

Papers

Intracellular accumulation of the amyloidogenic L68Q variant of human cystatin C in NIH/3T3 cells

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Abstract

Aim—To study the cellular transport of L68Q cystatin C, the cystatin variant causing amyloidosis and brain haemorrhage in patients suffering from hereditary cystatin C amyloid angiopathy (HCCAA).

Methods—Expression vectors for wild-type and L68Q cystatin C were constructed and used to transfect mouse NIH/3T3 cells. Stable cell clones were isolated after cotransfection with pSV2neo. Clones expressing human wild-type and L68Q cystatin C were compared with respect to secreted cystatin C by enzyme linked immunosorbent assay (ELISA), and for intracellular cystatin C by western blotting and immunofluorescence cytochemistry. Colocalisation studies in cells were performed by double staining with antibodies against human cystatin C and marker proteins for lysosomes, the Golgi apparatus, or the endoplasmic reticulum, and evaluated by confocal microscopy.

Results—Concentrations of human cystatin C secreted from transfected NIH/3T3 cells were similar to those secreted from human cells in culture. In general, clones expressing the gene encoding L68Q cystatin C secreted slightly lower amounts of the protein than clones expressing wild-type human cystatin C. Both immunofluorescence cytochemistry and western blotting experiments showed an increased accumulation of cystatin C in cells expressing the gene encoding L68Q cystatin C compared with cells expressing the gene for the wild-type protein. The intracellularly accumulating L68Q cystatin C was insoluble and located mainly in the endoplasmic reticulum.

Conclusions—The cellular transport of human cystatin C is impeded by the pathogenic amino acid substitution Leu68→Gln. The resulting intracellular accumulation and increased localised concentration of L68Q cystatin C might be an important event in the molecular pathophysiology of amyloid formation and brain haemorrhage in patients with HCCAA.

Keywords: amyloidosis; cysteine proteinase inhibitor; cerebral haemorrhage

Hereditary cystatin C amyloid angiopathy (HCCAA) is transmitted in an autosomal dominant manner and it is characterized by deposition of amyloid in the central nervous system (CNS).¹ The clinical manifestations of HCCAA are caused by multifocal brain haemorrhages normally seen at 25–35 years of age and range from death shortly after the first stroke to several years of life crippled by multiple strokes causing paralysis and dementia.^{2,3} Although the main clinical manifestations of HCCAA are caused by amyloid in the CNS, amyloid deposits have also been detected in other organs, such as the lymph nodes, the submandibular salivary glands, and the skin.^{4,5}

HCCAA was first described in 1935 by Arnason,⁶ who based his studies on clinical symptoms and their distribution in families. Two members of the affected families underwent postmortem examinations in 1947 but histopathological investigations did not reveal the nature of the lesions in the cerebral arteries.⁷ The presence of amyloid deposits in the arterial walls of the brains of patients with HCCAA was first demonstrated in 1972.⁸

Protein sequencing has shown that the main component of the amyloid deposits extracted from brain vessels of patients with HCCAA is a variant of the cysteine proteinase inhibitor, cystatin C.⁹ Cystatin C is a low M_r protein consisting of 120 amino acid residues in a single polypeptide chain.¹⁰ It belongs to family 2 of the cystatin protein superfamily¹¹ and is found in all extracellular fluids examined,¹² with particularly high concentrations in cerebrospinal and seminal fluids.¹³ The gene encoding human cystatin C has been mapped to chromosome 20p11.2.^{14–16} The protein is synthesised as a preform containing a signal peptide of 26 amino acids for translocation into the endoplasmic reticulum, which indicates that the functions of the inhibitor are mainly extracellular.¹⁷ It is believed that the physiological role of cystatin C and other cystatins is to regulate the activity of endogenous proteinases, which are often secreted or leak from the lysosomes of dying or diseased cells. Cystatins also

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seem to have a role in the defence against microbial invasion.¹⁸

The amyloid protein extracted from patients with HCCAA differs from normal cystatin C in two respects. The first 10 amino acids of the N-terminal segment are missing¹⁹ and there is an amino acid substitution, Leu68→Gln, in the amyloid protein.^{10 17 20} It is probable that the Leu68→Gln substitution causes HCCAA because genetic studies have shown that a T→A point mutation in the codon for Leu68 in exon 2 of the cystatin C gene is found exclusively in affected members of HCCAA families, and not in healthy subjects from the same families, nor in DNA from a large group of unrelated healthy individuals.²¹ This substitution was detected as an extra 630 base pair (bp) Alu I fragment as a result of the loss of an Alu I restriction site found in the normal gene.²¹ For easy and rapid routine diagnosis of HCCAA, a polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) assay can be used.²²

The molecular mechanisms leading to amyloid deposition in patients with HCCAA are not known, although recent progress in the production of the Leu68→Gln variant of cystatin C (L68Q cystatin C) in *Escherichia coli* has allowed initial in vitro studies of the functional and physicochemical properties of the protein variant.²³ The results from these studies indicate a stability difference between wild-type and L68Q cystatin C as a possible important factor for amyloid formation, but in vitro formation of amyloid has not been shown. To elucidate whether changed intracellular processing or secretion of the cystatin C variant could be an underlying cause of HCCAA, we have studied mouse cells expressing wild-type and L68Q substituted human cystatin C genes.

Materials and methods

CONSTRUCTION OF EXPRESSION PLASMIDS

The full length human cystatin C cDNA, C6a,¹⁷ was ligated into the EcoR I site of pUC18. A clone containing the cDNA insert positioned with the 5' end directed towards the unique restriction sites of the multiple cloning region of the plasmid was selected by restriction mapping and was designated pC6a-B. The same cDNA insert, but modified at the 5' end to encode the *E coli* OmpA signal peptide instead of the natural signal peptide, has been used in the *E coli* expression plasmid pHD313.²⁴ The modified cDNA insert of pHD313 has been altered by oligonucleotide directed mutagenesis to replace the Leu68 codon by a Gln codon in plasmid pCmutH5 to create an *E coli* expression plasmid for L68Q cystatin C production.²³ A 511 bp Pst I/EcoR I fragment corresponding to the 3' half of the cDNA including the coding region for residues 54–120 was excised from pCmutH5. This fragment was ligated into pC6a-B, that had been digested with Pst I/EcoR I and dephosphorylated, to obtain plasmid pMA13 with a normal signal peptide–L68Q cystatin C encoding insert.

