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CYSTATIN C—PROPERTIES AND USE AS DIAGNOSTIC MARKER

Anders O. Grubb

Department of Clinical Chemistry, University Hospital, Lund, Sweden

1. Protease-Antiprotease Equilibria

The healthy human body might be described schematically as being composed of several dynamic equilibria. All diseases might be considered as disturbances in one or more of these dynamic equilibria. The balance between protein production and degradation is one of these equilibria, which are crucial to health, and several systems for control of both production and degradation are known. Degradation of

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proteins is brought about by proteolytic enzymes, proteases, which, based on their catalytic mechanisms, can be assorted to four major classes: the serine-, cysteine-, aspartic-, and metallo-proteases. The activities of human serine proteases of, *inter alia*, the coagulation, fibrinolytic, and complement systems have for several decades been known to be regulated by a large number of proteinaceous serine protease inhibitors of, for examples, the Kunitz, Kazal, and serpin types. In contrast, relatively few inhibitors of the other three major classes of proteases have been described. However, extensive research during the last 15 years has identified a group of human inhibitors for papain-like cysteine proteases comprising at least 11 different inhibitors. This group of inhibitors constitutes a new superfamily of human proteins, which is named the cystatin superfamily. The present discourse will focus on one of the most well characterized inhibitors, cystatin C, and provide some information on its biochemical properties, its role in normal and abnormal physiological processes, as well as on its use as a diagnostic marker.

2. Biological Roles of Papain-like Cysteine Proteases

A major part of the cysteine proteases are evolutionary related to the structurally well-defined cysteine protease papain and are therefore called papain-like cysteine proteases (B6, B7, R4). The human cysteine proteases of this family are mainly localized in lysosomes and play key roles in the intracellular degradation of proteins and peptides (cathepsins B, H, and L) (B4, G3). They also participate in the proteolytic processing of prohormones (D5) and proenzymes (T1) and seem to be involved in the penetration of normal tissues by macrophages (I1, R5) as well as by several types of malignant cells (C5, P8, S8, S9). Such proteases (e.g., cathepsin K) are also pivotal in the degradation and remodeling of bone (D2, D4, G1, L5) and may be instrumental in controlling the MHC class II trafficking in dendritic cells (cathepsin S) (P6).

Papain-like cysteine proteases are present not only in animals and plants but also in bacteria, fungi, and protozoa (R4), and the proteases of, for example, *Entamoeba histolytica, Trypanosoma congolese, Leischmania mexicana, Trichomonas vaginalis,* and *Plasmodium falciparum* seem to be involved in the replication, migration, and food digestion of these organisms (B24, C2, L17, N8, R4, R9, R10). Proteolytic processing of polyproteins by virally encoded proteases, *inter alia* cysteine proteases (R4) is required for replication of several viruses (K7, L4, O2).

3. The Cystatin Superfamily of Inhibitors of Papain-like Cysteine Proteases

The occurrence of proteins which inhibit papain-like cysteine proteases has been known at least since 1946, when Grob demonstrated that blood serum (G7) and

isolated fractions thereof (G8) inhibit papain. In retrospect, it might be concluded that Grob detected the inhibitory capacities of the plasma proteins cystatin C, low- and high-molecular-weight kininogens, and α_2-macroglobulin. However, it was not until the 1980s and 1990s that the majority of the multitude of presently identified cysteine protease inhibitors were isolated and structurally and functionally characterized (A1, B1, B5, F2, F3, I2, N3, N4, R6, R7, T9). The most comprehensive class of cysteine protease inhibitors is the cystatin superfamily of inhibitors. The name "cystatin" was originally given to a low-molecular-weight inhibitor of papain isolated from chicken egg white (B5, B8), since this inhibitor was a *cyst*eine protease *in*hibitor. The middle section, *stat,* of cystatin might also be considered as alluding to the capacity of cystatins to arrest the activity of cysteine proteases, since *stat-* is the supine stem of the Latin verb *sisto,* meaning "to arrest." In 1986 it was agreed that "cystatins" should be used to denote the entire superfamily of cysteine protease inhibitors structurally and functionally related to chicken cystatin (B3).

Cystatins have been demonstrated not only in higher organisms but also in a large number of plants and lower organisms including rice seeds, *Drosophila,* and *Candida albicans* (D3, K6, T8).

4. The Human Cystatins

The amino acid sequence of human cystatin C (Fig. 1) was determined in 1981 (G11) and, since it did not display any significant homology with the sequences of any protein of the superfamilies known then, it was evident that it belonged to a new protein superfamily. Retrospectively, it can be seen that the amino acid sequence of cystatin C was the first sequence of a cystatin to be determined (B5). The function of cystatin C as an inhibitor of cysteine proteases was identified about 2 years later, when the sequence of chicken cystatin was determined, showing that the two proteins had a sequence identity of 44% (B2, B25, S2, T10). Studies during the two last decades have identified 10 further human cysteine protease inhibitors, which display strong sequence homologies to cystatin C and chicken cystatin and, consequently, belong to the human cystatin superfamily. The human cystatin family therefore presently comprises 11 identified proteins (Table 1). Two of these, cystatins A and B, form the family 1 cystatins and are mainly, or exclusively, intracellular proteins, while cystatins C, D, E, F, S, SA, and SN are mainly extracellular and/or transcellular proteins and constitute the family 2 cystatins. The family 3 cystatins, high- and low-molecular-weight kininogen, contain three cystatin domains each (S1) and are mainly intravascular proteins, which, in addition to being inhibitors of cysteine proteases, are precursor molecules for the production of the vasoactive kinins, bradykinin and kallidin (M9). High-molecular-weight kininogen also participates in the contact-phase activation of the endogenous blood coagulation cascade (M9). The three cystatin domains (called D1–D3)

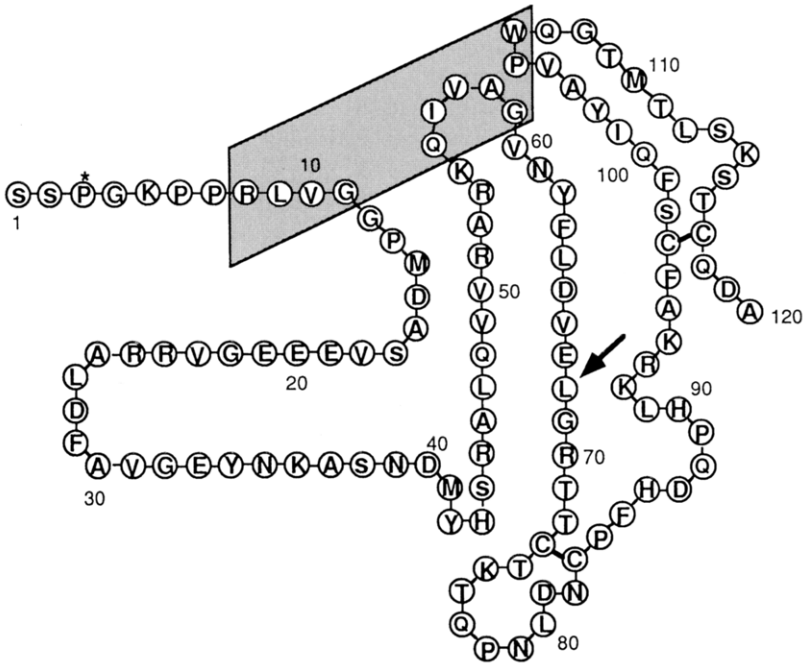

FIG. 1. Amino acid sequence and schematic structure of human cystatin C. The shaded area marks the inhibitory site for papain-like cysteine proteases, which does not overlap with the inhibitory site for mammalian legumains comprising, *inter alia,* the Asn^{39} residue. The arrow indicates the Leu^{68} residue, which is replaced with a Gln residue in the cerebral hemorrhage producing cystatin C variant. The asterisk marks the Pro^{3} residue, which is partly hydroxylated.

of the kininogens inhibit not only papain-like cysteine proteases (D2 and D3) but also calpains (D2). Figure 2 displays a schematic illustration of the evolutionary relationships among all known inhibitory active cystatins and kininogen cystatin domains.

