

# Cystatins in Health and Diseases

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**Abstract** The Cystatins constitute a large group of evolutionary related proteins with diverse biological activities. They have been recently realized as instrumental in myriad of pathophysiological conditions. They have been implicated in various pathological conditions. The degree of malignancy of various types of cancer cells has been found to be inversely associated with the expression of cystatins. Cystatins have been found to have various antimicrobial, antiviral and immunomodulatory properties. Keeping in view as their being prospective drug targets and anti-disease options this review explores the role of cytoplasmic and cell secreted cystatins in various human diseases.

**Keywords** Cystatins · Glomerular filtration rate · Amyloid angiopathy · Cancer · Immunomodulation

## Introduction

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 equilibria. The balance between protein production and degradation is one of these equilibria, which are crucial to health. Degradation of proteins is brought about by proteolytic enzymes, proteases, which based on their catalytic mechanisms can be sorted into four major classes; the serine, cysteine, aspartic and metallo-proteases.

Cysteine proteases comprise a group of proteolytic enzymes that cleave the peptide bonds by the use of a reactive cysteine residue at the catalytic site. The action of these proteolytic enzymes is biologically controlled by proteinase inhibitors. Cystatins constitute a powerful regulatory system for endogenous cysteine proteinases which are often secreted or leaking from the lysosomes of dying or diseased cells. Cystatins are proteins that tightly bind and inhibit the harmful effect of cysteine proteinases (Ekiel et al. 1997). These proteins are all related by structure and function to an inhibitor of cysteine proteinases which was first described in egg white and called as chicken egg white cystatins. Cystatins have been evolutionary related forming the “Cystatin Superfamily”. The members of a protein superfamily were grouped into three families on the basis of their location, size and complexity of polypeptide chains (Barrett et al. (1986a, b).

*Members of family 1*, the stefins are found primarily intracellularly, contain about 100 amino acid residues (~11 KDa) and lack disulfide bonds. Known proteins of this family have been named “human stefin”, “human cystatin A”, “rat cystatin”, “human cystatin B” and rat cystatin B”.

*Members of family 2*, the cystatins are found primarily in body fluids and in tissues also. These contain about 120 amino acid residues (~14 KDa) and two intrachain disulfide bonds. The salivary cystatins, cystatin C and chicken egg white cystatin belong to this family.

*Family 3* comprises the plasma kininogens and may therefore also be called the kininogen family. Three types of Kininogens, the low molecular weight kininogens (LMWK), high molecular weight (HMWK) and T-kininogens are single chain glycoproteins with molecular weight of ~50–1,200 KDa. They contain additional disulfide bonds and are also glycosylated. Cystatins have an

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important regulatory role in normal body processes involving cysteine proteinase activity. Because these cysteine proteinases exist in all living organisms and because they are involved in various biological and pathological processes the control of their protease function by cystatins is of cardinal importance. An increasing attention is being paid to the physiological significance of these natural inhibitors in pathological processes. The reason for this growing interest is that uncontrolled proteolysis can lead to irreversible damage and detrimentally affect human health by facilitating diseases. This review focuses on the possible role of cystatins, natural and specific inhibitors of cysteine proteinases in pathological processes.

### Cystatin C: A New Serum Marker for Glomerular Filtration Rate

Cystatin C is found in all body fluids and tissues of mammals. It is a jack-of-all trades, playing a broad role in many functions. A large number of normal and pathological processes are controlled by the balance between proteinases and their inhibitors. The physiological role of cystatins is believed to be the protection of cells forming appropriate endogenous or exogenous proteolysis by the regulation of cysteine proteinases both host and of microbial origin.

Levels of cystatin C in various body fluids is used as a barometer of disease. Cystatin C is a non-glycosylated, low molecular mass (13 KDa) protein produced by all nucleated cells. Its low molecular mass and its high isoelectric point (pI) allow it to be freely filtered by the glomerular membrane. The serum concentration of cystatin C has been shown to correlate with Glomerular Filtration Rate GFR, See Table 1 in combination with a stable production rate, has been found to be a sensitive marker of GFR (Jung and Jung 1995; Simnonse et al. 1985; Grubb et al. 1985; Grubb 1992).

Serum levels of cystatins C are independent of weight, muscle, mass, age (>/year) or variable than those with

creatinine. Furthermore measurements can be made and interpreted from a single random sample.

Chronic kidney disease is a risk factor for heart failure (HF). Although cystatin (C) can detect early kidney-dysfunction, little is known about the association between cystatin (C) and HF. Djousse et al. (2008) demonstrated that higher levels of cystatin C are associated with an increased HF and that such association may be limited to hypertensive individuals.