The expression vector pBW60 was constructed from pUC19. The human growth hormone polyadenylation signal region obtained as a Sma I/EcoR I fragment from

pHD184²⁵ was first subcloned into Sma I/EcoR I digested pUC19, followed by subcloning of the human ubiquitin promoter as a Hind III fragment from pHD184, resulting in the expression vector pBW60.

The cDNA inserts of plasmids pC6a-B and pMA13 were excised with EcoR I, rendered blunt ended with Klenow DNA polymerase, and subcloned into BamH I digested and Klenow treated pBW60, resulting in the two expression plasmids pBW69 and pBW70 expressing wild-type and L68Q substituted human cystatin C, respectively. The correct plasmid DNA inserts were verified by sequencing.

TRANSFECTION AND CLONING OF MOUSE NIH/3T3 CELLS

NIH/3T3 cells (ATCC; CRL 1658) were transfected with 20 µg pBW69 or pBW70 DNA and cotransfected with 2 µg pSV2neo DNA by the calcium phosphate coprecipitation procedure as described by Graham and van der Eb.²⁶ For each transfection, ~ 3 × 10⁵ cells were seeded in to a 5 cm Petri dish and on the next day the cells were given fresh medium and incubated for one hour. DNA precipitates were then added dropwise to the medium. After five hours the cells were washed with phosphate buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl and 3 mM KCl) and fresh medium was added. The following day the cells were trypsinised and diluted into 24 well dishes. Selection for cells with stably integrated plasmid DNA with 600 µg/ml geneticin sulphate (G418s; Sigma, St Louis, Missouri, USA) was initiated the day after trypsinisation. After one to two weeks of selection, wells containing single cell clones were selected for further propagation.

CELL CULTURES

NIH/3T3 cell clones were cultivated at 37°C in an atmosphere of 5% CO₂ in 75 cm² culture flasks (Costar, Cambridge, Massachusetts, USA), in six well plates (Falcon; Becton Dickinson Labware, Lincoln Park, New Jersey, USA) or, for microscopy, on glass coverslips (Nunc, Roskilde, Denmark). The medium used was Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, Paisley, Strathclyde, UK), antibiotics (10 U/ml penicillin and 10 µg/ml streptomycin; Life Technologies), and 200 µg/ml of active G418s (Calbiochem, La Jolla, California, USA) in the case of transfected cells.

PREPARATION OF SAMPLES FROM CELL CULTURES

Cells were plated in 100 mm Petri dishes and grown in 10 ml DMEM supplemented with 10% fetal calf serum. At semi-confluency, the medium was changed and 5 ml fresh medium was added. The cells were grown for 24 hours and then harvested. The conditioned medium was centrifuged at 300 ×g for 10 minutes to pellet non-adherent cells, then again at 10 000 ×g for 10 minutes to pellet cell debris and particulate matter, after which the supernatant was recovered and stored at –20°C. The cells in the flasks were harvested in two different ways. In one, adherent cells were washed

once with Hank's balanced salt solution (HBSS; Life Technologies) and lysed by the addition of 1 ml of 0.2% (vol/vol) Triton X-100 supplemented with a preservation cocktail (final concentration of 5 mM benzamidine hydrochloride, 15 mM NaN₃, and 10 mM EDTA). The cell lysate was then recovered by scraping. In the other protocol, the cells were washed with HBSS and then incubated for five minutes at 37°C in 5 ml of calcium/magnesium free HBSS. After this incubation, the cells became detached and were pelleted by centrifugation, resuspended in 500 µl of PBS supplemented with the preservation cocktail above, and lysed by sonication (Branson Sonifier Cell Disruptor B15; Kebo Lab AB, Mölndal, Sweden). The cell lysates were centrifuged at 10 000 ×g for five minutes and the supernatants were removed. The remaining pellet of cell debris was boiled in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (75 mM Tris/HCl, pH 7.5, containing 2% (wt/vol) SDS, 5% (vol/vol) mercaptoethanol, and the preservation cocktail above) for 30 minutes or until the pellet was dissolved completely. The samples were stored at -20°C until analysed.

POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

SDS-PAGE was performed with 15% polyacrylamide gels and the buffer system of Laemmli.²⁷ The separated protein bands were transferred electrophoretically to poly(vinylidene)difluoride membranes (Immobilon-P; Millipore, Bedford, Massachusetts, USA).

For immunodetection of blotted cellular proteins, the membrane was washed for 30 minutes in TEN (25 mM Tris/HCl buffer, pH 7.6, containing 1 mM disodium-EDTA, and 150 mM NaCl), supplemented with 3% (wt/vol) skimmed milk, and 0.1% (wt/vol) NaN₃ to block endogenous peroxidases. This was followed by three 15 minute washes with TEN containing 3% skimmed milk to saturate the membrane completely. The membrane was incubated overnight with protein A purified, polyclonal rabbit antihuman cystatin C antibodies,¹² diluted to 1 µg/ml in TEN containing 3% skimmed milk. Horseradish peroxidase conjugated secondary antibodies (Dako A/S, Copenhagen, Denmark) and enhanced chemiluminescence (ECL Plus kit; Amersham International, Amersham, Buckinghamshire, UK) were used for detection, as recommended by the manufacturer. In all immunoblotting experiments, one lane in the original SDS polyacrylamide gel was loaded with a reference sample containing 25 ng recombinant human cystatin C.²³ By analysing a dilution series of recombinant cystatin C, the detection limit of the method was found to be 1 ng of cystatin C.

Actin was used as a loading control in all experiments. Filters developed previously for cystatin C were stripped and reprobed with a mouse monoclonal anti-actin antibody (clone C4; ICN Biomedicals Inc, Aurora, Ohio, USA), diluted 1/1000 in TEN with 3% skimmed milk, and incubated for two hours.

