human cystatin C decreases inhibition of papain and human cathepsins B, L, and H (16).

The variant cystatin C has an amino acid substitution (5, 6, 14) within the segment purported to contain the active site of all known cystatins (8). Comparison of the genes encoding cystatin C, isolated from normal tissue and from the brain of an HCHWA-I patient, revealed a mutation in the Icelandic gene resulting in the L68Q substitution (17). This mutation abolishes an *Alu*I restriction site in the cystatin C gene of HCHWA-I patients. Loss of this site was detected by restriction fragment length polymorphism only in HCHWA-I patients and cosegregated with the disease in every case (18). The same mutation was identified in the cystatin C gene of a patient with sporadic CAA (19). The concentration of cystatin C in the CSF of HCHWA-I patients is lower compared with normal controls (20). There are several reports of patients with cerebral hemorrhage related to CAA with low concentration of cystatin C in the CSF (21–23). Immunohistochemical examination of brain tissue obtained from two of these patients confirmed the diagnosis of CAA and identified co-deposition of cystatin C and amyloid β-protein. Since the cystatin C sequence was not determined, it cannot be ruled out that these patients had a mutation in this gene. Thus, sporadic CAA in some patients may be associated with mutations in the cystatin C gene. The frequency of these mutations is yet to be determined.

The mechanisms by which soluble precursor proteins are processed to result in the deposition of insoluble amyloid fibrils are unknown. Several observations indicate that aberrant expression or processing of the human β-amyloid precursor protein may lead to the development of Alzheimer's disease (for a review, see Ref. 24). Patients with trisomy 21 (Down's syndrome patients) overexpress the protein and tend to develop Alzheimer's disease-like pathology early in life. β-Amyloid precursor protein missense mutations within or flanking the amyloid β-protein region were identified in families with early onset familial Alzheimer's disease. While one mutation causes an increase in the amount of secreted amyloid β-protein, three other substitutions seem to lead to an increased proportion of secreted amyloid β-protein ending at residue Ile^{42} rather than Val^{40}. This finding may be pathologically relevant, since there

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[1] The abbreviations used are: HCHWA-I, hereditary cerebral hemorrhage with amyloidosis, Icelandic type; CAA, cerebral amyloid angiopathy; CSF, cerebrospinal fluid; DFP, diisopropyl fluorophosphate; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Z-Phe-Arg-AMC · HCl, benzyloxycarbonyl-phenylalanine-arginine-7-amino-4-methylcoumarin-HCl.

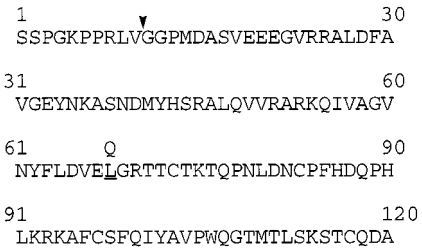

FIG. 1. **The amino acid sequence of cystatin C.** The cleavage site of the NH_2-terminal decapeptide is indicated by an *arrowhead*. The site of the HCHWA-I amino acid substitution L68Q is *underlined*.

is evidence that the longer peptide is more fibrillogenic and enhances the aggregation of shorter peptides *in vitro*. Data suggest that two other substitutions, which segregate with CAA, enhance the peptide's fibrillogenicity. Although extensive research has been conducted on the amyloidogenicity of the amyloid β-protein, relatively little is known about the processes that lead to cystatin C amyloid formation. The precise nature of the changes due to the amino acid substitution in cystatin C is unknown, although it is clear that it modifies certain physical properties of the protein, resulting in accelerated cerebrovascular amyloid deposition. Recombinant HCHWA-I cystatin C variant was shown to aggregate spontaneously to a significant extent (25). We studied the possibility that the amino acid substitution found in HCHWA-I patients may affect the production and/or secretion of cystatin C or alternatively result in abnormally processed or incorrectly folded protein. The comparison of expression, secretion, and stability of the variant cystatin C with the wild type protein showed that both are processed and secreted to a similar extent in heterologous cells. However, the secreted wild type protein is resistant to extracellular proteolysis, whereas the secreted variant cystatin C is quickly degraded both in conditioned media and CSF.

EXPERIMENTAL PROCEDURES

Establishment of Stably Transfected Cell Lines—The full-length normal or HCHWA-I variant cystatin C genes (17) contained within *Hin*dIII sites were subcloned into pRK5 vector and cotransfected with pSV40neo into human culture cell lines using Lipofectamine transfection reagent (Life Technologies, Inc.). Stably transfected cells were selected with Geneticin (G418; Life Technologies, Inc.). Overexpression and secretion of cystatin C were confirmed by Western blot analysis of cell lysates and cell culture media with anti-cystatin C polyclonal antibody (Axell). Minimally, two distinct clones of each stably transfected cell line were used for each type of experiment. All the studies described were carried out with both human astrocytic glioma U251 and human kidney 293 transfected cell lines.

