STROKE IN ICELANDIC PATIENTS WITH HEREDITARY AMYLOID ANGIOPATHY IS RELATED TO A MUTATION IN THE CYSTATIN C GENE, AN INHIBITOR OF CYSTEINE PROTEASES

By EFRAT LEVY, CARLOS LOPÉZ-OTIN, JORGE GHISO,
DAVID GELTNER, AND BLAS FRANGIONE

From the Department of Pathology and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

Familial amyloid angiopathy, or hereditary cerebral hemorrhage with amyloidosis in patients from Iceland (HCHWA-I)[1], is an autosomal dominant form of amyloidosis that leads to hemorrhagic and thrombotic strokes causing death before the age of 40 yr (1-3). 128 affected members in eight families originating from the same geographic area in Iceland have been identified. The histopathology of HCHWA-I brains demonstrates generalized amyloid angiopathy, similar to cases with HCHWA of Dutch origin (4) and cases of Alzheimer disease, but they are unassociated with neuritic plaques or tangles (2, 5). The amyloid protein extracted from leptomeninges vessel wall of patients with HCHWA-I was shown to be a variant of cystatin C (6). Very recently, amyloid deposits were also found in tissues outside the central nervous system (7).

Human cystatin C (8) (previous nomenclature: γ-CSF [9], post-γ protein [10], γ-trace [11], and post-gamma globulin [12]) is a low-molecular mass protein shown to be a potent inhibitor of lysosomal cysteine proteinases (8, 13). It is present in many tissues and all body fluids (13-15). Cystatin C is secreted into tissue culture media by monocytes as well as by several primary cells, including brain cells and diverse established cell lines. It was suggested that downregulation of its secretion by monocytes may play a role in inflammation (16). The superfamily of cysteine proteinase inhibitors encompasses at least three distinct families: the stefins, cystatins, and kininogens (17, 18). The cystatin C protein isolated from the cerebrovascular amyloid was found to lack the first 10 NH$_2$-terminal amino acid residues and one amino acid substitution was demonstrated to be present at position 68 (glutamine for leucine), when compared with urinary cystatin C (19). The cystatin C concentration in the cerebrospinal fluid in patients with HCHWA-I is abnormally low (14). It is conceivable that a mutation had occurred leading to the production

1 *Abbreviation used in this paper:* HCHWA-I, hereditary cerebral hemorrhage with amyloidosis, Icelandic type.

of an unusual cystatin C protein that is abnormally degraded, bound, and/or pre-cipitated. To clarify this point, genomic DNA libraries were constructed from nor-mal human tissue and from the brain of an Icelandic patient with HCHWA-I. The gene encoding cystatin C was isolated from both libraries and sequenced. A pre-liminary study has already been reported (20).

Materials and Methods

The normal human cystatin C gene was previously isolated from a human genomic DNA library constructed from high molecular weight genomic DNA prepared as described (21) and cloned into a bacteriophage λ charon 4A vector (20, 22). A second human genomic DNA library was prepared from the brain of an Icelandic female with HCHWA who died at the age of 38 yr (GG) (19). 20-kb DNA fragments were generated by partial Sau 3AI digestion of the genomic DNA. The isolated arms of λ EMBL4 DNA (Promega Biotec, Madison, WI) generated by digestion with Bam HI were ligated with the fragmented DNA and packaged into bacteriophage λ particles using an in vitro packaging system (Stratagene, La Jolla, CA). About 3 × 10^5 recombinant bacteriophage from both libraries were screened by low strin-gency hybridization with synthetic oligonucleotides after replica plating onto nitrocellulose filters (23). The oligonucleotides were 5'-labeled with γ-[^{32}P]ATP and T4 polynucleotide ki-nase as described (24). Approximately 5 × 10^5 cpm/ml of radioactive oligonucleotide probe was used for hybridization (20). Hybridized filters were washed to a final stringency of 0.2 × SSC, 0.1% at 42°C (1× SSC = 0.15 M NaCl, 0.015 M Na$_3$ citrate, pH 7.5).