ASSAY OF CYSTATIN C IN CULTURE MEDIA

Determinations of human cystatin C concentrations were performed by an enzyme linked immunosorbent assay (ELISA) using a polyclonal antiserum and a monoclonal antibody against human cystatin C as capturing and detection reagents, respectively, as described previously.^{28, 29} The monoclonal antibody used in the ELISA is specific for human cystatin C and shows little or no crossreaction with mouse cystatin C.³⁰ Mouse cystatin C was quantified by a similar, previously described ELISA, using biotinylated rabbit antimouse cystatin C IgG as the detecting antibody.³⁰

ASSAY OF TOTAL CELLULAR PROTEIN

Protein assays were performed in microtitre plates using the Coomassie binding method³¹ modified as described previously.³⁰

IMMUNOCYTOCHEMICAL STAINING

Fixation and subsequent steps for the intracellular labelling were performed at room temperature. Briefly, the cells were fixed with 3.7% (wt/vol) paraformaldehyde (crystalline; Sigma) in PBS for 10 minutes and washed three times with PBS. Non-specific binding sites were blocked by incubating cells with 0.2% (wt/vol) bovine serum albumin (Sigma) for 45 minutes. After blocking, the cells were incubated for two hours with primary antibodies diluted in PBS containing 0.1% (wt/vol) saponin (Sigma). Primary antibodies used were as follows: for cystatin C, either rabbit polyclonal or mouse monoclonal antihuman cystatin C IgG at 20 µg/ml; for double staining, the primary antibodies against human cystatin C were mixed with antibodies against markers of the endoplasmic reticulum (monoclonal mouse antiprotein disulphide isomerase (PDI) StressGen Biotechnologies Corp, Victoria, British Columbia, Canada),^{32, 33} the Golgi apparatus (monoclonal mouse antirat liver 58 kDa protein; Sigma),³⁴ or lysosomes (rabbit polyclonal antihuman liver cathepsin B

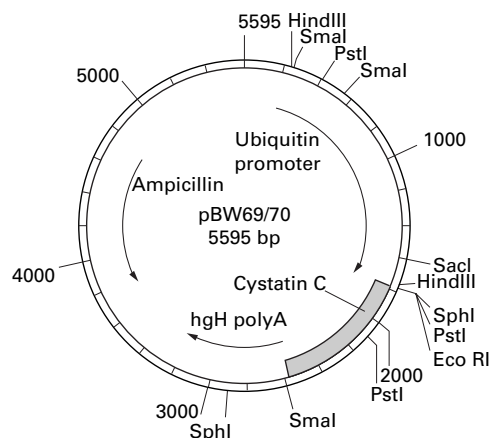


Figure 1 Expression plasmid constructs. The pUC19 derived plasmid, pBW69, contains the full length coding sequence for human cystatin C^o and the poly(A) region from the human growth hormone gene, and is under control of the human ubiquitin promoter. Plasmid pBW70 differs from pBW69 only in the codon for cystatin C residue 68, for which the HCCAA related point mutation (CTG→CAG) was introduced, resulting in the amino acid substitution Leu68→Gln in cystatin C. The positions of unique restriction sites are indicated.

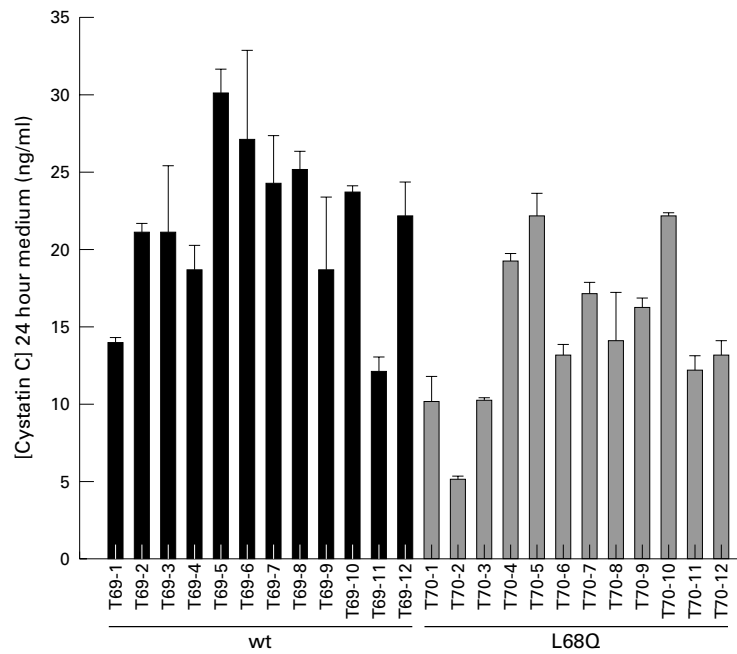


Figure 2 Secretion of human cystatin C from transfected NIH/3T3 cells. Mouse NIH/3T3 cells were transfected with the expression vectors pBW69 (black bars) or pBW70 (grey bars). For each construct, 12 cell clones were obtained after G418s selection and cultured for 24 hours at an approximate density of 10^6 cells/cm². The cystatin C concentrations in the conditioned media were measured by an enzyme linked immunosorbent assay specific for human cystatin C. The mean values from duplicate measurements are shown. wt, clones expressing the human wild-type cystatin C gene; L68Q, clones expressing the L68Q cystatin C gene.

IgG³⁵, or rabbit polyclonal antihuman placenta cathepsin D IgG,³⁶ a kind gift of Dr C Isidoro, University of Turin, Italy). In controls, primary antibodies were replaced by corresponding pre-immune sera. Depending on the primary antibodies used, the secondary antibodies were either fluorescein (FITC) labelled donkey anti-rabbit IgG or Texas red labelled donkey antimouse IgG (both from Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA); at 25 µg/ml in PBS containing 0.1% saponin and 5% (wt/vol) normal donkey serum (Jackson Immunoresearch Laboratories). The cells were again treated with 3.7% (wt/vol) paraformaldehyde for two minutes and washed three times with PBS. After rinsing with distilled water, the coverslips were mounted on slides