Immunoblot Analysis—Cells stably transfected with the normal or HCHWA-I variant cystatin C genes were grown to near confluency by 24 h. The conditioned medium was collected and spun at 4,500 × *g* for 10 min at 4 °C to remove cellular debris. The cells were harvested in phosphate-buffered saline, pH 7.3, homogenized with a Polytron homogenizer, and centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatants of spun media and cell homogenates were aliquoted and stored at −70 °C until further use. Equal amounts of total proteins from cell homogenates or equal volumes of media based upon the relative concentration of total proteins in cell homogenates were applied to each lane to enable controlled comparison of cystatin C levels. The samples were boiled in sample buffer (1% SDS, 3% glycerol, 1.5% β-mercaptoethanol, and 20 mM Tris-HCl, pH 6.8) and separated by 10% Tricine-Tris-polyacrylamide gel electrophoresis (PAGE). For immunoblot analysis of nonreduced proteins, the samples were mixed with sample buffer without β-mercaptoethanol and were not subjected to boiling prior to separation on gels. The proteins were electrophoretically transferred (2 h at 200 mA at 4 °C) to nitrocellulose membrane (Bio-Rad) using 10 mM CAPS buffer, pH 11.0 containing 10% methanol. The membranes were blocked with 5% nonfat dry milk and 3% bovine serum albumin in 10 mM Tris, 150 mM sodium chloride, pH 7.5 (TBS) with 0.1% Tween 20 overnight at room temperature and then incubated with rabbit anti-cystatin C antibodies (1:300) (Axell) for 2 h at room temperature. Horseradish peroxidase-linked anti-rabbit IgG (Amersham Pharmacia Biotech) was used as secondary antibody (1:10,000). Membranes were washed with TBS with 0.1% Tween 20 after incubation with primary and secondary antibodies. Immunoblots were visualized on DuPont Reflection Autoradiography film by enhanced chemiluminescence as specified by the manufacturer (Pierce).

Mass Spectrometry—Cells stably transfected with the normal or HCHWA-I variant cystatin C genes or vector alone were incubated in medium without serum to near confluency by 24 h. The conditioned media and cell homogenates were harvested as described above. The supernatants of spun media and cell homogenates were dialyzed against distilled H_2O in Spectra/Por membrane (molecular weight cut-off of 3,500) (Spectrum Medical Industries), concentrated with Centricon 10 (Amicon), and subjected to matrix-assisted laser desorption mass spectrometry using the dried droplet method (26). The matrix used was α-cyano-4-hydroxycinnamic acid (Sigma). The linear, time-of-flight mass spectrometer used was custom built at the Skirball Institute at the New York University Medical Center (27).

Amino Acid Sequence Analysis—Cells stably transfected with the normal or HCHWA-I variant cystatin C genes were grown to near confluency by 24 h. The conditioned media were collected, cleared by spinning at 4,500 × *g* for 10 min at 4 °C and incubated at room temperature or 37 °C for 0 or 4 days. The cells were lysed with lysis buffer (150 mM sodium chloride, 10 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% cholic acid, 5 mM EDTA, 52 μM leupeptin, 0.1 μM pepstatin A, and 2 mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000 × *g* for 10 min at 4 °C. Cellular and secreted proteins were immunoprecipitated with anti-cystatin C antibody and Protein A-Sepharose (Amersham Pharmacia Biotech) for 4 h at 4 °C. Cystatin C was also immunoprecipitated from CSF obtained from normal individuals. The pellet was washed twice with lysis buffer and once with TBS, boiled in sample buffer, separated on 10% Tricine-Tris-PAGE, and transferred to Immobilon-P membranes (Millipore Corp.) in CAPS buffer. Membranes were stained with 0.1% Coomassie Blue R-250 (Bio-Rad) in 40% methanol, 1% acetic acid. The bands were excised and sequenced on a Procise-basic 492 microsequencer, and the resulting phenylthiohydantin amino acid derivatives were identified using on-line model 140C Microgradient Delivery System analyzer and the standard program (Applied Biosystems).

Purification of Cystatin C from Tissue Culture Media—Stably transfected cells were grown to near confluency and incubated in medium without serum for 24 h. The conditioned media were harvested as described above and dialyzed against distilled H_2O in Spectra/Por membrane (molecular weight cut-off of 3,500) (Spectrum Medical Industries). Following lyophilization, the media were suspended in 25 mM ethanolamine buffer, pH 9.4, and applied to a DEAE-Sephadex A-50 (Amersham Pharmacia Biotech) column equilibrated in the same buffer at 4 °C. Under these conditions, cystatin C did not bind to the column and eluted with the flow-through, while most of the other proteins present in the medium absorbed onto the column. Fast and mild purification conditions are essential for isolation of full-length cystatin C. Fractions were monitored by UV spectrometry at 280 nm and immunoblot analysis. Microsequence analysis was performed to confirm the isolation of full-length cystatin C. The purity of cystatin C in the samples was determined by mass spectrometry and by staining the Tricine-Tris gels with 0.05% Coomassie Blue R-250 (Bio-Rad) in 49% methanol, 5% acetic acid.

Congo Red Staining—Stably transfected cells were grown on coverslips for up to 8 days in culture. The cells were fixed in ethanol and stained for 1 h with 0.2% Congo Red dissolved in 80% ethanol saturated with sodium chloride, pH 11.0, and 2.5 mM sodium hydroxide. After being washed with 80% ethanol, slides were visualized under polarized light.

Inhibitory Activity Assay—Stably transfected tissue culture cells were grown to near confluency by 72 h. Nontransfected cells were used under the same conditions as negative controls. Conditioned media were collected and centrifuged at 4,500 × *g* for 10 min and concentrated on Centricon 3 (Amicon). Cathepsin activity was tested by a fluorometric assay on a Fluoroskan II plate reader (excitation wavelength, 383 nm; emission wavelength, 460 nm) using the synthetic methyl coumaryl substrate Z-Phe-Arg-AMC · HCl. Activity was assayed in concentrated media corresponding to 1 and 4 μg of total protein in cell lysates. The media were incubated in 200 μl of 100 mM sodium phosphate-buffered saline, pH 6.0, containing 2 mM EDTA, 2 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin A, and 20 μM Z-Phe-

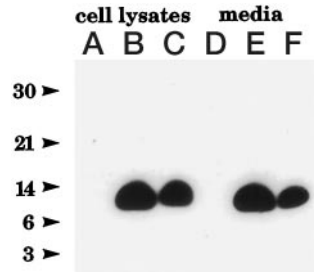

FIG. 2. **The wild type and HCHWA-I variant cystatin C expressed and secreted by stably transfected human astrocytic glioma U251 cells.** Immunoblot analysis is shown with anti-cystatin C antibody of cell homogenates (*lanes A–C*) and culture media (*lanes D–F*) of cells transfected with vector alone (*lanes A* and *D*), the wild type (*lanes B* and *E*), and variant cystatin C (*lanes C* and *F*). The *left lane* shows molecular mass standards, in kilodaltons.