Individuals infected with HIV had substantially worse kidney function when measured by cystatin C level compared with HIV negative controls, whereas mean creatinine levels and estimated glomerular filtration are similar. Cystatin C measurement could be a useful clinical tool to identify HIV-infected person at increased risk for kidney and cardiovascular diseases (Odden et al. 2007).

### Role of Cathepsins and Cystatins in Patients with Recurrent Miscarriage

Cathepsins and their endogenous inhibitors cystatins were evaluated in tissue and serum of patients with recurrent miscarriage. Deciduas and villi were surgically collected from 22 patients and 12 healthy women. Immunohistochemistry was performed with antibodies against cathepsin, stefin A, (cystatin A, stefin B, cystatin B) and cystatin C. The concentrations of cathepsins, stefins and cystatin C were measured by enzyme-linked immunosorbent assay. Serum level of cystatin C in 85 Japanese women with recurrent miscarriage was also measured. Staining of cathepsin B, D, H, L, stefin B and cystatin C was observed in the cytoplasm of epithelial cells in decidua.

Stefin A was found to be expressed on the surface of the trophoblast. The concentration of cathepsin B and H in patients decidua was found to be significantly higher than in control individuals. The serum level of cystatin C was found to be significantly lower in patients than in control individuals. These findings suggest that the regulation of the cathepsin cystatin system may play an important role in patients with recurrent miscarriage (Tamao et al. 2005). A study was carried out by Despina et al. (2007) to investigate circulating levels of cystatin C (an endogenous marker of renal function) in mothers, fetuses and neonates from intrauterine growth restricted (IUGR; characterized by impaired nephrogenesis) and appropriate for gestational age (AGA) pregnancies. Cystatin C levels did not correlate with gestational age and did not differ between males and females. Fetal cystatin C serum levels are lower in the IUGR group, significantly decrease after birth and do not correlate with maternal levels in both groups. However cystatin C levels positively correlate with respective creatinine and urea levels in the perinatal period.

**Table 1** Correlation between GFR and creatinine Or Cystatin C concentration in 51 patients

Number of patients	All	Normal	Reduced <sup>a</sup>
Number of patients	51	27	24
1/cystatin C vs. GFR	0.87 <sup>b</sup>	0.39 <sup>c</sup>	0.91
1/creatinine vs. GFR	0.71	0.17 <sup>d</sup>	0.83
1/cystatin C vs. creatinine	0.73	0.19 <sup>d</sup>	0.79

<sup>a</sup> Ichexol clearance <80 ml/min per 1.73 m<sup>2</sup>

<sup>b</sup>  $P < 0.05$  better than the value for 1/creatinine vs. GFR

<sup>c</sup>  $P = 0.03$

<sup>d</sup> Not significant

### Cystatins and Amyloid Fibrillation

Human stefin B is a cysteine proteinase inhibitor. It is an intracellular protein expressed in many types of cells, located in the cytoplasm and nucleus. It has been found as part of a multi protein complex specific to the central nervous system (Giaimo et al. 2002). The main pathology for this proteinase inhibitor is its role in monogenic epilepsy, a progressive myoclonus epilepsy of type 1 (EPM1), termed unverricht Lund borg disease. The disease was previously linked to human chromosome 21 q 22.3. The gene encoding of cystatin B was shown to be localized in this region and levels of messenger RNA encoded by this gene were found to be decreased in cells from affected individuals. Two mutations a 3' splice site mutation and a stop codon mutation, were identified in the gene encoding cystatin B in EPM1 patients but were not present in unaffected individuals. These results provide evidence that mutations in the gene encoding cystatin B are responsible for the primary defect in patients with EPM 1 (Len et al. 1996).

Cystatin C, a serum measure of renal function, has been reported as a strong predictor of risk of death and cardiovascular events in elderly people. Ni et al. (2007) found that total plasma cystatin C levels were significantly higher in patients than controls. Elevated cystatin C levels were independently associated with both ischemic and hemorrhagic stroke and cystatin C was a strong predictor for risk of cardiovascular events and death.

### Cystatins and Cerebral Amyloid Angiopathy

Hereditary cystatin C amyloid angiopathy (HCCAA) is a rare, fatal amyloid disease in young people in Iceland caused by a mutation in cystatin C, which is an inhibitor of several cysteine proteinases such as cathepsins, S, B and K.

Mutated cystatin C forms amyloid, predominantly in brain arteries and arterioles, but also to a lesser degree in tissues outside the central nervous system such as skin, lymph nodes, testis, spleen, submandibular salivary glands and adrenal cortex. The amyloid deposition in the vessel walls causes thickening of the walls leading to occlusion or rupture and resulting in brain hemorrhage (Palsdottir et al. 2006).