Table 1 Cystatin C expression and growth of transfected NIH/3T3 cells

Cell	Day	Protein (mg)	Mouse cystatin C (ng/mg protein)	Human cystatin C (ng/mg protein)
Non-transfected	1	0.012 (0.001)	6650 (907)	< 50
	2	0.050 (0.009)	4420 (155)	< 20
	3*	0.100 (0.006)	5210 (198)	< 10
	4	0.228 (0.014)	3550 (250)	< 5
T69-5	1*	0.024 (0.004)	4840 (1660)	3190 (59)
	2	0.063 (0.001)	5560 (753)	3360 (279)
	3	0.166 (0.018)	5760 (178)	3010 (109)
	4	0.379 (0.029)	4100 (464)	1710 (136)
T70-5	1	0.017 (0.002)	3857 (377)	3106 (85)
	2	0.053 (0.002)	4610 (394)	2560 (73)
	3	0.089 (0.005)	5570 (257)	2210 (30)
	4	0.188 (0.003)	3280 (140)	1360 (109)

Eight flasks of each of the transfected NIH/3T3 clones T69-5 and T70-5 were cultured at 37°C, at an initial seeding of $\sim 1 \times 10^6$ cells in 14 ml DMEM containing 10% fetal calf serum. The medium and cells in two flasks of each clone were harvested after 1, 2, 3, and 4 days, corresponding to ~25%, 50%, 75%, and 100% confluent cells, respectively. The cells were homogenised and cellular protein was quantified by a dye binding assay. Accumulation of human cystatin C and mouse cystatin C in the media was measured by enzyme linked immunosorbent assay. The mean (SD) values for duplicate measurements on the two flasks from each timepoint are given.

* Duplicate measurements from one flask.

with SlowFade antifade reagent (Molecular Probes, Eugene, Oregon, USA) and observed with a Zeiss LSM-310 confocal laser scanning microscope.

Results

An expression plasmid containing a full length human cystatin C cDNA (pBW69) was constructed and a parallel construct containing the Leu68→Gln mutation in the cDNA segment (pBW70) was also made (fig 1) to allow comparative studies of the cystatin C variant causing HCCAA. The ubiquitin promoter used in these constructs is highly conserved evolutionarily and is of the house-keeping type, which should allow use of the plasmids in various cell types from different higher animals. This was verified by initial expression experiments, demonstrating secretion of high concentrations of human cystatin C (10–200 ng/~ 10^6 cells/24 hours) from transfected mouse NIH/3T3 cells, hamster CHO cells, and monkey COS-7 cells (results not shown). Thus, transfection with expression plasmids pBW69 and pBW70 resulted in concentrations of secreted human cystatin C from the mouse, hamster, and monkey cells fully comparable with those reported for human cell lines in culture (such as colon carcinoma cells³⁷ or lung cancer cells³⁹).

Mouse NIH/3T3 cells were selected for detailed studies of possible differences between human wild-type and L68Q cystatin C in cellular transport and secretion. Cotransfection of pBW69 or pBW70 with a selection marker plasmid (pSV2neo) allowed the isolation of stable NIH/3T3 clones. Twelve clones from each cotransfection were grown, and their cystatin C secretion was measured by an ELISA specific for human cystatin C (fig 2). The results demonstrated clearly that high concentrations of the amyloidogenic L68Q variant of cystatin C were secreted from the NIH/3T3 cells, although the amounts secreted were slightly lower than from the clones expressing wild-type human cystatin C. The mean values (SEM; n = 12) for secretion from the wild-type and L68Q cystatin C expressing clones were 21.5 (1.5) and 14.4 (1.5) ng/ 10^5 cells/24 hours, respectively; these results were highly significant using the Mann-Whitney test ($p \leq 0.005$).

Clones T69-5 and T70-5 were chosen for further studies because they secreted the highest concentrations of wild-type and L68Q cystatin C, respectively. The cells were cultured for four days, with samples taken each day to investigate whether expression varied with the degree of confluency of the cells, and whether the pBW69 and pBW70 transfected cells showed any changes in growth rate as a result of cystatin C expression. Measurement of total cell protein in the samples, taken as a measure of the number of cells, indicated a slightly slower growth for the L68Q cystatin C expressing cells than for the human wild-type cystatin C expressing cells or the non-transfected cells (table 1). However, the amount of human cystatin C secreted from each cell appeared similar for the T70-5 and T69-5 cells. The secretion of mouse cystatin C