Arg-AMC · HCl. All samples were analyzed with or without 5 μM L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane at 37 °C for 1–24 h. L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane is an inhibitor of cysteine proteinases including cathepsins B, H, and L (28). All treatments were carried out in duplicate.

Pulse-Chase Experiments—Stably transfected tissue culture cells were incubated with 300 μCi/ml [^{35}S]methionine/cysteine EXPRE^{35}S^{35}S (DuPont) in methionine/cysteine-free medium with 5% dialyzed serum for a pulse of 20 min at 37 °C. Following two washes with phosphate-buffered saline, pH 7.3, the cells were chased in complete medium with 150 μg/ml methionine at 37 °C for different periods. The media were collected and spun at 4,500 × *g* for 10 min at 4 °C. The cells were washed twice in phosphate-buffered saline, pH 7.3, and lysed in lysis buffer. 100 μg of total protein of cell lysates and about 200 μl of media (volume in each sample relative to amounts of protein in lysates) were immunoprecipitated with anti-cystatin C antibody and Protein A-Sepharose (Amersham Pharmacia Biotech) for 4 h at 4 °C. The immunoprecipitated proteins were washed with lysis buffer and TBS and separated on 10% Tricine-Tris-PAGE. The gels were enhanced with Amplify (Amersham Pharmacia Biotech) and exposed to x-ray films. The protein bands were scanned using Adobe Photoshop and quantitated using the NIH Image program. Relative intensity of the bands was calculated as percentage of the intensity of the protein band in cell lysates at time zero of the chase.

To test for internalization of cystatin C from culture media, nontransfected cells were incubated for 24 h at 37 °C with media of stably transfected [^{35}S]methionine/cysteine-labeled cells, which were harvested 3 h into the chase. The incubation was performed in the presence or absence of 42 μM leupeptin, a lysosomal protease inhibitor. To determine the proteolytic effects of conditioned media on cystatin C in the absence of cells, media that had been conditioned by different nontransfected cell lines for 48–72 h were mixed with media of stably transfected [^{35}S]methionine/cysteine-labeled cells, that were harvested 3 h into the chase (1:1 to 25:1 volumes). The mixtures were incubated at 37 °C in the presence or absence of protease inhibitors. As a control, media of labeled cells were mixed with medium never exposed to cells. Media of labeled cells were also mixed with normal CSF. Cystatin C was immunoprecipitated from the various mixtures and analyzed by gel electrophoresis as described above. The labeled bands were scanned using Adobe Photoshop and quantitated using the NIH Image program. Relative intensity of the bands was calculated as percentage of the intensity of the protein band at the beginning of the incubation (time zero).

RESULTS

In order to examine the production, secretion, and stability of wild type and variant cystatin C, human astrocytic glioma U251 and human kidney 293 cell lines were stably transfected with the cystatin C genes. Immunoblot analysis of cell homogenates and culture media of the transfected cells separated under reducing conditions revealed a band of approximately 14 kDa, corresponding to cystatin C (Fig. 2). Similar results were obtained under nonreducing conditions. Analysis of cell homogenates of several stably transfected cell lines, with high levels of overexpression of cystatin C, showed a band of approximately 28 kDa in addition to the 14-kDa band. The higher molecular weight band may represent a dimeric form of cystatin C. A single 14-kDa band corresponding to the monomeric protein was observed in culture media of all these cell lines.

Sequence analysis of the cystatin C forms produced and secreted by the cells transfected with the wild type or variant genes revealed only full-length cystatin C, starting at Ser^1, in cell lysates and conditioned media (Fig. 1). Even when media conditioned for 24 h by cells stably transfected with the wild type gene were harvested and incubated at 37 °C or room temperature for 4 days, no NH_2-terminal decapeptide truncation was observed. Similarly, we found only the full-length protein in CSF of normal individuals.

Parallel analysis of the inhibitory activity of the normal and variant cystatin C from tissue culture media revealed that both proteins effectively inhibited the proteolytic activity of the cathepsins secreted by the cells (data not shown). This is in agreement with previous reports demonstrating that the amino acid substitution does not significantly affect the inhibitory activity of the full-length recombinant cystatin C (25).

Analysis of cell homogenates and culture media of the cystatin C-transfected cell lines by mass spectrometry revealed proteins consistent with the mass of wild type and variant cystatin C (13,343 and 13,362 daltons, respectively). Cell homogenates also contained proteins with mass corresponding to the exact double of these molecular weights (Fig. 3). These results are consistent with the immunoblot observation that cell homogenates with high concentration of cystatin C contained a dimeric form of cystatin C.

To study the effect of cystatin C concentration on dimerization, samples containing increasing amounts of purified proteins were subjected to Western blot analysis under reducing conditions. A single 14-kDa band corresponding to the monomeric protein was observed in samples containing low protein concentration (Fig. 4). Increased concentration of cystatin C resulted in the appearance of a 28-kDa band, representing the dimeric form of cystatin C (Fig. 4). Interestingly, the HCHWA-I variant of cystatin C formed dimers at lower concentrations (75–100 ng) than the wild type protein (250–500 ng). These data demonstrate that both the wild type and variant cystatin C proteins form concentration-dependent dimers resistant to dissociation by β-mercaptoethanol and SDS.

Congo Red staining did not reveal intracellular or extracellular staining in any of the cells cultured for up to 8 days (data not shown). Therefore, it seems that neither the normal nor the variant cystatin C proteins accumulated or formed fibrils in the cells or culture media in the conditions and cell types studied.