Oligonucleotides were chosen on the basis of the amino acid sequence of human cystatin C (14, 19). Where the amino acid sequence did not allow the unambiguous selection of nu-cleic acid residues, the actual choice of nucleotide sequence was based on the codon bias ob-served in a number of human mRNA sequences (25). Three oligonucleotides, a 33-mer cor-responding to a sequence coding for amino acids 33–43, a 31-mer encoding amino acids 81–91, and a 30-mer encoding amino acids 102–111, were synthesized. Positively hybridizing clones were plaque purified and DNA prepared. The extent of sequences encoding the human cys-tatin C gene was determined by restriction mapping and Southern blotting (26) using the three oligonucleotides as probes (20). Fragments specifically hybridizing with each of the probes were subcloned into pUC18 or directly into M13.

Exonuclease Bal 31 was used as described (27) to generate a set of overlapping deleted fragments from each subclone, which were cloned into bacteriophage M13 and sequenced by the dideoxy-chain termination method (28) with sequenase (United States Biochemical Corp., Cleveland, OH) (29). Sequence information obtained from the normal cystatin C gene enabled direct subcloning of the variant DNA fragments from bacteriophage λ into bacterio-phages M13. Identification of restriction fragments for subcloning and sequencing was deter-mined by hybridization of the labeled oligonucleotides to dried agarose gels (30).

Results and Discussion

Sequences coding for human cystatin C were previously isolated from a human genomic library cloned into bacteriophage λ. Restriction maps of the λ clones con-taining the normal human cystatin C gene (20) facilitated the subcloning of frag-ments for sequence analysis. The three synthetic oligonucleotide probes detected three noncontiguous fragments (as indicated in Fig. 1), contained within a Hind III restriction fragment of 9.5 kb. The 1.5-kb Hind III/Bgl II fragment detected by the 33-mer probe and the 2.8-kb Bgl II/Bam HI fragment detected by the 30-mer probe were subcloned into pUC18 for sequence analysis. The 500-bp Eco RI/Pst I fragment giving positive hybridization with the 31-mer probe was directly sub-cloned into M13mp8 and sequenced (20). The regions sequenced are indicated in Fig. 1; the sequence data are shown in Fig. 2 together with the encoded amino acid
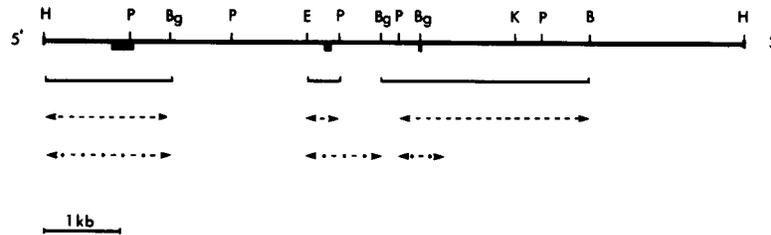
FIGURE 1. Restriction map of the Hind III fragment containing the cystatin C gene. Restriction enzymes shown are: Bam HI (B), Bgl II (Bg), Eco RI (E), Hind III (H), Kpn I (K), and Pst I (P). The positions of cystatin C coding sequences are indicated by filled boxes. The three fragments subcloned for further analysis are indicated by brackets. Arrows (◄--►) delineate the regions of the human cystatin C sequenced. Arrows (◄--·►) delineate the regions sequenced from the genomic DNA derived from the HCHWA-I patient.

sequence. Comparison of sequences with the amino acid sequence of cystatin C allows the reading frame to be established unambiguously. This alignment also allows the localization of the splice acceptor and donor sites of the three cystatin C exons.