5. Identification of Target Enzymes for Cystatins

All human cystatins are assumed to have major biological roles as inhibitors of one or more target proteases of human and/or nonhuman origin. Identification of target proteases of biomedical relevance is, however, difficult. Only few examples of clear-cut identifications of target proteases for inhibitors are known, and these identifications usually rely on experiments by Nature and not by Man. Two examples are the identifications of granulocyte elastase as a target enzyme

TABLE 1
THE HUMAN CYSTATIN SUPERFAMILY

Family 1 (intracellular cystatins)	Family 2 (extracellular and/or transcellular cystatins)	Family 3 (intravascular cystatins)
Cystatin A	Cystatin C	LMW-kininogen
Cystatin B	Cystatin D	HMW-kininogen
	Cystatin E	
	Cystatin F	
	Cystatin S	
	Cystatin SA	
	Cystatin SN	

Synonyms: cystatin A (R7): Acid cysteine proteinase inhibitor, epidermal SH-proteinase inhibitor, Stefin A; cystatin B (R6): neutral cysteine proteinase inhibitor, Stefin B; cystatin C: See Section 8 in main text; cystatin E: cystatin M (S10); cystatin F: leukocystatin (H2); cystatins S, SA, and SN (I2): salivary cysteine proteinase inhibitor; cystatin SN: cystatin SU (A1) (Grubb, A., unpublished results); LMW- and HMW-kininogens (M9): α-cysteine proteinase inhibitor, α-thiol proteinase inhibitor.

for α_1-antitrypsin revealed by the pathophysiology of α_1-antitrypsin deficiency (emphysema and liver cirrhosis) (O3) and of thrombin as a target enzyme for antithrombin revealed by the pathophysiology of antithrombin deficiency (thromboembolism) (C3). No serious disease states in which the major pathophysiological events can be ascribed to the lack of the specific inhibitory capacity of a cystatin have so far been described. Individuals with complete deficiencies of high- and low-molecular-weight kininogens have been described, but they do not seem to suffer from any serious pathophysiological abnormality (C7). Mutations in the genes for cystatins B and C produce progressive myoclonus epilepsy (B12, L1, L2, P2, P3) and hereditary cerebral hemorrhage (A6, G2, P1), respectively, but the present knowledge of the pathophysiology of these disorders does not allow the identification of target proteases for any of these cystatins. Although therefore no unequivocal identification of target proteases for human cystatins has been feasible yet, it is possible to select, or exclude, candidate target proteases for the different cystatins based upon theoretical considerations (B13) as well as upon comparisons with known examples of target proteases for, *inter alia,* those serine protease inhibitors mentioned above. One requirement that must be fulfilled by a cystatin to render it a biomedical significant inhibitor of a potential target enzyme is that the molar concentration of the cystatin must be higher than that of the active protease at the site in the body where the protease is released. A second requirement is that the equilibrium constant for dissociation of a protease–cystatin complex must be low to secure a negligible amount of free proteolytic activity at equilibrium. Finally, the association rate constant for the formation of the protease–cystatin complex

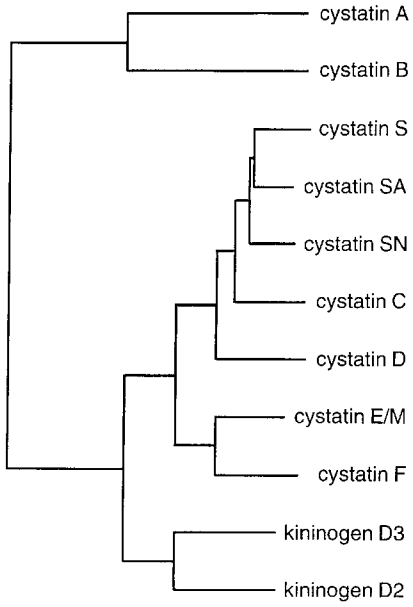

FIG. 2. Schematic diagram of the evolutionary relationships among all known inhibitory active human cystatins and kininogen cystatin domains. The phylogenetic tree was constructed using "Growtree," included in the GCG software package (version 8.1; Genetic Computer Group, Inc., Madison, WI). The phylogenic distances were obtained according to the method of Kimura (K4). The reconstruction of the tree was done by the unweighted-pair group method using arithmetic averages.

must be high so that any free protease activity is rapidly quenched. Since all human cystatins display a unique set of equilibrium and association rate constants when tested against a limited collection of cysteine proteases, each cystatin has a unique inhibitory spectrum (Table 2). However, these inhibitory spectra are usually overlapping, and it is quite probable that some cystatins might share target proteases.

6. Distribution of Cystatins in Body Fluids

The distribution in body fluids of the different cystatins is remarkably different (Fig. 3). For example, while cystatin C is present in appreciable amounts in all investigated body fluids, cystatins S, SN, and SA are virtually confined to saliva, tears, and seminal plasma (A1). Cystatin D is present only in saliva and tear fluid (A1, F3). In some body compartments, e.g., spinal fluid, cystatin C represents more than 90% of the total molar concentration of cysteine protease

TABLE 2
EQUILIBRIUM CONSTANTS FOR DISSOCIATION OF COMPLEXES BETWEEN HUMAN CYSTATINS AND CYSTEINE PROTEASES K_i (nM)

Cystatin	Cathepsin B	Cathepsin L	Cathepsin S	Cathepsin H	Papain
A	8.2	1.3	0.05	0.31	0.019
B	73	0.23	0.07	0.58	0.12
C	0.25	< 0.005	0.008	0.28	0.00001
D	>1000	25	0.24	0.28	1.2
E	32	—	—	—	0.39
F	>1000	0.31	—	—	1.1
S	—	—	—	—	108
SN	19	—	—	—	0.016
SA	—	—	—	—	0.32
L-kininogen	600	0.017	—	0.72	0.015

Data from (A9, L11, N3, N4, S1).

inhibitors, while in other compartments, e.g., blood plasma, it only represents a few percent of the total cysteine protease inhibitory capacity (A1). Moreover, the total cysteine protease-inhibiting capacity varies also considerably among different body compartments. For example, the total papain-inhibiting capacity of blood plasma is about 12 μmol/L, while that of cerebrospinal fluid is less than 1 μmol/L (A1). Since each body fluid displays a unique set of cystatins, it is also clear that the different body fluids display unique cysteine protease inhibitory spectra, although these partially overlap, like the inhibitory spectra of the individual cystatins. Table 3 shows that different cysteine proteases with different catalytic properties are controlled by separate cystatins in the various body fluids.

7. Additional Functions Attributed to Cystatin C

In addition to being an inhibitor of papain-like cysteine proteases, cystatin C has recently been shown be an efficient inhibitor of some of the cysteine proteases of another family of cysteine proteases, called the peptidase family C13, with human legumain as a typical enzyme (C6). Human legumain has, like cathepsin S, been proposed to be involved in the class II MHC presentation of antigens (M3). It has also been shown that the cystatin C inhibitory site for mammalian legumain does not overlap with the cystatin C inhibitory site for papain-like cysteine proteases (Fig. 1) and that the same cystatin C molecule therefore is able to simultaneously inhibit one cysteine protease of each type (A10).

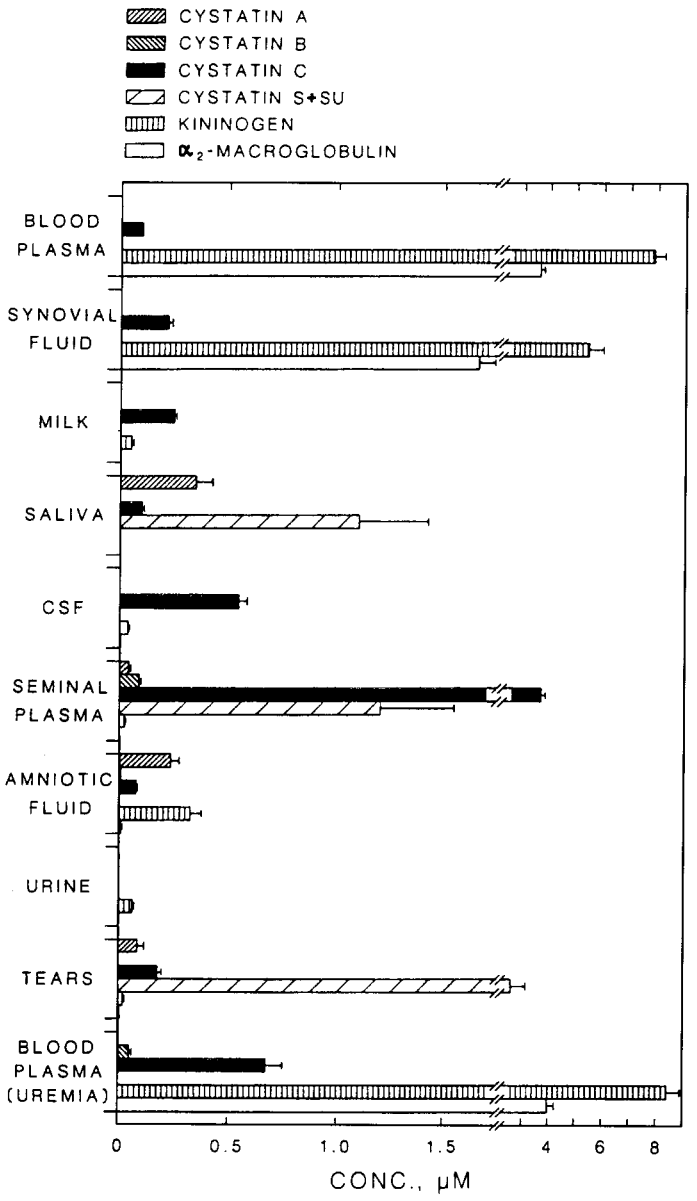

FIG. 3. Molar concentrations of cystatins and α_2-macroglobulin in 10 human body fluids.