To examine the temporal profile of cystatin C production and secretion, pulse-chase studies were carried out. Cells were metabolically radiolabeled for 20 min by the addition of [^{35}S]methionine/cysteine to the medium followed by chase for various lengths of time. Both normal and variant cystatin C were expressed and cleared from the cells at the same rate and to a similar extent (Fig. 5). The levels of the normal and variant cystatin C secreted into the culture media were similar, with maximum accumulation by about 3 h into the chase. However, while the normal cystatin C secreted into the culture media was stable for at least 5 days, the levels of the mutated protein were reduced in the media, and only traces were observed after 2 days of chase (Fig. 6). It should be noted that the cells were viable and metabolically active during the entire treatment course, and levels of other proteins remained unchanged over the same period, indicating that cellular death or general proteolysis were not responsible for the observed decrease in variant cystatin C.

To determine whether the wild type and variant cystatin C are transported via the same intracellular pathway, pulse-chase experiments were carried out in the presence of brefeldin

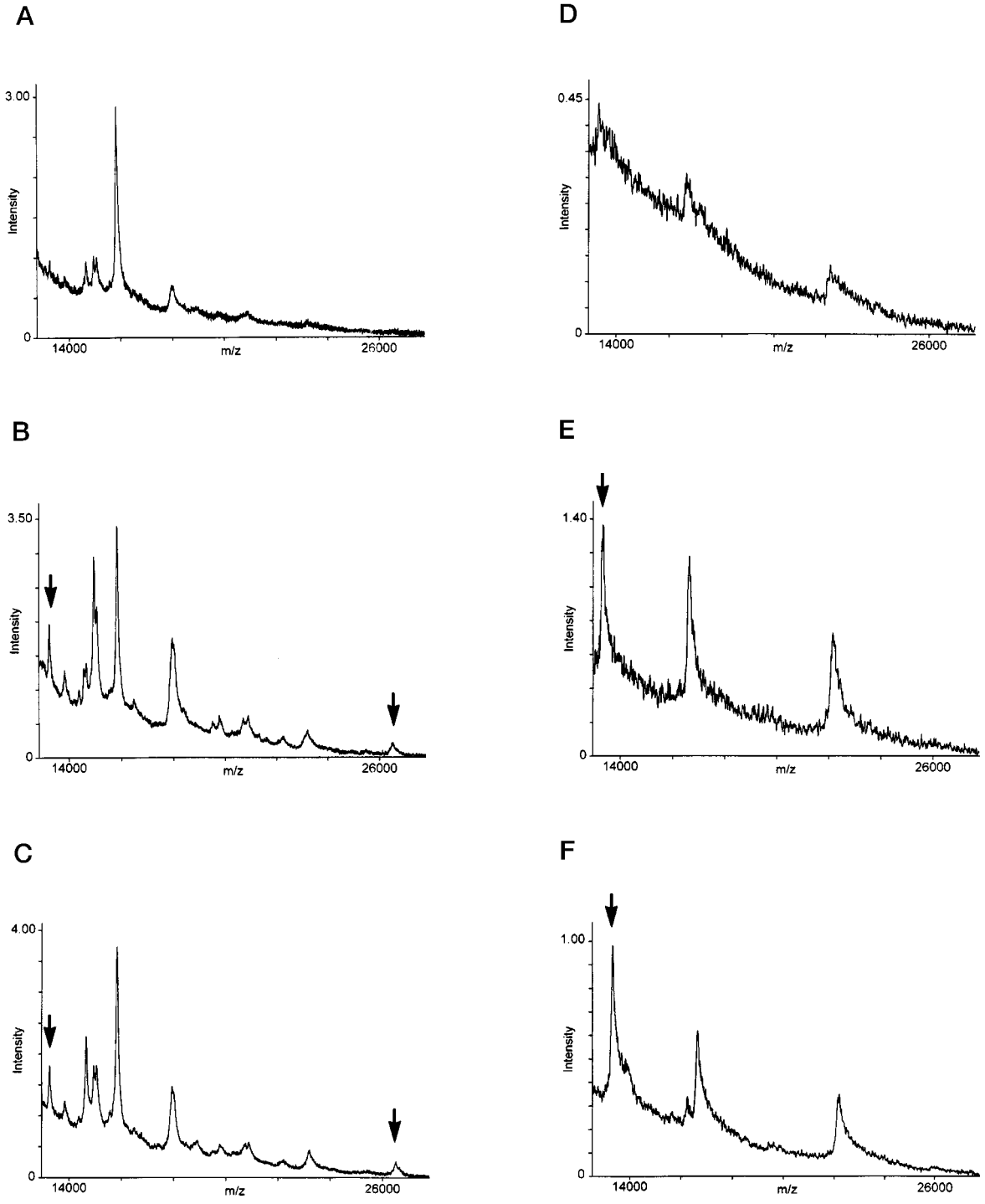

FIG. 3. **Identification of the wild type and HCHWA-I variant cystatin C expressed and secreted by stably transfected human 293 cell lines.** Matrix-assisted laser desorption/ionization mass spectrometry of cell homogenates of cells stably transfected with pRK5 vector (*A*), wild type cystatin C (*B*), and the variant cystatin C (*C*). Mass spectrometry of culture media of cells stably transfected with pRK5 vector (*D*), wild type cystatin C (*E*), and the variant cystatin C (*F*). The monomeric and dimeric forms of cystatin C are marked.

A or at low temperature. Brefeldin A blocks protein transport from the endoplasmic reticulum and causes redistribution of Golgi components to the endoplasmic reticulum (29). Similarly, low temperature greatly reduces transport through the endoplasmic reticulum. Both brefeldin A and low temperature blocked the secretion of normal and variant cystatin C into the culture media (Fig. 7). These data suggest that both the wild type and variant proteins are produced and transported by the same intracellular constitutive secretory pathway.