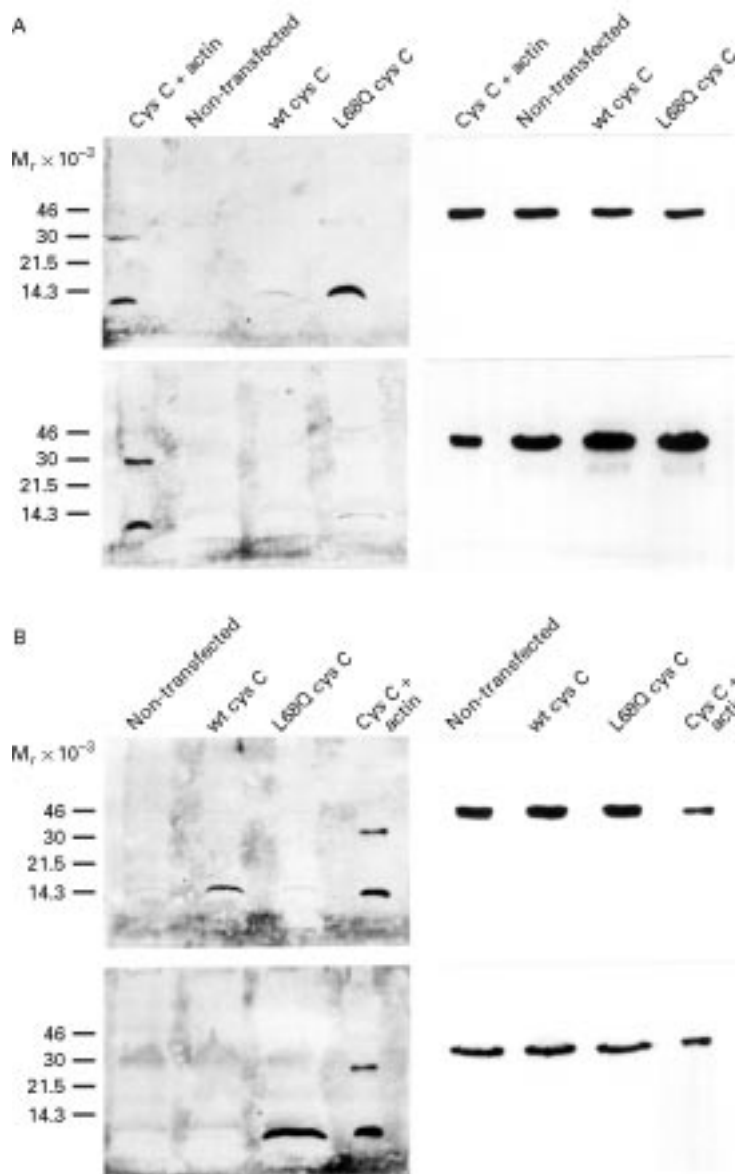


Figure 3 Immunoblotting of intracellular cystatin C. Intracellular cystatin C and actin, recovered from clones T69-5 and T70-5 by lysing the cells with 0.2% Triton X-100 (A) or after sonication (B), were analysed by immunoblotting after SDS-PAGE in a 15% polyacrylamide gel. Extracts were generated by the two methods for non-transfected NIH/3T3 cells, cells from clone T69-5 (wt cys C) and cells from clone T70-5 (L68Q cys C). All cells were harvested from 48 hour cultures, when the cells were semi-confluent. The cell extracts were separated into soluble (upper gel and blot) and insoluble (lower gel and blot) fractions by centrifugation. Aliquots of the cell extract fractions containing 25 µg total protein (according to a dye binding assay) were loaded into the electrophoresis lanes. An aliquot of 20 µl of a control sample consisting of a Triton X-100 extract from non-transfected NIH/3T3 cells (20 µg total protein) to which 25 ng recombinant human cystatin C had been added was included in all gels (cys C + actin). The positions corresponding to the migration of prestained size marker proteins (Rainbow™ coloured protein molecular weight markers; Amersham) are indicated to the left. The left hand side of the figure shows filters developed with a polyclonal antiserum against human cystatin C. The same filters developed with a monoclonal antibody against actin are shown on the right. The 30 kDa band seen in the control samples on the filters developed with the antiserum against human cystatin C is a dimeric form of cystatin C, which is formed upon boiling of the recombinant protein.

from the transfected NIH/3T3 cells was also analysed, as a measure of normal cell function, and was found to be similar for clones T69-5, T70-5, and the non-transfected cells. The same growth experiment was performed with clones T69-1, T69-2, T70-1, and T70-2, with results supporting the conclusion that the cells transfected with pBW70 and expressing human

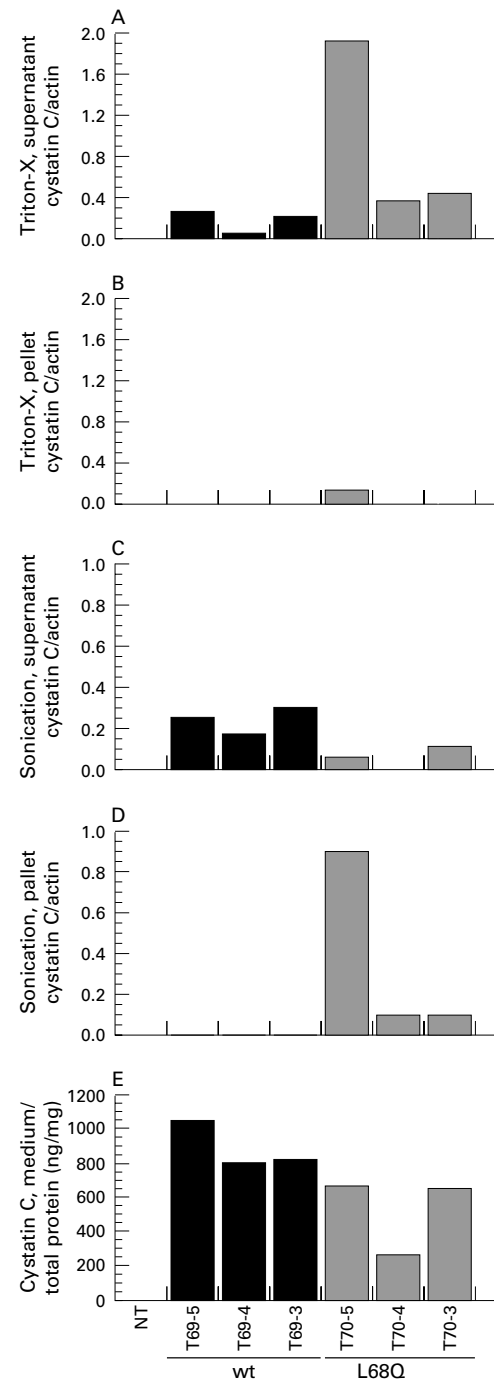


Figure 4 Semiquantitative analysis of intracellular cystatin C in NIH/3T3 cells expressing human wild-type and L68Q cystatin C genes. Cystatin C and actin bands in immunoblots similar to the ones shown in fig 3 were analysed by densitometric scanning. For experimental details, see Materials and methods section. Three NIH/3T3 clones expressing the wild-type human cystatin C gene (T69-3, T69-4, and T69-5; black bars) and three clones expressing the L68Q cystatin C gene (T70-3, T70-4, and T70-5; grey bars) were analysed. (A) and (B) show soluble and insoluble immunoreactive cystatin C in Triton X-100 generated cellular extracts, respectively. (C) and (D) show soluble and insoluble cystatin C, respectively, in cellular extracts obtained by sonication. (E) Shows secreted human cystatin C from the same cell cultures. NT, non-transfected cells.

L68Q cystatin C (clones T70-1, T70-2, and T70-5) grow slightly slower in culture than cells transfected with the wild-type human cystatin C expression plasmid (results not shown).