TABLE 3

HALF-LIVES ($t_{1/2}$, S) OF FREE (A) HUMAN CATHEPSIN B AND (B) PAPAIN ON INTERACTIONS WITH CYSTATINS IN HUMAN BODY FLUIDS

	$t_{1/2}$ (s)				
	Cystatin A (12,000)	Cystatin B (12,000)	Cystatin C (15,000)	Cystatin S/SN (15,000)	Kininogen (60,000)
(a) Cathepsin B					
Blood plasma	> 740	> 1900	3.5	> 650	6.3
Synovial fluid	> 740	> 1900	1.7	> 650	9.1
Milk	> 740	> 1900	1.5	> 650	960
Saliva	39	> 1900	3.8	4.6	> 5900
Cerebrospinal fluid	> 740	> 1900	0.69	> 650	1200
Seminal plasma	320	190	0.10	4.3	1500
Amniotic fluid	59	2100	4.9	> 650	150
Urine	> 740	> 1900	> 9.9	> 650	760
Tears	150	3900	2.1	2.0	3000
Blood plasma (uremia)	> 740	330	0.56	> 650	5.9
(b) Papain					
Blood plasma	> 11	> 2.1	0.74	> 5.5	0.006
Synovial fluid	> 11	> 2.1	0.36	> 5.5	0.008
Milk	> 11	> 2.1	0.31	> 5.5	0.84
Saliva	0.6	> 2.1	0.80	0.039	> 5.2
Cerebrospinal fluid	> 11	> 2.1	0.14	> 5.5	1.1
Seminal plasma	4.9	0.21	0.021	0.036	1.3
Amniotic fluid	0.90	2.4	1.0	> 5.5	0.13
Urine	> 11	> 2.1	> 2.1	> 5.5	0.67
Tears	2.2	4.3	0.43	0.017	2.6
Blood plasma (uremia)	> 11	0.37	0.12	> 5.5	0.005

Calculations were made according to the equation $t_{1/2} = \ln 2/(k_{+1} \times [I])$, where k_{+1} denotes association rate constant and $[I]$ is inhibitor concentration. The molecular weights used in the calculations are given in parentheses. Cystatin S was assumed to have the same k_{+1} value as cystatin SN. A k_{+1} value of 1.0×10^7 M^{-1} s^{-1} for cystatin C was used for calculations of the $t_{1/2}$ for free papain at the cystatin C concentrations found in the fluids. Data from (A1).

Cystatin C has also been suggested to possess biological functions, presumably unrelated to its protease-inhibiting potential. For example, human cystatin C has been described to play a regulatory role in inflammatory processes, *inter alia*, by down-regulation of the phagocytosis-associated respiratory burst reaction displayed by polymorphonuclear neutrophils as well as by down-regulation of their chemotactic response (L6, L7). Chicken cystatin, and thus probably human cystatin C, has also been shown to up-regulate nitric oxide release from peritoneal macrophages (V1). However, these suggested additional functions of cystatin C remain to be confirmed.

8. Previous Designations for Cystatin C

The protein now generally designated with the functional name cystatin C was first discovered in 1961 (B26, C8, M1) and its function as a cysteine protease inhibitor was thus unidentified for more than 20 years. As a consequence, several trivial names were used for the same protein, and it is important to know these for complete retrieval of data on cystatin C by bibliographic studies. The following trivial names can be found in the literature: γ-trace, post-γ-globulin, gamma-CSF, post-gamma protein, γ_c-globulin, δ aT, and high alkaline fraction (HAF) (B26, C1, C8, H9, H10, K5, L3, M2, M4, S11).

9. Structure of Human Cystatin C and Its Concentration in Body Fluids

The complete amino acid sequence of the single polypeptide chain of human cystatin C was determined in 1981 (G11) and later corroborated by identification and sequencing of the corresponding cDNA (Fig. 4) (A3) and gene (A5, A7).

The three-dimensional structure of cystatin C is not yet determined, although some crystallographic data are available (K8), but it can be presumed that it is similar to that described for the homologous protein chicken cystatin (B19) and this has, at least partially, been confirmed by NMR studies (E1). A schematic structure for human cystatin C is given in Fig. 1.

Studies of truncated forms of cystatin C (A8) and of cystatin C variants produced by site-directed mutagenesis (B18, H4, H5, H6, L12, M7), as well as identification of sequence similarities between all cystatins, have indicated that the inhibitory center of cystatin C for papain-like cysteine proteases comprises three peptide segments, Arg^{8}-Leu^{9}-Val^{10}-Gly^{11}, Gln^{55}-Ile^{56}-Val^{57}-Ala^{58}-Gly^{59}, and Pro^{105}-Trp^{106}. Peptidyl derivatives, structurally based upon the aminoterminal segment of the inhibitory center, have been synthesized and shown to be efficient inhibitors of cysteine proteases (G9, H3). Some of these peptidyl derivatives have also displayed antibacterial and antiviral properties (B16, B17).

Table 4 displays some of the physicochemical properties of cystatin C as well as its normal concentration in body fluids.

10. Serum/Plasma Cystatin C as a Marker for Glomerular Filtration Rate (GFR)

No investigations have demonstrated that the diagnostic usefulness of the serum level of cystatin C is different from that of the plasma level of cystatin C. The term

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                                                                    22
                                                  GGGCGCAGCGGGTCCTCTCTAT

                                                                    95
CTAGCTCCAGCCTCTCGCCTGCGCCCCACTCCCCGCGTCCCGCTCCTAGCCGACC ATG GCC GGG CCC CTG CGC
                                                  Met Ala Gly Pro Leu Arg
                                                  -26                 -21

                                                                   155
GCC CCG CTG CTC CTG CTG GCC ATC CTG GCC GTG GCC CTG GCC GTG AGC CCC GCG GCC GGC
Ala Pro Leu Leu Leu Leu Ala Ile Leu Ala Val Ala Leu Ala Val Ser Pro Ala Ala Gly
                                                                     -1

                                                                   215
TCC AGT CCC GGC AAG CCG CCG CGC CTG GTG GGA GGC CCC ATG GAC GCC AGC GTG GAG GAG
Ser Ser Pro Gly Lys Pro Pro Arg Leu Val Gly Gly Pro Met Asp Ala Ser Val Glu Glu
1                                                                   20

                                                                   275
GAG GGT GTG CGG CGT GCA CTG GAC TTT GCC GTC GGC GAG TAC AAC AAA GCC AGC AAC GAC
Glu Gly Val Arg Arg Ala Leu Asp Phe Ala Val Gly Glu Tyr Asn Lys Ala Ser Asn Asp
                                                                    40

                                                                   335
ATG TAC CAC AGC CGC GCG CTG CAG GTG GTG CGC GCC CGC AAG CAG ATC GTA GCT GGG GTG
Met Tyr His Ser Arg Ala Leu Gln Val Val Arg Ala Arg Lys Gln Ile Val Ala Gly Val
                                                                    60

                                                                   395
AAC TAC TTC TTG GAC GTG GAG CTG GGC CGA ACC ACG TGT ACC AAG ACC CAG CCC AAC TTG
Asn Tyr Phe Leu Asp Val Glu Leu Gly Arg Thr Thr Cys Thr Lys Thr Gln Pro Asn Leu
                                                                    80