The reduction in the secreted variant cystatin C may be due to either uptake by cells or extracellular degradation. Since no increase in cellular protein was observed over time (Fig. 6), the variant of cystatin C does not appear to be removed by cellular uptake. To confirm this observation, nontransfected cells were incubated with [^{35}S]methionine/cysteine-labeled media of cells stably transfected with the normal or variant genes. No internalized cystatin C was observed. Since the majority of the internalized proteins are degraded in lysosomes, the experi-

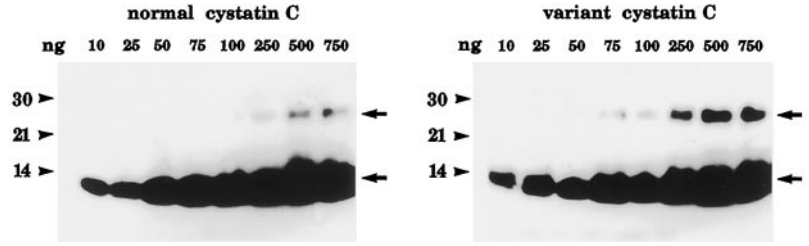

FIG. 4. **Dimerization of cystatin C depends on protein concentration.** Immunoblot analysis with anti-cystatin C antibody of the wild type and variant cystatin C proteins purified from culture media of stably transfected 293 cells. The *arrows* denote the monomeric and dimeric proteins. The amount of purified protein in each lane is indicated. The *left lane* shows molecular mass standards, in kilodaltons.

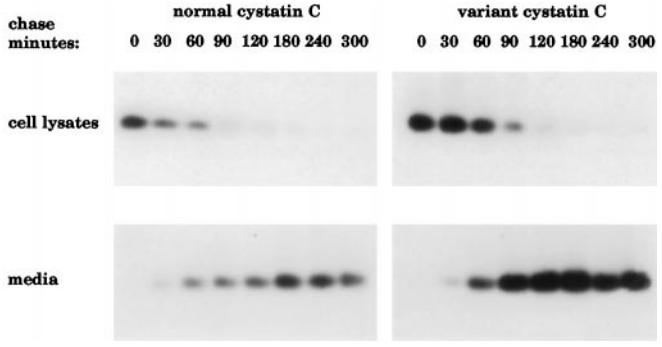

FIG. 5. **The temporal profile of cystatin C expression and secretion by human tissue culture cells stably transfected with the wild type or HCHWA-I variant cystatin C genes.** Cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates and media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films.

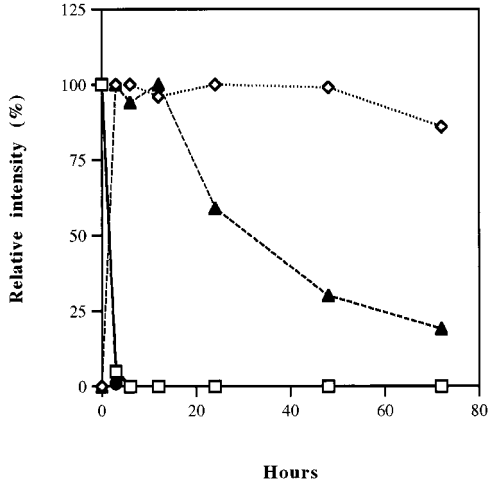

FIG. 6. **The variable stability of the secreted cystatin C in culture media.** Stably transfected human tissue culture cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates and media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as percentage of the intensity of the protein band of cell lysates at the beginning of the chase (time 0). The results from a single representative experiment are presented. Less than 10% variation was observed between experiments. □, normal cystatin C in cell lysates; ◇, normal cystatin C in medium; ●, variant cystatin C in cell lysates; ▲, variant cystatin C in medium.

ment was performed in the presence of leupeptin, a lysosomal serine and cysteine protease inhibitor (30). No internalized cystatin C was observed under these conditions (data not shown).

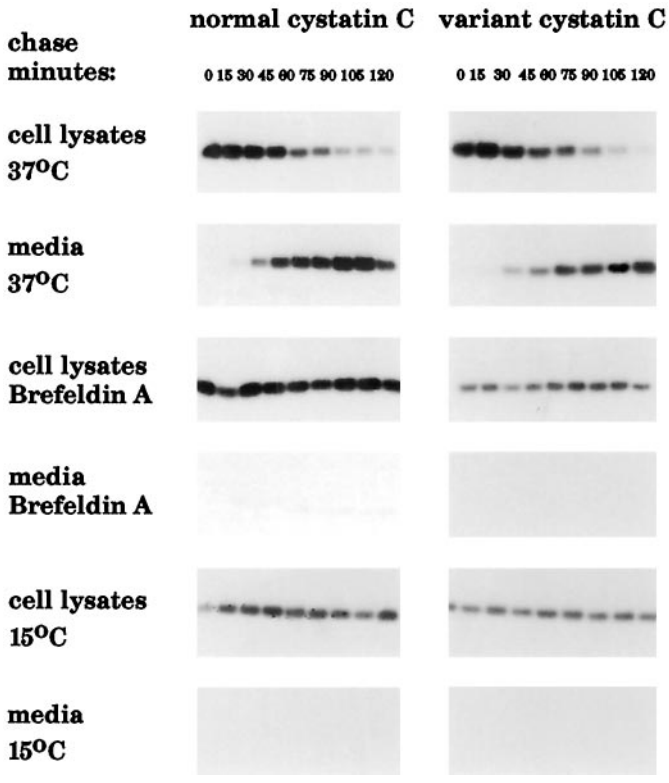

FIG. 7. **Effect of brefeldin A or low temperature block on secretion of normal and variant cystatin C.** Pulse-chase experiments in the presence of brefeldin A or chase at 15 °C. Cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for different time periods. Cell lysates and media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films.