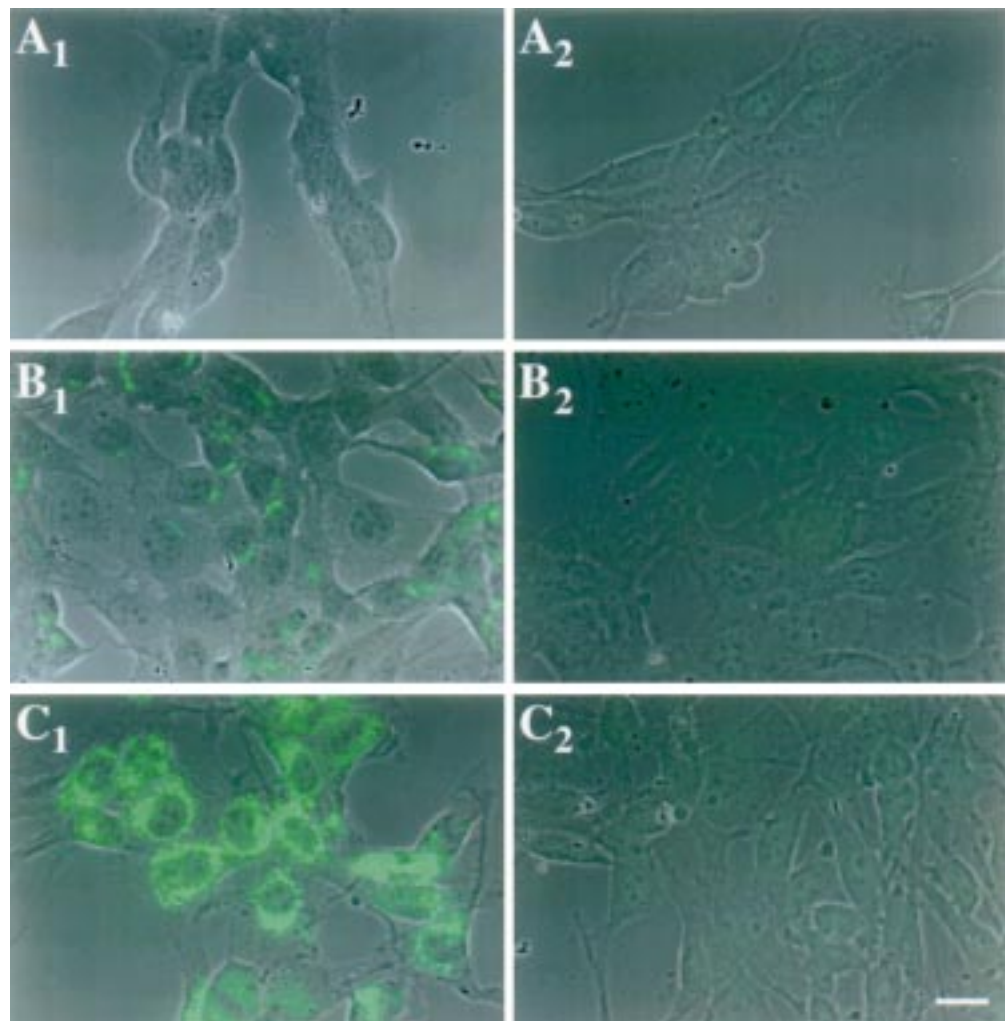


Figure 5 Intracellular localisation of transfected wild-type and mutant human cystatin C in NIH/3T3 fibroblasts. Parental mouse NIH/3T3 fibroblasts (A₁, A₂) and their wild-type (B₁, B₂; clone T69-5) and mutant (C₁, C₂; clone T70-5) human cystatin C cDNA transfected counterparts were analysed for intracellular cystatin C by immunocytochemistry. The primary antibody consisted of a monospecific rabbit polyclonal IgG directed against human cystatin C (A₁, B₁, and C₁). The secondary antibody was a fluorescein conjugated, donkey antirabbit IgG. Immunofluorescence images were superimposed on the corresponding phase contrast images. Background staining only was seen in the corresponding controls (A₂, B₂, and C₂), in which the primary antibody was replaced with rabbit pre-immune IgG. Bar, 20 μ m.

Initial experiments to quantify soluble intracellular cystatin C by ELISA indicated a higher concentration of L68Q cystatin C in clone T70-5 cells than of human wild-type cystatin C in clone T69-5 cells. However, the cystatin concentrations in cell extracts were on the border of the sensitivity limit of the ELISA, rendering exact calculations of ratios between intracellular and secreted amounts of cystatin C from the transfected cells impossible. Therefore, cell lysates obtained from clones T69-5 and T70-5 were analysed by SDS-PAGE, followed by immunoblotting using antibodies against human cystatin C (fig 3). Immunostaining of the same filters with an antibody against actin was used to verify equal loading in each lane. When the cells were lysed with Triton X-100 (fig 3A), almost all intracellular human cystatin C in clone T69-5 and T70-5 cells was dissolved and recovered in the supernatant (fig 3A), with only small amounts of cystatin C detectable in the insoluble fraction (fig 3A). The experiment indicated strongly that the amount of L68Q cystatin C present in

clone T70-5 cells was much higher than that of wild-type human cystatin C in clone T69-5 cells. When intracellular cystatin C was analysed after disruption of the cells by sonication (fig 3B), a further difference between clone T70-5 cells expressing the L68Q cystatin C gene and clone T69-5 cells expressing the wild-type human cystatin C gene was evident. The wild-type cystatin C was found in the soluble fraction (fig 3B) whereas the L68Q variant was found in the insoluble fraction (fig 3B). The experiment also indicated that there was a quantitative difference between the amount of L68Q and wild-type cystatin C present intracellularly.

To analyse further the differences between human wild-type and L68Q cystatin C in intracellular quantity and localisation, and to verify that the differences seen were not caused by a random clone variability between T69-5 and T70-5 cells, the western blotting experiments described above were repeated with two additional pBW69 transfected NIH/3T3 clones (T69-3 and T69-4) and two additional pBW70