                                                                   455
GAC AAC TGC CCC TTC CAT GAC CAG CCA CAT CTG AAA AGG AAA GCA TTC TGC TCT TTC CAG
Asp Asn Cys Pro Phe His Asp Gln Pro His Leu Lys Arg Lys Ala Phe Cys Ser Phe Gln
                                                                   100

                                                                   515
ATC TAC GCT GTG CCT TGG CAG GGC ACA ATG ACC TTG TCG AAA TCC ACC TGT CAG GAC GCC
Ile Tyr Ala Val Pro Trp Gln Gly Thr Met Thr Leu Ser Lys Ser Thr Cys Gln Asp Ala
                                                                   120

                                                                   593
TAG GGGTCTGTACCGGGCTGGCCTGTGCCTATCACCTCTTATGCACACCTCCCACCCCCTGTATTCCCACCCCTGGAC

                                                                   672
TGGTGGCCCCTGCCTTGGGGAAGGTCTCCCCATGTGCCTGCACCAGGAGACAGACAGAGAAGGCAGCAGGCGGCCTTTG

                                                                   751
TTGCTCAGCAAGGGGCTCTGCCCTCCCTCCTTCCTTCTTGCTTCTCATAGCCCCGGTGTGCGGTGCATACACCCCCACC

                          777
TCCTGCAATAAAATAGTAGCATCCCC
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FIG. 4. Nucleotide and deduced amino acid sequence of a cDNA clone encoding human precystatin C. Numbering of the nucleotide sequence starts at the first nucleotide and proceeds in the 5′ to 3′ direction. Amino acid numbering begins with residue 1 of the mature protein (G11) and the putative hydrophobic signal sequence thus comprises residues −26 to −1. The Kozak initiation consensus and the polyadenylation signal are underlined.

TABLE 4
PHYSICOCHEMICAL PROPERTIES OF HUMAN CYSTATIN C AND ITS CONCENTRATION IN BODY FLUIDS

Polypeptide chains: One, with 120 amino acid residues
Glycosylation: None
Molecular mass: 13,343 Da (nonhydroxylated); 13,359 Da (hydroxylated proline residue at position 3)
Isoelectric point: 9.3
Electrophoretic mobility: γ_3 (agarose gel electrophoresis at pH 8.6)
Extinction coefficient: 1.22×10^4 (mol^{-1} liter cm^{-1}) = 9.1 (280 nm, 1%, 1 cm)
Amino acid sequence: SSPGK PPRLV GGPMD ASVEE EGVRR ALDFA VGEYN KASND MYHSR ALQVV RARKQ IVAGV NYFLD VELGR TTCTK TQPNL DNCPF HDQPH LKRKA FCSFQ IYAVP WQGTM TLSKS TCQDA
Disulfide bonds: Between residues 73 and 83 and between residues 97 and 117
Gene location: Chromosome 20 at p.11.2
DNA sequence: The nucleotide sequence data are available from the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number X52255
Half-life: About 20 min (experimentally determined for human cystatin C in rat plasma. The similarity in distribution volume and renal clearance between human cystatin C and acknowledged markers of human glomerular filtration, i.e., iohexol and ^{51}Cr-EDTA, suggests that the substances are eliminated at the same rate in humans with a half-life of approximately 2 h in individuals with normal renal function)
Concentrations in body fluids of healthy adults (mg/liter; mean and range):
  Blood plasma: 0.96; 0.57–1.79
  Cerebrospinal fluid: 5.8; 3.2–12.5
  Urine: 0.095; 0.033–0.29
  Saliva: 1.8; 0.36–4.8
  Seminal plasma: 51.0; 41.2–61.8
  Amniotic fluid: 1.0; 0.8–1.4
  Tears: 2.4; 1.3–7.4
  Milk: 3.4; 2.2–3.9

"serum cystatin C" will therefore in the following paragraphs, unless otherwise indicated, also refer to the plasma level of cystatin C.

10.1. PRODUCTION OF CYSTATIN C

Determination of the structure of the human cystatin C gene and its promoter has demonstrated that the gene is of the housekeeping type, which indicates a stable production rate of cystatin C by most nucleated cell types (A7). The presence of a hydrophobic leader sequence in precystatin C (Fig. 4) strongly indicates that the protein normally is secreted (A3, A7). Indeed, immunochemical and Northern blot studies of human tissues and cell lines have shown that cystatin C and/or its mRNA is present in virtually all investigated cell types (A7, J1, L9, L10, S7). Likewise, investigations of the production of cystatin C by human cell lines in culture have displayed that nearly all cell lines investigated secrete cystatin C

(B14, C4, N4) (Abrahamson, M., personal communication). Studies of the serum level of cystatin C in large patient cohorts have failed to correlate the serum level to any pathophysiological state besides those affecting the glomerular filtration rate, which also is compatible with a stable secretion of cystatin C from most human tissues (G12, K9, N2, S6). However, some reports have described that stimulation of macrophages *in vitro* down-regulates their secretion of cystatin C (C4, W1), but inflammatory conditions are not generally associated with decreased serum levels of cystatin C.

10.2. Catabolism of Cystatin C

Blood plasma proteins with molecular masses below 15–25 kDa are generally almost freely filtered through the normal glomerular membrane and then almost completely reabsorbed and degraded by the normal proximal tubular cells. This should consequently also be true for cystatin C with a molecular mass of 13 kDa and with a probable ellipsoid shape with axes of about 30 and 45 Å (B19). Indeed, studies of the handling of human cystatin C in the rat have shown that the plasma renal clearance of cystatin C is 94% of that of the generally used GFR-marker ^{51}Cr-EDTA and that cystatin C thus is practically freely filtered in the glomeruli (T4). At least 99% of the filtered cystatin C was found to be degraded in the tubular cells. Figure 5 shows the rat plasma concentration of intact human ^{125}I-cystatin C and ^{51}Cr-EDTA relative to the initial concentrations after intravenous injection. Figure 6 displays the plasma disappearance of cystatin C in normal and nephrectomized rats and indicates that the renal plasma clearance of cystatin C is about 85% of the total plasma clearance (renal + extrarenal). When the GFR of a set of rats was variably lowered by constricting their aortas above the renal arteries, the renal plasma clearance of cystatin C correlated strongly with that of ^{51}Cr-EDTA (Fig. 7), with a linear regression coefficient of 0.99 and with the y intercept not being statistically different from 0 (T4). This observation clearly implied an insignificant peritubular uptake of cystatin C. Immunohistochemical and Northern blot studies of human kidneys have also strongly indicated that human cystatin C normally is degraded by proximal tubular cells after its passage through the glomerular membrane (J1).

10.3. Clinical Use of Serum Cystatin C as a GFR Marker

The knowledge that most human tissues produce cystatin C and that it, being a low-molecular-mass protein, is removed from plasma by glomerular filtration, suggested that its plasma, or serum, level might be a potentially good marker for GFR. Early investigations demonstrated that serum cystatin C, indeed, was a marker for GFR, at least as good as serum creatinine in the populations investigated (G12, S6). These studies also showed that the serum cystatin C level was a

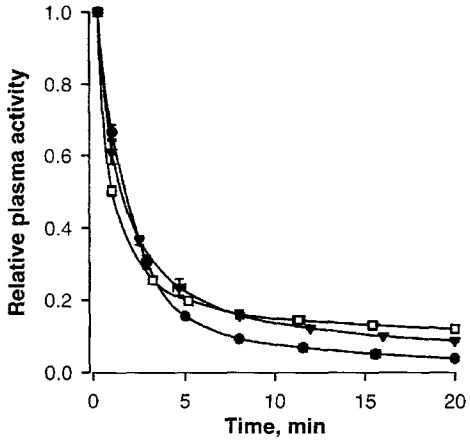

FIG. 5. Plasma concentration of intact ^{125}I-cystatin C(•), ^{51}Cr-EDTA (□) and ^{131}I-aprotinin(▼) relative to the initial plasma concentration after intravenous injection in 12 rats. Error bars show ±1 SEM, when larger than the symbols. Aprotinin is a 6.5-kDa microprotein with a pI of 10.5.

better GFR marker than the serum levels of the other low-molecular-mass proteins investigated, β_2-microglobulin, retinol-binding protein, and complement factor D (G12, S6). However, in these early studies the cystatin C concentration was determined by enzyme amplified single radial immunodiffusion (L15). This procedure is slow, requiring at least 10–20 h, and has a relatively high coefficient of variation (about 10%), which decrease the usefulness of the obtained serum cystatin C value

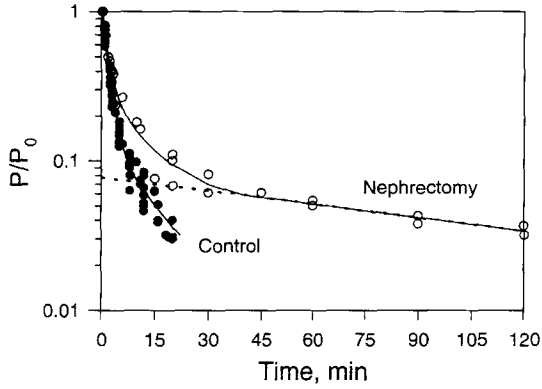

FIG. 6. Plasma disappearance of intact ^{125}I-cystatin C in nephrectomized (○) and control (•) rats. The monoexponential regression line of the plasma concentration, P/P_0, against the time, t, between 60 and 120 min in nephrectomized rats is indicated by a dotted line.