To determine whether the variant of cystatin C is removed from the culture media due to proteolytic degradation, we studied the effects of conditioned media on cystatin C in the absence of cells. Media which had been conditioned by different non-transfected cell lines were mixed with media containing labeled cystatin C. The mixed media were examined for levels of cystatin C after incubation at 37 °C for different time periods. The level of wild type cystatin C did not show significant change when conditioned media containing labeled cystatin C were incubated for up to 4 days with 293 or U251 conditioned media. However, in similar experiments, the level of variant cystatin C was reduced by 50% within a day, and only 10–15% was detected thereafter (Fig. 8*A*). Immunoblot analysis with anti-cystatin C antibody of conditioned media of human kidney 293 cells stably transfected with the normal or variant cystatin C and incubated for different time periods at 37 °C confirmed the stability of the normal protein and disappearance of the variant protein from conditioned media in the absence of cells. Only the

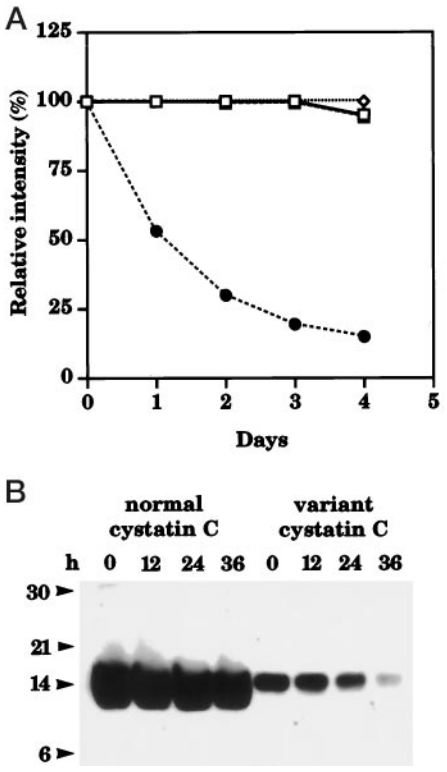

FIG. 8. **The HCHWA-I variant of cystatin C is degraded by conditioned culture media.** *A*, conditioned media of human kidney 293 cells were mixed with media containing labeled cystatin C. The media mixes were incubated for different time periods at 37 °C. Media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films. The protein bands were scanned and quantitated. The relative intensity of the bands was calculated as percentage of the intensity of the protein band at the beginning of the incubation (time zero). The results from a single representative experiment are presented. Less than 10% variation was observed between experiments. □, normal cystatin C in conditioned medium; ◇, variant cystatin C in control medium; ●, variant cystatin C in conditioned medium. *B*, immunoblot analysis with anti-cystatin C antibody of conditioned media of human kidney 293 cells stably transfected with the normal or variant cystatin C, incubated for different time periods at 37 °C.

14-kDa band is observed in these experiments. No specific degradation fragments of the variant cystatin C are observed (Fig. 8*B*). The levels of the variant as well as the wild type cystatin C were unchanged when the incubation was performed in the presence of control medium never exposed to cells or at room temperature. Protease activity capable of degrading variant cystatin C was also found in CSF of normal individuals and in media conditioned by many other cell types. However, a very low level of activity is secreted by cells of megakaryocytic and neuronal origins (Table I).

To characterize the enzyme activity responsible for the degradation of the variant cystatin C, proteinase inhibitors (representative of the four major classes of proteinases) were used individually or as a mixture (Table II). The proteinase activity was not affected by metal-chelating agents, inhibitors of thiol proteases, aspartyl proteases, aminopeptidases, and other lysosomal hydrolases. While cysteine protease inhibitors partially inhibited the activity, inhibitors of trypsin and numerous serine proteases did not inhibit the enzyme activity. In contrast, the serine proteinase inhibitor, diisopropyl fluorophosphate (DFP), blocked the degradation of variant cystatin C. The level of inhibition increased with increased concentration of DFP (Fig. 9). While 0.1 mM DFP inhibited more than 80% of the protease activity in human kidney 293 cells medium and 70%

TABLE I
The proteolytic activity present in conditioned media of different cell lines

Conditioned media were mixed with media containing labeled variant cystatin C for 72 h at 37°C. Media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as the percentage of the intensity of the protein band formed in the presence of medium not conditioned by any cells (mean ± S.D.; n = 3–5). No degradation was observed when experiments were performed with wild type cystatin C.

Source of protease	Relative intensity
Unconditioned medium	100 ± 0
CSF	45 ± 17
Human kidney 293	23 ± 18
Monkey kidney COS-1	12 ± 16
Mouse connective tissue L929	14 ± 20
Human astrocytoma U251	20 ± 17
Human monocytes THP-1	21 ± 16
Rat skeletal muscle L6	22 ± 20
Bovine arterial endothelium	40 ± 3
Human macrophages RAW264.7	46 ± 15
Human microglia N13	31 ± 10
Human megakaryocytes DAMI	97 ± 5
Human megakaryocytes MEG01	74 ± 10
Human megakaryocytes CHRF288	90 ± 13
Human neuroblastoma IMR-32	66 ± 32
Human neuroblastoma CHP-126	80 ± 24
Human neuroblastoma NT2	98 ± 2
Human neuroblastoma SHSY5Y	100 ± 0
Mouse neuroblastoma CCL-131	100 ± 0

in cerebrospinal fluid, the same concentration of inhibitor had only about a 40% effect on the protease activity in human astrocytic glioma U251 cell line medium. A higher concentration of DFP (1 mM) is necessary to reach almost 80% inhibition of the protease activity in the U251 cell line medium. These data suggest that a serine protease is involved in the degradation of the variant cystatin C, leading to reduction of its concentration in CSF of HCHWA-I patients.