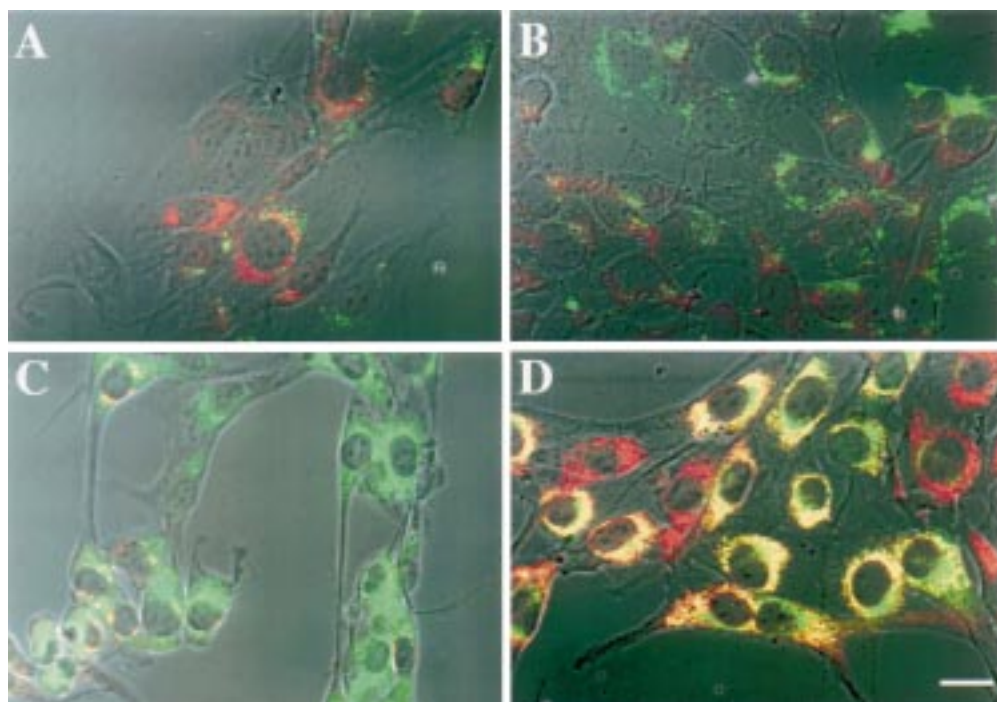


Figure 6 Colocalisation of mutant human cystatin C with a marker for the endoplasmic reticulum. NIH/3T3 fibroblasts transfected with the mutant human cystatin C cDNA (clone T70-5) were analysed for possible colocalisation of intracellular cystatin C with markers for lysosomes (cathepsins D and B), the Golgi apparatus (58 kDa protein), and the endoplasmic reticulum (protein disulphide isomerase). Immunofluorescence images for the two fluorophores (see below) were superimposed on the corresponding phase contrast images. The yellow colour indicates possible colocalisation of the two antigens. The primary antibodies were: (A) a mouse monoclonal antibody directed against human cystatin C and a rabbit polyclonal IgG directed against human cathepsin D; (B) a mouse monoclonal antibody directed against human cystatin C and a rabbit polyclonal IgG directed against human cathepsin B; (C) a rabbit polyclonal antibody directed against human cystatin C and a mouse monoclonal directed against p58; and (D) a rabbit polyclonal antibody directed against human cystatin C and a mouse monoclonal directed against protein disulphide isomerase. The secondary antibodies for cystatin C were Texas red conjugated, donkey antimouse IgG in (A) and (B) (red) and fluorescein conjugated, donkey antirabbit IgG in (C) and (D) (green). The secondary antibodies for cathepsins D and B in (A) and (B), respectively, (green) were fluorescein conjugated, donkey antirabbit IgG. The secondary antibodies for p58 and protein disulphide isomerase in (C) and (D), respectively, (red) were Texas red conjugated, donkey antimouse IgG. Bar, 20 μ m.

transfected clones (T70-3 and T70-4). The immunostained cystatin C bands in blots like the ones shown in fig 3 were quantified by densitometric scanning, normalised against the intensity of a standard cystatin C sample included in the original SDS polyacrylamide gels, and corrected for minor differences in sample loading by similar scanning of immunostained actin in the samples. The results are summarised in fig 4. The amount of intracellular, Triton X-100 soluble L68Q cystatin C was higher than that of wild-type cystatin C in all clones analysed (fig 4A). The amount was clearly highest in cells from clone T70-5, the clone also showing the highest secreted concentrations of L68Q cystatin C (fig 2). After Triton X-100 lysis and centrifugation, only clone T70-5 cells showed small amounts of human cystatin C in the pellet fraction (fig 4B), which probably reflects incomplete washing of the pellet, rather than the presence of truly Triton X-100 insoluble material. After sonication of the cells, all the intracellular human cystatin C in the three clones expressing the wild-type cystatin C gene was recovered in the soluble fraction (fig 4C), whereas the main part of the intracellular cystatin C in the three clones expressing the L68Q variant was found in the insoluble fraction (fig 4D). The amount of cystatin C secreted from the six clones was also reanalysed under the conditions of this experiment (fig 4E), veri-

fying that the cystatin C concentrations secreted from the clones expressing the wild-type human cystatin C gene were slightly higher than those from the cells expressing L68Q cystatin C. Smaller differences in secreted concentrations were apparent when the cystatin C concentrations were related to total cellular protein of the cultures instead of the initial number of cells. This seemed to reflect the slower growth of the L68Q cystatin C expressing clones, and was especially pronounced for the clone displaying the highest level of L68Q cystatin C synthesis and containing the highest amount of intracellular cystatin C, clone T70-5.

An initial comparison of pBW69 and pBW70 transfected NIH/3T3 cells by immunocytochemical staining for cystatin C and conventional microscopy indicated a quantitative difference, with more pronounced staining in clone T70-5 cells transfected with pBW70 than in clone T69-5 cells. Confocal microscopy, using a polyclonal antiserum against human cystatin C, was used to visualise the quantitative differences (fig 5). Some wild-type human cystatin C was seen in the perinuclear region of clone T69-5 cells (fig 5B₁). In contrast, the clone T70-5 cells expressing L68Q cystatin C exhibited intense intracellular staining for cystatin C (fig 5C₁). The same results were obtained when a monoclonal antibody against the human inhibitor was used for

staining of the T69-5 and T70-5 cells. Corresponding results, although less pronounced quantitatively, were obtained for other transfected clones displaying lower expression levels of human cystatin C (T69-3 and T69-4 expressing the wild-type human cystatin C gene; T70-3 and T70-4 expressing the L68Q cystatin C gene) (not shown).