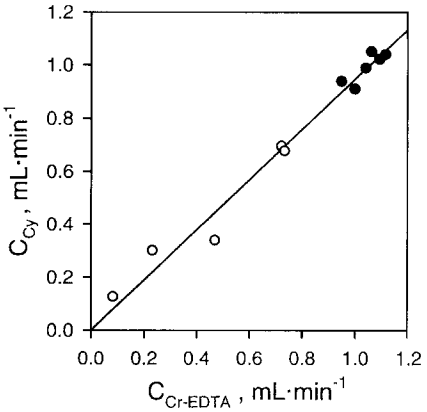

FIG. 7. Renal plasma clearance of ^{125}I-cystatin C (C_{cy}) compared to that of ^{51}Cr-EDTA ($C_{Cr-EDTA}$) in rats with normal glomerular filtration (•), and in rats with renal blood flow reduced to 25–50% of control by constricting the aorta above the renal arteries (○). The clearance measurements were completed 2.5–6.0 min after tracer injection. $C_{cy} = 0.944 \times C_{Cr\text{-}EDTA}$. $r = 0.989$.

as a GFR marker in the clinical routine. The development, about 10 years later, of automated particle-enhanced immunoturbidimetric methods, which were rapid as well as more precise, therefore significantly improved the possibility of using serum cystatin C as a GFR marker in clinical routine work (K9, N2). So did the introduction of a sandwich enzyme immunoassay for the determination of serum cystatin C (P4). Since the automated particle-enhanced immunoturbidimetric procedure for determination of serum cystatin C was introduced in 1994, the vast majority of all studies of the use of serum cystatin C as a GFR marker have relied upon the commercially available version of this procedure. A commercially available automated particle-enhanced immunonephelometric method has also recently been described (E2, F1, M10).

Serum creatinine is ubiquitously used as an indicator for GFR despite the knowledge that a substantial proportion of patients with reduced GFR display serum creatinine levels within the normal range and that even a 50% reduction of GFR not infrequently is associated with a normal concentration of serum creatinine (L8, P5, S3). The usefulness of serum creatinine as a marker for GFR is limited by, *inter alia*, the influence of an individual's muscle mass on the production rate of creatinine (H8, P5, S5), by the tubular secretion and reabsorption of creatinine, by the dietary intake of creatine and creatinine, and by analytical difficulties (P5). These significant limitations in the use of serum creatinine as an indicator for GFR has made it of interest to search for better indicators for GFR. Several recent studies have compared the use of serum cystatin C and creatinine as markers for GFR as determined by "golden standard" procedures based upon determinations of the

plasma clearance of injected low-molecular-mass substances such as Cr^{51}-EDTA, ^{99m}Tc-DTPA, and iohexol. These studies have indicated either that serum cystatin C is a better GFR marker than serum creatinine, particularly for individuals with small to moderate decreases in GFR, in the so-called creatinine-blind GFR range, or that the two parameters are of equal value as GFR indicators (B20, B22, H7, J4, K9, N2, N6, P4, P7, R2, R3, S12, S13, T6, V2). Figures 8 and 9 illustrate one

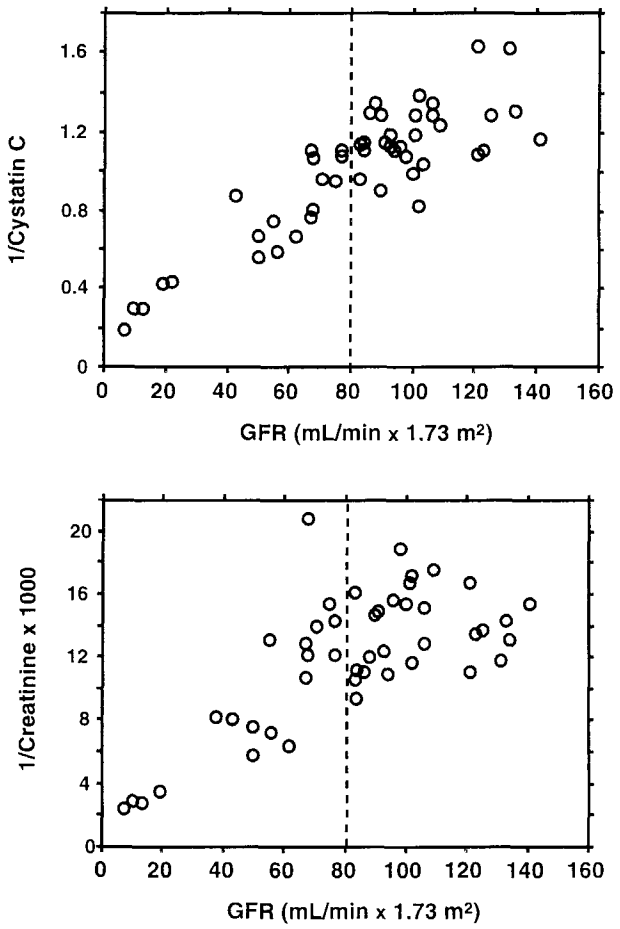

FIG. 8. Correlation between glomerular filtration rate and (top) reciprocal serum cystatin C (mg/liter) or (bottom) reciprocal serum creatinine (μmol/liter) in 27 male and 24 female patients;- - - - is the lower reference limit for glomerular filtration rate. The difference in the diagnostic capacity of serum cystatin C and serum creatinine to identify patients in the "creatinine-blind" area with a glomerular filtration rate of 60–80 ml/min $\times 1.73$ m^2 is obvious in this investigation of a population of patients with various renal conditions.

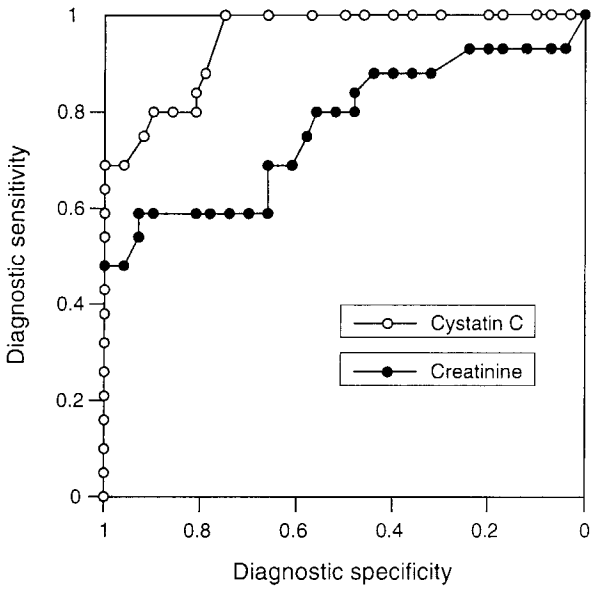

FIG. 9. Nonparametric ROC plots for the diagnostic accuracy of serum cystatin C and creatinine in distinguishing between normal and reduced glomerular filtration rate ($\geq$ and $<$ 80 ml/min$\times$1.73 m^2, respectively) in 51 patients with various renal conditions (the same population as the one displayed in Fig. 8).

study indicating the usefulness of serum cystatin C in the creatinine-blind GFR range. Nearly all investigations have emphasized that serum cystatin C, in contrast to serum creatinine, is uninfluenced by gender and muscle mass. Several studies indicate that virtually the same reference values might be used for serum cystatin C for males and females from 1 year of age, up to 50 years of age, when the age-related decline in GFR becomes significant (B20, B22, H7, L14, N6, R3).

Serum cystatin C has also been described to be a better predictor than serum creatinine of fasting total homocysteine serum levels, probably because of its closer correlation to GFR (B23, N7).