DISCUSSION

HCHWA-I is characterized by accelerated amyloid deposition in the cerebral and spinal vessel walls leading to hemorrhagic strokes early in life (1, 2). The main component of the amyloid deposited in the brain of HCHWA-I patients is cystatin C with an amino acid substitution, L68Q (5, 6, 14). The events leading from the mutation to amyloid formation and deposition, to cerebral hemorrhage, and to specific decrease in cystatin C level in the CSF are not known. In order to understand the consequences of the amino acid substitution, we studied cell lines stably overexpressing the normal or the variant genes. While a cell of origin for amyloid fibrils is not apparent, it has been proposed that cystatin C in serum and CSF is synthesized and secreted by monocytes and glial cells, respectively (31, 32). Furthermore, monocytes/macrophages and glial cells have been implicated in the development of many kinds of amyloidosis (31–35). Therefore, human astrocytic glioma cell line U251 was used for permanent overexpression of the wild type and the mutated cystatin C. Human kidney 293 cell line was used to test whether the results obtained are cell type-specific. No difference between the two cell types was observed.

The cystatin C isolated from the leptomeninges of HCHWA-I patients was found to be NH_2-terminally truncated, starting at Gly^{11} (5, 6, 14). Amino-terminal sequence analysis of the cystatin C forms produced and secreted by the cells transfected with the wild type and mutated genes revealed only full-length cystatin C, starting at Ser^1. The cystatin C synthesized by monocytes of carriers of the mutation is also full-length, since it comigrated electrophoretically with native cystatin C (36).

TABLE II
Effect of protease inhibitors on degradation of cystatin C

Medium conditioned by 293 cells was mixed with media containing labeled variant cystatin C. The media mixes were incubated for 72 h at 37°C with different protease inhibitors. Media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as the percentage of the intensity of the protein band formed in the presence of medium not conditioned by any cells (100%) (mean ± S.D. n = 3–5). No degradation was observed when experiments were performed with labeled wild type cystatin C.

Inhibitors	Concentration	Relative intensity
No inhibitor		33 ± 13
Inhibitors of metalloproteinases		
EDTA	5 mM	35 ± 3
EGTA	10 mM	25 ± 9
1,10-Phenanthroline[a]	2 mM	32 ± 5
4,7-Phenanthroline	3 mM	45 ± 10
Inhibitor of aspartylproteinases		
Pepstatin A	10 mM	33 ± 6
Inhibitors of thiolproteinases		
PCMBS[b]	0.5 mM	38 ± 1
E-64[c]	1 mM	49 ± 12
Cystatin A	0.1 mg/ml	43 ± 7
α-Macroglobulin	1 mg/ml	37 ± 1
Inhibitors of amino/carboxyproteinases		
Bestatin	1 mM	28 ± 11
Captopril	1 mM	28 ± 7
Inhibitors of serineproteinases		
Benzamidine	2.5 mM	37 ± 1
Phenylmethylsulfonyl fluoride	2 mM	37 ± 1
Aprotinin[d]	1 mg/ml	22 ± 12
TLCK[e]	0.1 mM	38 ± 0
TPCK[f]	1 mM	36 ± 11
Leupeptin	0.2 mM	37 ± 1
Soybean trypsin inhibitor	0.5 mg/ml	33 ± 8
DFP	10 mM	100 ± 0

[a] 4,7-Phenanthroline is an inactive isomer of 1,10-phenanthroline.
[b] *p*-Chloromercuribenzenesulfonic acid.
[c] *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane.
[d] Aprotinin used at 22 trypsin inhibitory units/mg of protein.
[e] N^{α}-*p*-Tosyl-L-lysine chloromethyl ketone).
[f] *N*-Tosyl-L-phenylalanine chloromethyl ketone.

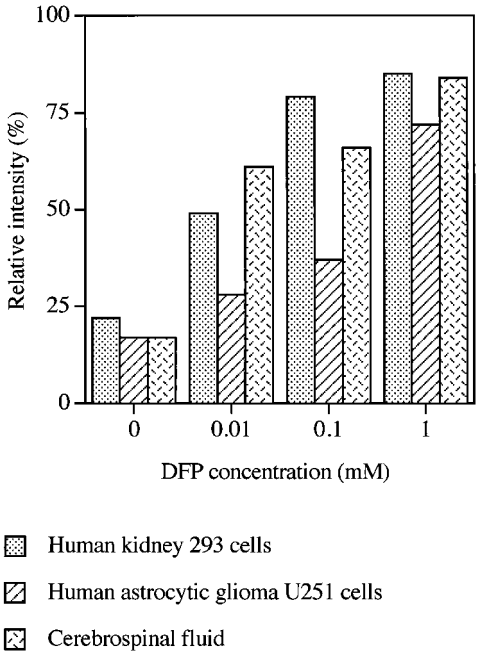

FIG. 9. **The serine proteinase inhibitor, DFP, blocks the protease activity present in conditioned media and in cerebrospinal fluid.** Conditioned media of human kidney 293 or human astrocytic glioma U251 cells or cerebrospinal fluid were mixed with media containing labeled variant cystatin C. The media mixes were incubated for 72 h at 37 °C with different concentrations of DFP. Media proteins were immunoprecipitated with anti-cystatin C antibody, separated on Tricine-Tris-PAGE, and exposed to x-ray films. The protein bands were scanned and quantitated. Relative intensity of the bands was calculated as percentage of the intensity of the protein band at the beginning of the incubation (time zero).

Moreover, we found only the full-length protein in CSF of normal individuals. The absence of truncated cystatin C in CSF is in accord with results of isoelectric focusing and sequence analysis of cystatin C in the CSF of HCHWA-I patients (37, 38). The protease responsible for the truncation of the NH_2-terminal 10 amino acids has not been identified. It was proposed that a serine proteinase with specificity of elastase could be involved, since it is capable of generating cystatin C lacking the first 10 amino acids *in vitro* (5, 6, 14, 16). The cleavage of the NH_2-terminal decapeptide of the amyloid may occur *in vivo* during long term exposure of the protein to proteases before, during, and/or after amyloid fibril formation.