The cystatin C gene encodes a polypeptide of 146 amino acids. The first 26 amino acids encoded by the open reading frame specify a secretory peptide signal sequence (31). The gene contains two intervening sequences that interrupt the coding region at amino acids 55 and 93. The Kozak initiation consensus (32) sequence abuts the probable ATG initiator codon. A putative TATA motif, ATAAA, which is common to a large number of eukaryotic promoters, is present 119 bp upstream to the ATG start codon. 241 bp downstream to the TAG stop codon, there is the hexanucleotide AATAAA, a consensus polyadenylation signal that precedes the site of polyadenylation in many eukaryotic cellular mRNAs (33).

The 5' region, upstream from the TATA box, is remarkable for its high content of G residues (58%), which frequently occur as long uninterrupted tracts. In agreement with sequence data for cystatin SN (34), this region is highly purine rich (~83% A+G). A 31-bp direct repeat sequence occurs at nucleotides −257 to −226 and −238 to −207 relative to the ATG start codon. Twice in the 5' untranslated sequence, at position −730, as well as −146, are heptamer sequences that are high affinity binding sites for the transcription factor Sp1 (35, 36). Interestingly, the promoter of the hamster PrP gene, the product of which gives rise to brain amyloid deposits in scrapie-infected animals, also contains three potential binding sites for the transcription factor Sp1 (37). The sequence TGGGG is repeated many times in the cystatin C gene, including 12 times in the 5' untranslated region. Although the significance of this sequence is unknown, it has been observed that this pentamer is present three times in a 27-base-long DNA synthetic oligonucleotide, which binds in vitro to duplex DNA at a single site within the 5' end of the human c-myc gene, resulting in the repression of c-myc transcription (38). The sequence GGGGTGGGG is present three times in the 5' untranslated region of the cystatin C gene and once in the 3' flanking region of the third exon. This CACCC box is predominantly found in globin genes in several copies, and in both orientations, and is considered an enhancer element (39-41).

Salivary cystatins share a high degree of amino acid (34) and nucleotide (42) se-

FIGURE 2.   Sequence of the gene encoding the human cystatin C. Nucleotides in the 5' untranslated region are numbered relative to the ATG start codon. Numbering of the amino acids deduced from the nucleotide sequence starts with residue 1 of the mature protein. Numbering of the amino acid sequence of the secretory signal sequence is relative to the mature protein. An upstream TATA motif and the polyadenylation signal are underlined, as is the site of point mutation. spl binding sequences and a direct repeat are indicated by dashed lines. Dots indicate regions of intervening sequences not sequenced and not put to scale. Shown here are 250 nucleotides downstream to the polyadenylation signal, out of 2,000 nucleotides sequenced, as indicated in Fig. 1.

quence homology with cystatin C. Alignment of the gene encoding cystatin C and cystatin SN (34) for maximum homology (data not shown) demonstrates that the salivary cystatin genes and the cystatin C gene share the same gene organization. The two introns present in the cystatin C gene correspond precisely in location to the two introns in the salivary cystatins.

Kitamura et al. (43) have shown that the kininogen gene is composed of eleven exons, of which the first nine exons can be characterized by a triple repeat of three exons. Each repeat comprises a cysteine proteinase inhibitor–like domain (18). Comparision of our data of the cystatin C gene, as well as that of the salivary cystatins (34) to the three repeated domains of the kininogen gene, demonstrates conservation of exon-intron structure between the cystatins and kininogen in support of the notion that they are evolutionarily related (13, 17, 18). Comparison of our data to the DNA sequences of cystatin SN (34) demonstrates that the second exons of these genes are especially homologous (85% sequence homology), while the first and third exons are 68% and 69% homologous. It has been suggested that the segment between amino acids 53 and 69, located in the second exon, may be the active site of the cysteine proteinase inhibitors, since it is the most conserved region in all known cystatins (8, 13).

Comparison of the sequences derived from genomic DNA also demonstrates that these genes share a high degree of sequence homology beyond donor and acceptor splicing sites of RNA. The highest homology of untranslated sequences ($\sim$80%) was found within the intervening sequences on both sides of the third exon. These results place the cystatin C and the salivary cystatins in the same gene family.