10.4. Reference Values for Serum Cystatin C

Establishment of reference values of general use requires general availability of a well-defined calibrator. The availability of such a calibrator also facilitates accreditation of procedures for quantitative determination of the corresponding analyte. Recombinant human cystatin C can easily be produced and isolated and used for establishing reliable calibrators (A2, D1). A first step toward an international calibrator for cystatin C has been taken by the production of a solution of recombinant human cystatin C of high purity, determining the concentration of this

TABLE 5
REFERENCE INTERVALS FOR SERUM CYSTATIN C

Adults (male + female; 20–50 years): 0.70–1.21 mg/liter[a]
Adults (male + female; above 50 years): 0.84–1.55 mg/liter[a]
Children (male + female; 1–18 years): 0.70–1.38 mg/liter[b]
Children (male + female; 1–16 years): 0.63–1.33 mg/liter[c]

[a]Reference (N6).
[b]Reference (B20).
[c]Reference (H7).

solution by quantitative amino acid analysis and spectrophotometric analysis and then diluting it with cystatin C-free human serum to physiological concentrations (K9). Based upon the use of such a cystatin C calibrator and a commercially available automated particle-enhanced immunoturbidimetric method, several studies of reference values for serum cystatin C, comprising populations of both adults and children have been published (B20, B22, H7, K9, L14, N6). The results for adults have generally shown that there is no sex differences for any age group and that the well-known decrease in GFR with age is mirrored by an increase in the cystatin C level with age (Fig. 10). However, the decrease of GFR with age is slow before 50 years of age, and it has been suggested (N6) that it is sufficient for most practical purposes to use separate reference values only for the age groups 20–50 and above 50 years of age (Table 5).

The results for children (Fig. 11) have demonstrated that the cystatin C level, in contrast to the creatinine level, was constant for children beyond the first year and with no difference between the sexes (B20, B22, H7, R3). The recommended reference values for children beyond 1 year of age were virtually identical to those

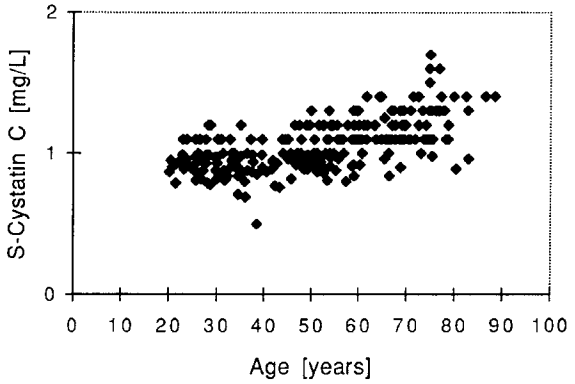

FIG. 10. Serum cystatin C in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. No sex difference was found.

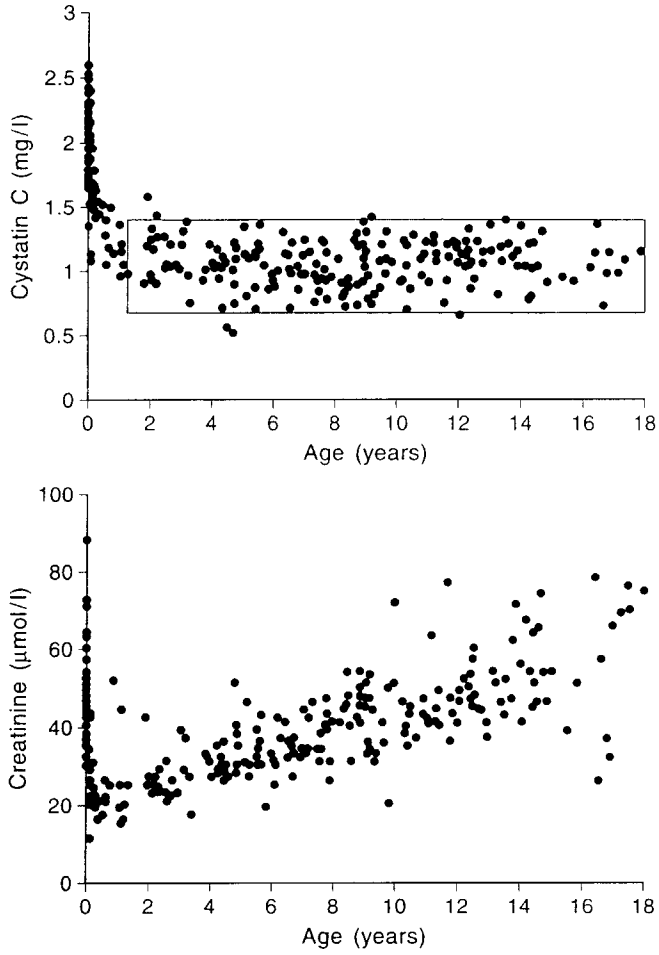

FIG. 11. Serum cystatin C (top) and creatinine (bottom) in relation to age in a population of 258 children, 1 day–18 years old, and without evidence of kidney disease. The boxed area represents the serum cystatin C reference interval for children over 1 year.

recommended for adults 20–50 years of age, so it might be justifiable to use the same values for both age groups (Table 5). It should be emphasized, however, that efficient international use of serum cystatin C as a GFR marker requires the establishment of a generally available international cystatin C calibrator. The relatively large variation in the reference values for serum cystatin C suggested so far (R1, R3) is most probably due to the use of different, often ill-described, cystatin C calibrators.

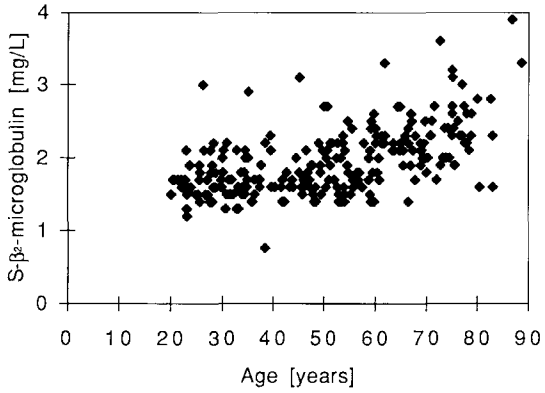

FIG. 12. Serum β_2-microglobulin in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. No sex difference was found, but the influence of age on glomerular filtration rate and thus on serum β_2-microglobulin is evident.

Since all plasma proteins with molecular masses below 15–25 kDa are almost freely filtered through the normal glomerular membrane, their serum concentrations in a person will be strongly influenced not only by their production rates, but also, and often to at least the same extent, by the person's GFR. Thus, when the production rates of such low-molecular-mass proteins are of interest for evaluation of biomedical processes, the diagnostic specificity of the ratios between the serum concentrations of such proteins and cystatin C can be expected to be higher than that of the serum concentration of each specific protein. For example, the influence of age on GFR and thus on the serum β_2-microglobulin level (Fig. 12) is not seen for the ratio between the serum levels of β_2-microglobulin and cystatin C (Fig. 13) (N6). This ratio might thus be a more specific marker for cell proliferation than the isolated serum β_2-microglobulin level.

10.5. Limitations in the Use of Serum Cystatin C as a Marker for GFR

When the first automated particle-enhanced immunoturbidimetric method was introduced, it was claimed to be undisturbed by hypertriglyceridemia (K9). However, widespread clinical use of it since then has demonstrated that the results are influenced by sample turbidity caused by, *inter alia,* chylomicronemia, which might produce both falsely low and high values for serum cystatin C (Nilsson-Ehle, P., submitted manuscript; Grubb, A., unpublished results). The influence of chylomicronemia on the analytical procedure might partly explain the relatively large biological variation reported for serum cystatin C (K3) as well as the outcome of some studies which have failed to show any advantage of serum cystatin C over serum creatinine as a GFR marker. Studies of the biological variation of serum

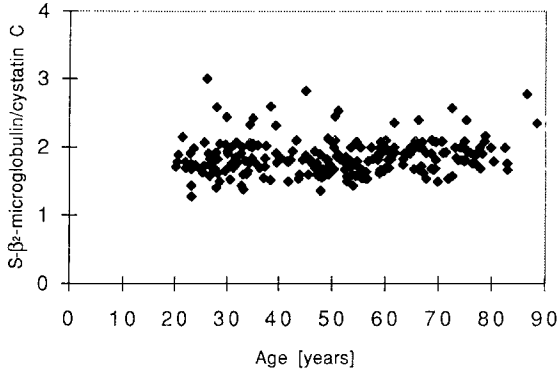

FIG. 13. Serum β_2-microglobulin/cystatin C ratio in relation to age in a population of 121 healthy women and 121 healthy men, 20–89 years old. The serum β_2-microglobulin/cystatin C ratio is, in contrast to the serum levels of each separate protein, not influenced by the age-dependent decrease in glomerular filtration rate.

cystatin C, using nonturbid samples, indicate that it is comparable to that of serum creatinine (N5) (Nilsson-Ehle, P., submitted manuscript).