To analyse further the localisation of the intracellular cystatin C accumulating in cells expressing the L68Q variant, double staining with antibodies against cystatin C and marker proteins of lysosomes, the Golgi apparatus, and the endoplasmic reticulum was performed. The results showed no great overlap between staining for cystatin C and the lysosomal enzymes, cathepsin B and cathepsin D (fig 6A and B). A small amount of L68Q cystatin C colocalised with the 58 kDa protein of the Golgi apparatus (fig 6C). The predominant localisation of the intracellular L68Q cystatin C was in the endoplasmic reticulum, as indicated by double staining for cystatin C and protein disulphide isomerase (fig 6D). Confocal microscopy of the same double staining of clone T69-5 cells demonstrated only a small overlap between the (much less intense) wild-type human cystatin C staining and the staining for the endoplasmic reticulum marker protein (results not shown). Taken together, these results indicate strongly that L68Q cystatin C is retained and accumulates in the endoplasmic reticulum as a consequence of the L68Q substitution.

Discussion

To help elucidate the molecular background to HCCAA we constructed an expression vector (pBW60) containing the human ubiquitin promoter, a multiple cloning site with recognition sites for 10 commonly used restriction enzymes, followed by a poly(A) sequence from the human growth hormone gene, which has been shown to act as an efficient signal for RNA processing. The human ubiquitin promoter is a strong constitutive promoter; the amino acid and gene sequences of ubiquitin are well conserved evolutionarily and the gene is expressed in all cells in all eukaryotic organisms tested.⁴⁰ Thus, the ubiquitous expression of a gene inserted in the cloning region of pBW60 would be expected. This is also supported by transfection experiments with other constructs, in which the ubiquitin promoter has been used to drive expression in a variety of cell lines, such as CHO,⁴⁰⁻⁴² CA77,^{40, 42} and MDCK.⁴³ Indeed, our cystatin C expression plasmids based on the pBW60 vector resulted in the synthesis of human wild-type (pBW69) and L68Q cystatin C (pBW70) at concentrations comparable with that of wild-type mouse cystatin C in NIH/3T3 cells (table 1), which in turn are at least as high as those of wild-type cystatin C in cultured human monocytes,⁴⁴ human lung,³⁹ and colon carcinoma cell lines.^{37, 38} Thus, our experimental system mimics the *in vivo* situation when wild-type or L68Q cystatin C genes are expressed in the cells of patients with HCCAA.

Our results concerning transfected NIH/3T3 clones show clearly that the human L68Q cystatin C variant is secreted from these cells, at concentrations almost as high as from cells expressing the wild-type human cystatin C gene. This shows that the L68Q variant has the capacity to fold properly *in vivo*, in agreement with *in vitro* results for *E coli* produced L68Q cystatin C.²³ This also means that significant amounts of L68Q cystatin C should be present extracellularly in patients with HCCAA. However, it has been suggested by recent mass spectroscopy studies of cystatin C in cerebrospinal fluid and cultured monocytes from patients with HCCAA that this is not the case.⁴⁵ One reason for these apparently conflicting results could be the fact that the mass spectroscopy study was based on capture of cystatin C by solid phase coupled Cm-papain. Our *in vitro* studies have shown that L68Q cystatin C dimerises rapidly under physiological conditions^{23, 46} and, moreover, that this dimerisation involves interactions between the enzyme interacting sides of two cystatin C molecules, leading to inactivation of the cystatin as a proteinase inhibitor.⁴⁶⁻⁴⁸ Thus, the Cm-papain capture approach probably caused exclusion of the L68Q variant from the samples studied by mass spectroscopy. On the other hand, cultured monocytes from patients with HCCAA, who are heterozygous for the L68Q mutation and thus contain alleles both for wild-type and L68Q cystatin C, secrete significantly lower concentrations of cystatin C when compared with monocytes from healthy individuals.⁴⁴ Likewise, immunochemical analysis has shown that the cerebrospinal fluid concentration of cystatin C in patients with HCCAA is significantly lower than in healthy unrelated individuals.⁴⁹ This indicates, perhaps, that the L68Q cystatin C produced by cells in the brain aggregates shortly after secretion in the *in vivo* environment, although the variant clearly remains soluble when secreted in our cell culture system.

Even though the main fraction of L68Q cystatin C was secreted from the fibroblast-like cells used in our experimental system, we could demonstrate a pronounced intracellular accumulation of the cystatin C variant in the cells. This is in agreement with the results from studies of monocytes from patients with HCCAA, for which ELISA measurements showed an increased ratio between intracellular and secreted cystatin C, and for which a more intense immunostaining of cystatin C was observed, compared with monocytes from healthy individuals.⁴⁴ Because the NIH/3T3 clones expressing wild-type and L68Q cystatin C mimic human cells homozygous for the wild-type and mutated cystatin C alleles, respectively, we could draw further conclusions about differences in cellular transport of the two cystatin variants by comparing the two different clone types. Apart from resulting in a clear increase in the total amount of intracellular cystatin C in each cell, a qualitative difference for the intracellular cystatin C as a result of the Leu68→Gln substitution was indicated, in that L68Q cystatin C was found in the

insoluble fraction after sonication of the cells, whereas wild-type human cystatin C was soluble. This could result from the intracellular aggregation of a fraction of the synthesised L68Q cystatin C before secretion. In support of this explanation, we have shown that in vitro refolded recombinant L68Q cystatin C spontaneously forms insoluble aggregates upon incubation at body temperatures, although rather slowly.²³ By comparing cells transfected with expression vectors for human wild-type and L68Q cystatin C by confocal microscopy after double staining with antibodies against human cystatin C and marker proteins of different cellular compartments, we obtained results which indicated strongly that the fraction of L68Q cystatin C accumulating intracellularly is retained in the endoplasmic reticulum. This is the compartment where several secretion defective protein variants (for example, pathological variants of α_1 -antitrypsin⁵⁰⁻⁵¹) accumulate. Even though the endoplasmic reticulum is also a known site for degradation of misfolded proteins,⁵²⁻⁵³ the pronounced cystatin C immunostaining noted in our cells, which synthesised human L68Q cystatin C at concentrations similar to those of human cells, indicates that the cystatin variant accumulates intracellularly, and results in a local high concentration. Because concentration is a critical factor both for the triggering of and the rate of amyloid formation,⁵⁴ this might be a key event in the pathophysiology of HCCAA.

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