The cystatin C deposited as amyloid fibrils in the wall of small blood vessels in the brain of Icelanders showed a single amino acid substitution (glutamine instead of leucine at position 68) (19), as compared with normal cystatin C. Sequencing of the cystatin C variant isolated from the genomic DNA library of the HCHWA-I patient demonstrated that the amino acid substitution is the result of a point mutation. A single base substitution was found, CAG instead of CTG, in amino acid position 68 of the protein. This mutation abolishes an Alu I restriction site in the cystatin C gene of HCHWA-I patients. Loss of this site was detected by RFLP using Alu I restriction endonuclease and a cystatin C cDNA probe (44 and unpublished observations). The Alu I RFLP marker was only found in HCHWA-I patients and cosegregated with the disease in every case. The nucleic acid sequence obtained from the HCHWA-I patient was otherwise 100% homologous to the normal cystatin C gene, in the coding as well as noncoding regions sequenced.

It was shown that the first 10 amino acids are missing from the cystatin C protein isolated from patients' amyloid (19). Since sequence analysis indicates that the 5' end of the structural gene isolated from the HCHWA-I patient is intact, the truncated cystatin C amyloid is probably caused by postranslational enzymatic cleavage. In this context it is of interest to note that multiple salivary cysteine proteinase inhibitors (45, 46), as well as cystatin C obtained from urine, seminal fluid, and cerebrospinal fluid isolated from normal individuals (unpublished observation), were found to have heterogeneous NH_2 termini. Identical untranslated regions 5' to the normal and mutated genes argue against the possibility of aberrant promoter sequences, causing abnormal expression of the gene.

The variant cystatin C gene differs from the normal gene in a single nucleotide, resulting in a single amino acid substitution in the highly conserved region of the

cystatin gene family (8, 13), increasing the hydrophilicity of this region in the HCHWA-I amyloid subunit. Furthermore, the presence of Gln instead of Leu makes the peptide bond Gln-Gly, a potential site for the formation of β hairpin type-I' (19, 47). The mutation in the structural gene for cystatin C may be the primary defect in this inherited disorder but its relationship to the formation and deposition of the amyloid fibrils in HCHWA-I patients is unclear. The mutation and/or degradation may render the cystatin C protein resistant to normal protein turnover, or alternatively, create novel binding sites that may be more susceptible to proteolytic breakdown and polymerization.

Summary

Cystatin C is an inhibitor of lysosomal cysteine proteases and consists of 120 amino acids. A variant of cystatin C lacking the first 10 NH$_2$-terminal residues and having one amino acid substitution at position 68 forms amyloid deposits mainly in the walls of brain arteries, causing fatal strokes in Icelandic patients with familial cerebral hemorrhage secondary to a form of an autosomal dominant amyloidosis. To understand the molecular basis of the genetic defect, the gene encoding cystatin C was isolated from genomic DNA libraries made from normal tissue and the brain of an Icelandic patient with hereditary cerebral hemorrhage with amyloidosis (HCHWA-I). The data indicate that the cystatin C gene encodes a polypeptide of 146 amino acids, of which the first 26 correspond to a secretory peptide signal sequence. The gene contains two intervening sequences that interrupt the coding region at amino acids 55 and 93. Comparison with genes encoding salivary cystatins and kininogen proteins show sequence homology and conservation of exon-intron structure. Except for a mutation in the second exon (CAG instead of CTG in the normal gene, resulting in the substitution of glutamine for a leucine residue), the gene cloned from the brain of the Icelandic patient is identical to the normal cystatin C gene. Thus, HCHWA-I is the first familial type of amyloidosis related to a point mutation in a gene encoding for an inhibitor. The mutation in the structural gene encoding cystatin C appears to be the primary defect in this inherited disorder causing amyloid fibril formation and accumulation followed by cerebral hemorrhage.

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