It has also been observed that some, but not all, rheumatoid factors interfere in some of the presently used particle-enhanced immunoturbidimetric methods and produce erroneously high results (Grubb, A., unpublished results).

It should also be emphasized that although the precision of the automated particle-enhanced immunometric methods is higher than that of the enzyme-amplified single radial immunodiffusion first used to quantitate serum cystatin C, it is still lower than that for most methods for determination of serum creatinine. Moreover, the data for the intraindividual variation of serum cystatin C strongly indicate that a higher precision of the method would markedly improve the clinical usefulness of serum cystatin C determinations according to the criterion of Cotlove (N6).

10.6. Recommended use of Serum Cystatin C as a GFR Marker

Available evidence indicates that serum cystatin C is a better marker for GFR than serum creatinine, particularly for the identification of an initial small decrease in GFR, i.e., in the so-called creatinine-blind GFR range. The most efficient use of this knowledge in clinical practice requires that quantitative methods of good precision, undisturbed by sample turbidity, are used. At least some of the presently available particle-enhanced immunometric methods seem to fulfil the first criterion of acceptable precision, but nonturbid fasting samples should preferably be used until new methods undisturbed by turbidity are developed.

Combined use of serum cystatin C and creatinine produces the best possible information on GFR in situations where more accurate, but invasive and more expensive, clearance determinations cannot be performed for biomedical or economic reasons. If both serum cystatin C and creatinine are within the relevant reference limits, the risk of missing a decrease in GFR will be minimal.

In situations when the GFR has been determined by accurate invasive clearance methods, either serum cystatin C or creatinine might be used to follow changes in GFR. However, as long as the precision of the methods for creatinine determination is higher than that of the methods for cystatin C determination, there are presently no valid reasons to use serum cystatin C for this purpose, particularly since creatinine determinations generally are cheaper than cystatin C determinations.

It should be observed that there is a clear possibility that some renal disease processes might differently affect the filtration of cystatin C, a positively charged 13,000-Da molecule, and the filtration of creatinine, an uncharged 113-Da molecule. Indeed, although serum cystatin C has been described to be a better GFR marker than serum creatinine for patients with renal transplants (R8), transplanted and untransplanted patients with the same reduction in inulin clearance have been reported to display different levels of serum cystatin C (B21). Future studies might therefore show that the use of several GFR markers, differing in physicochemical properties, might be optimal for the noninvasive monitoring of kidney function.

11. Urine Cystatin C as a Marker for Proximal Tubular Damage

Since cystatin C is a low-molecular-mass protein, it is almost freely filtered through the normal glomerular membrane and then nearly completely reabsorbed and degraded by the normal proximal tubular cells. The urine level of cystatin C is therefore low in healthy individuals. The mean urine cystatin C in an adult healthy population has been estimated to 0.095 mg/liter or 8.0 mg/mol creatinine with a range of 0.033–0.29 mg/liter or 5.2–13.3 mg/mol creatinine (L15). Proximal tubular dysfunction results in impaired reabsorption of low-molecular-mass proteins and increased urinary excretion of cystatin C can therefore be used as a sensitive marker for disease processes affecting proximal tubular cells (A1, C10, K1, K2, L15, L16, T5). However, the practical use of the urine level of cystatin C as a marker for tubular dysfunction is hampered by two facts. First, the upper reference limit for the urine concentration of cystatin C is so low (L15) that presently available rapid and cheap immunochemical methods cannot be used to demonstrate the small increases in the urinary levels of cystatin C which signal initial, and often reversible, stages of proximal tubular dysfunction. Second, cystatin C is proteolytically degraded in a significant proportion of native urine samples

(G13, T7). Although these two difficulties can be overcome by the use of sensitive, more sophisticated quantitative methods and the addition of preservatives to urine samples (A1, T2), respectively, this state of affairs argues against the practical use of urine cystatin C as a marker for proximal tubular dysfunction (G13). The urine level of free protein HC (*alias* α_1-microglobulin) seems to be a more practical marker for proximal tubular dysfunction as the upper reference limit is high (about 8 mg/liter or 700 mg/mol creatinine) and since protein HC is stable in most native urine samples (G13, T2, T3). But it cannot be excluded that future studies will display that the diagnostic potentials of urine cystatin C and urine protein HC might differ in some disease states, since cystatin C is positively charged at the pH range of urine, while protein HC is negatively charged.

12. Cystatin C and Cerebral Hemorrhage

Cystatin C amyloid deposits have been demonstrated to be associated with two types of brain hemorrhage. One type is the dominantly inherited form of cerebral hemorrhage, which is caused by a mutation in the cystatin C gene and displays amyloid deposition of the cystatin C variant but no co-deposition of amyloid β-protein. The other type comprises the cerebral hemorrhage conditions, which are connected with cerebral deposition of amyloid β-protein and display co-deposition of wildtype cystatin C in the amyloid.

12.1. Cystatin C and Hereditary Cystatin C Amyloid Angiopathy

The Icelandic physician Árni Árnason described in 1935 several families suffering from high incidences of cerebral hemorrhage affecting young adults and showed that the disease displayed autosomal dominant inheritance (Fig. 14) (A12). Afflicted individuals generally suffered from their first cerebral bleeding before 40 years of age and subsequently from recurrent multifocal cerebral hemorrhages. It was not possible to identify carriers of the trait in the affected families, but Gudmundsson *et al.* showed in 1972 that the disease was associated with amyloid deposits mainly located in the media of the walls of cerebral medium-sized arteries and suggested "hereditary cerebral hemorrhage with amyloidosis (HCHWA)" as a suitable name for the syndrome (G14). The N-terminal sequence of a main component of the amyloid fibril was determined in 1983 and found to be identical to the sequence of cystatin C starting at residue 11 (C9). Subsequent immunohistochemical studies corroborated that cystatin C was a major component of the amyloid deposits (L13) and showed that also vessels and other tissues outside the central nervous system contained cystatin C amyloid deposits (B9, L13). In 1984 immunochemical quantitation of the level of cystatin C in cerebrospinal fluid demonstrated that virtually all carriers of the trait for the disease, independent of whether they

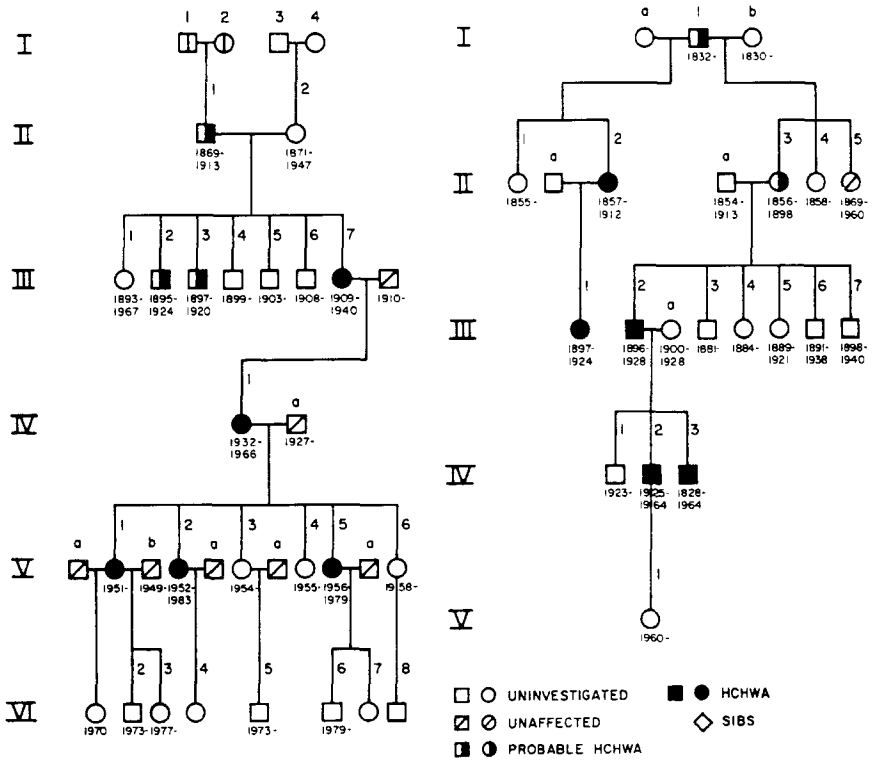

FIG. 14. Transmission in two families of the allele causing hereditary cystatin C amyloid angiopathy, HCCAA (also called hereditary cerebral hemorrhage with amyloidosis, HCHWA).