The wild type and variant cystatin C were constitutively secreted by human glial and kidney culture cells stably transfected with the cystatin C genes, in agreement with previous studies in a variety of cell cultures (39–41). Both had a relatively short intracellular half-life (about 73 min). The mature, active form of cystatin C was secreted as a monomer into the culture medium. Dimeric forms of the protein were found in homogenates of several cell lines overexpressing cystatin C. Some degree of intracellular dimerization was previously identified by chromatography on AcA-44 column of lysates of Chinese hamster ovary cells transfected with the wild type cystatin C cDNA (42). *In vitro* dimerization directly correlated with cystatin C concentration in the sample. The variant cystatin C formed dimers at concentrations lower than those necessary for dimerization of the wild type protein. Upon dimerization, cystatin C looses its cysteine protease inhibitory activity, indicating that the association may involve the active site (43). Cystatin C may acquire a nonfunctional dimeric form while in specific cellular compartments and upon secretion dissociate into its active monomeric form.

It was previously shown that recombinant normal cystatin C dimerizes when exposed to denaturing agents, low pH, or high temperature (43). Although these conditions are similar to those leading to protein unfolding, it was demonstrated that cystatin C dimers are formed by association of native, properly folded proteins (43). The dimers and aggregates of the variant recombinant protein were formed at normal body temperature, nearly 25 °C lower than that needed for the wild type cystatin C dimerization (25). The most likely site for physiological dimerization would be in acidic cellular compartments. The pH in lysosomes is in the range of 4.6–5.0, which corresponds to the pH where recombinant cystatin C dimerized to some extent *in vitro*. Consistent with this, a possible role of lysosomes in amyloid fibril formation has been proposed (44, 45). The lysosome could be the site of partial protein denaturation, yielding an intermediate that can associate into amyloid fibrils faster than it can be degraded. Similarly, it was shown that partial denaturation of transthyretin under conditions that mimic the acidic environment of a lysosome is sufficient to effect amyloid fibril formation by self-assembly of a conformational intermediate (46).

Wild type cystatin C secreted into the culture media was stable for at least 5 days. Conversely, the level of the cystatin C variant was significantly reduced in the media in 1 day, and only traces were observed after 2 days of culture. Protease inhibitory profile showed that the serine protease inhibitor, DFP, blocked the protease activity. Thus, a serine protease is involved in the observed reduction of the variant protein. This

activity is cell line-specific, since different levels of proteolytic activity are released by many cell types. Interestingly, a significant amount of protease activity capable of degrading variant cystatin C is also present in CSF of normal individuals.

The concentration of cystatin C in the CSF of HCHWA-I patients is lower compared with normal controls (20). While accumulation of this protein in vessel walls may explain its depletion from the CSF, reduced secretion or enhanced proteolysis may be additional explanations. It was previously shown that cultured peripheral blood mononuclear cells isolated from carriers of the mutated cystatin C gene (symptomatic as well as asymptomatic) had similar levels of intracellular cystatin C but lower average quantity of cystatin C in the culture media as compared with cultured monocytes of healthy controls (36, 47). Our results suggest that the level of the variant cystatin C secreted by monocytes is reduced extracellularly over time due to a secreted serine proteinase, since we demonstrate degradation of the variant protein, but not the normal one, in medium conditioned by human monocytes (THP-1). The data suggest that the low level of cystatin C in CSF of HCHWA-I patients is due to enhanced proteolysis of the variant protein.

Most native proteins are relatively resistant to cleavage, whereas unfolded proteins are usually good substrates for proteolysis. Thus, the rate at which a protein is degraded by a protease is indicative of its stability, enabling comparison of a mutant protein with its wild type counterpart. The fraction of molecules that are unfolded varies as a function of temperature for both proteins, but the transition for the mutant occurs over a lower temperature range than that for the wild type protein. Some proteins aggregate because they are unfolded or incompletely folded and thus escape proteolysis (48). It was suggested that particular amino acid replacements in the immunoglobulin light chain associated with light chain amyloidosis disrupt domain structure and stability. The ability of these mutants to aggregate correlated with the extent to which domain stability is decreased by denaturant-induced unfolding (49). The acid stability of transthyretin variants found in familial amyloid polyneuropathy correlated with the severity of the amyloid pathology where the control wild type protein is least pathogenic and most stable (50). Amino acid substitutions responsible for amyloid formation in lysozyme amyloidosis affect the stability of the protein and its tendency to aggregate (51). These data invoke a denaturation intermediate as the precursor to amyloid fibril formation.

The HCHWA-I amino acid substitution may affect cystatin C in the same manner. Dimers of the variant recombinant protein are formed at temperatures lower than those needed for the wild type cystatin C, suggesting that the substitution lowers the transition temperature for unfolding (25). We found that the variant cystatin C formed dimers at protein concentrations lower than those necessary for the wild type protein dimerization and demonstrated enhanced susceptibility to proteolysis. Protein folding and the maintenance of a stably folded structure are usually prerequisites for activity. Since the variant cystatin C retains its protease inhibitory activity it is possible that the amino acid replacement in HCHWA-I causes a relatively small change in conformation, insufficient to significantly alter its function. Nevertheless, the substitution may alter cystatin C tertiary structure, rendering it more susceptible to proteolysis, unfolding, and aggregation. The ability of the proposed misfolded amyloidogenic intermediate to assemble into characteristic amyloid fibrils will almost certainly depend on sequence elements that become exposed after partial or complete unfolding (52).

Self-assembly and amyloid fibril formation can occur from a conformational intermediate produced during denaturation. Under partially denaturing conditions, amyloidogenic proteins appear to be able to adopt alternative conformations, which render these proteins capable of self-assembly into amyloid fibrils (53). Cystatin C is another protein where decreased stability of the mutant protein correlates with its amyloidogenic nature. It remains to be determined whether partial unfolding of the protein is necessary for fibril formation.

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