had suffered from their first cerebral hemorrhage or not, could be identified by their low cystatin C concentration (G10, J3). Not only did this observation allow the identification of healthy carriers of the disease trait, but also suggested that the pivotal pathophysiological process was an abnormal metabolism of cystatin C. Subsequent studies showed that the complete sequence of the cystatin C polypeptide chain deposited as amyloid in one individual with brain hemorrhage differed in one position from the sequence previously determined for cystatin C isolated from a Swedish patient without cerebral hemorrhage (G2). It was therefore suggested that a point mutation responsible for this amino acid substitution, a glutamine residue replacing a leucine residue at position 68 of the cystatin C polypeptide chain, could constitute the genetic background to the disease, although it also might represent a disease-unrelated polymorphism (G2). The potential disease-causing mutation would, according to the sequence later determined for a full-length cDNA encoding cystatin C, destroy an *Alu* I-cleavage site in the cystatin C gene (A3). This

observation allowed the identification of a restriction fragment length polymorphism permitting identification of carriers of the mutation (P1) as well as the construction of a simple and rapid polymerase chain reaction (PCR)-based procedure for the same purpose (A6). When individuals from seven afflicted families were studied, it was observed that all individuals suffering from early-onset cerebral hemorrhage carried one mutated allele, while most healthy relatives and all unrelated persons did not (A6). The results of these studies therefore permitted the conclusion that the investigated mutation causes the disease and that all patients are heterozygous (A6, P1).

12.1.1. *Nomenclature*

Two designations for the disease originally described by Árnason are presently used, *viz.*, hereditary cystatin C amyloid angiopathy (HCCAA) and "hereditary cerebral hemorrhage with amyloidosis, Icelandic" (HCHWA-I). The first mentioned might be the preferred one, since it indicates the amyloid-forming protein and agrees with the recent observations that the amyloid depositions are neither confined to the cerebral vasculture (B9, L13), nor to Icelandic patients (G5). It is also an appropriate designation for the condition before the first cerebral hemorrhage has occurred.

12.1.2. *Clinical Considerations*

The disease should be considered when a normotensive, previously healthy individual below 40 years of age is hit by a severe cerebral hemorrhage and particularly if young relatives previously have suffered from brain hemorrhage. Recurrent cerebral hemorrhages generally occur and result in increasing motor disability and gradual loss of mental functions, but in some individuals the disease does not progress for several years and a few carriers of the mutated allele attain a normal life span (J2, O1). In a few cases the presenting symptom may be dementia rather than brain hemorrhage (J3). The disease is not uncommon in the Icelandic population, with more than 150 cases described during the last 70 years, but it is rare (G5) or not described in other populations (G4, M8).

12.1.3. *Diagnosis*

When HCCAA is suspected because of clinical observations and/or family history, the diagnosis can easily be verified by demonstrating a low cerebrospinal fluid level of cystatin C (G10, J3) or the presence of the mutated cystatin C allele producing the Leu 68→Gln cystatin C variant (A6, P1). The first-mentioned procedure requires lumbar puncture and, to secure a stable level of cystatin C in the sample, the addition of a serine protease inhibitor, e.g., benzamidinium chloride, directly when the sample is drawn (Grubb, A., unpublished results). The PCR-based procedure is more robust, does not require lumbar puncture, and can be used for prenatal diagnosis and is therefore presently the preferable diagnostic method.

12.1.4. *Molecular Pathophysiology*

It has been possible to produce the disease-causing Leu68→Gln cystatin C variant in an *Escherichia coli* expression system (A4). Parallel studies of Leu68→Gln cystatin C and the wild-type protein have revealed that although both are efficient inhibitors of cysteine proteases, they differ considerably in their tendency to dimerize and form aggregates. While wild-type cystatin C is monomeric and functionally active even after prolonged storage at elevated temperatures, Leu68→Gln cystatin C starts to dimerize and lose inhibitory capacity immediately after its isolation. The dimerization of Leu68→Gln cystatin C is highly temperature-dependent, with a rise in incubation temperature from 37 to 40°C resulting in a 150% increase in dimerization rate and a considerable concomitant rise in the formation of larger aggregates (Fig. 15). These observations might suggest

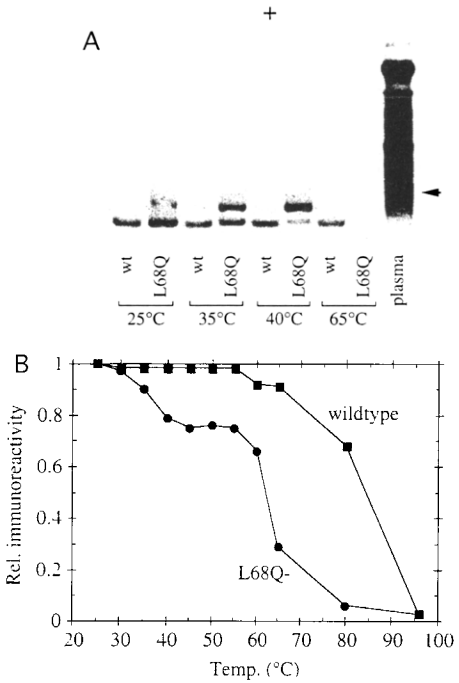

FIG. 15. Temperature stability of wild-type cystatin C and of L68Q-cystatin C, the cerebral hemorrhage-producing cystatin C variant. Samples of L68Q- and wild-type (wt) cystatin C were incubated for 30 min at various temperatures. (A) shows agarose gel electrophoresis at pH 8.6 of selected samples. The point of application and the anode are marked by an arrow and a plus sign, respectively. (B) shows the remaining cystatin C-immunoreactivity of sample supernatants after incubation and centrifugation as determined by single radial immunodiffusion.

a pathophysiological process in which Leu68→Gln cystatin C, due to its tendency to spontaneously aggregate, is partly trapped intracellularly and not secreted from the cell as efficiently as the wild-type protein. Continuous intracellular accumulation of Leu68→Gln cystatin C would, in combination with its specific physicochemical properties, lead to amyloid formation, cell damage, and death (A4). Recent *in vitro* studies have indeed shown that the intracellular processing of Leu68→Gln cystatin C differs from that of wild-type cystatin C (B15) and results in the formation of stable dimers that are partially retained in the endoplasmic reticulum (B11). It has also been asserted that no Leu68→Gln cystatin C is secreted from the cells of HCCAA patients (A13), but this has been questioned (B11). The temperature dependence of the aggregation of Leu68→Gln cystatin C might be of clinical relevance, since medical intervention to abort febrile episodes might reduce the *in vivo* formation of aggregates in carriers of the disease trait and thus possibly delay the point of time for their initial brain hemorrhage (A4).

12.2. Cystatin C and Cerebral Hemorrhage Conditions Connected with Deposition of Amyloid β-Protein

Cerebral amyloid angiopathy with wild-type, or a variant of, amyloid □-protein as the major amyloid constituent, is a condition with a high prevalence in the elderly and is also commonly found in patients with Alzhemier's disease or Down's syndrome (G6, H1, I3, M5, V3). The condition is associated with cerebral hemorrhage and may account for more than 10% of the brain hemorrhage cases in the elderly (I3). Immunohistochemical investigations of the amyloid deposits have demonstrated that all, or a considerable portion of them, display cystatin C immunoreactivity in addition to their amyloid β-protein immunoreactivity (B10, H1, I3, M5, M6, V3). Quantitative estimations have generally indicated that cystatin C is a minor constituent of the deposits, however (I3, M5, M6, V3). Efforts to demonstrate the presence of cystatin C variants, e.g., the one producing HCCAA, in the amyloid deposits have so far been unfruitful (A11, I4, M6, N1). It has been reported that the cerebrospinal fluid level of cystatin C is low in some of these conditions (S4), but if this observation can be used for diagnostic purposes is still uncertain. It is evident that the pathophysiological significance of the occurrence of cystatin C as a minor constituent in the amyloid deposits of these conditions remains to be determined.

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