### Cystatins and Cancer

Cathepsin B secretion may be important in penetration of the extra cellular matrix during metastasis, and cystatin C may be involved in regulating this process (Corticchiato et al. 1992; Dickinson 2002). The up regulation of cystatin F and down regulation of cystatin M associated with cancer is also indicative of a functional association. A correlation

between high serum levels of cystatin C and higher risk of death in colorectal cancer patients has been found (Kos et al. 2000).

### Inhibitory Properties of Low Molecular Mass Cysteine Proteinase Inhibitors from Human Sarcoma

Elevated activities of cysteine proteinases such as cathepsins B and L cancer procougarant have been linked to tumor malignancy. Elevated activities could be due to impaired regulation by the endogenous low molecular mass cysteine proteinase inhibitors (cystatins). It was found that the extract of cystatins from sarcoma was less effective against papain and cathepsin B than was extract from liver.

Inhibitory property of two members of the cystatin superfamily (stefin A and stefin B) was determined. It was found that stefin B from liver and sarcoma exhibited comparable inhibition of papain and cathepsin B. In contrast, stefin A from sarcoma exhibited a reduced ability to inhibit papain, human liver cathepsins B, H, L and human and murine tumor cathepsin. The  $k_i$  for inhibition of liver cathepsin B by sarcoma stefin A was 10-fold higher than that for inhibition of liver cathepsin B by liver stefin A, reflecting a reduction in the rate constant for association and an (increase) in the rate constant for dissociation. Lah et al. (1989).

### CMAP: A Novel Cystatin-Like Gene Involved in Liver Metastasis

A novel metastasis-associated gene was identified with a differential display system in murine carcinoma cells showing a high rate of metastasis to the liver. A human homologue was also identified using a PCR-based strategy. The protein coded by this gene was named cystatin like metastasis-associated protein (CMAP) and showed 22.1–28.1% homology to human family 2 cystatins. CMAP mRNA was selectively expressed in all murine liver metastatic tumors but not in any pulmonary metastatic tumors examined. Transfection of CMAP antisense DNA into highly metastatic liver cells greatly decreased their metastasis potential and CMAP expression indicating that CMAP is involved in liver metastatic ability after intravastation of malignant cells (Masashi et al. 1999).

### Cystatins and Injury

Cystatins C appears to be up-regulated in response to injury in the brain. Cystatin C protein was detected by immunohistochemistry in few of the hippocampal pyramidal cells of the normal rat brain, but was present in these cells

3 days after experimental ischemia (Palm et al. 1995). It was localized to morphologically degenerative neurons, and absent from morphologically viable neurons.

In all brains from Alzheimers disease affected individuals but not in majority of normal brains, strong localization of cystatin C protein was found in the pyramidal neurons regions of the brain most susceptible to cell death in this disease (Deng et al. 2001). The exact function of cystatin C in the brain and its role in injury are presently unknown. Its appearance would be consistent with a protective role, perhaps by blocking CP activity in damaged cells to allow for recovery or by acting as a growth factor. However, based on its association with damaged cells, it is conceivable that it may be a mediator of injury.

### Antimicrobial and Antiviral Activities

The cystatin isolated from horse-shoe crab hemocytes has antimicrobial activity against Gram-negative bacteria, with IC 50s against *Salmonella typhimrium*, *Escherchia coil* and *Klebsiella penumoniae* in the 80–100 µg/ml range (Agarwala et al. 1996).

Both chicken cystatin and human cystatins were found to inhibit growth of *P-gingivalis* with an IC 50 of 1.1. and 1.2 FM, respectively with an apparent bactericidal activity (Blank et al. 1996).

Cystatin C is also an effective inhibitor of replication of coronavirus, which can cause acute gastroenteritis, at slightly above physiological levels (Collins and Grubb 1998).

### Cystatin C in Heart

The role of cystatin C in the heart under physiological and pathological conditions remains to be established. The structure of the rat cystatins cDNA suggests that cystatin C is a secretory protein. Whether the inhibitor is released by the muscle fibers under physiological conditions or acts intracellularly is not known.

It is conceivable however that under pathological conditions, particularly in ischemia, the inhibitor is released and plays a role in modulating the activities of the extracellular cathepsins derived from monocytes and or inflammatory cells. Under such conditions, cystatin C or its fragment may also affect the chemotactic and phagocytic functions of granulocytes (Leung et al. 1990a, b).

### Immuno Modulation

Cystatins have been shown to have a wide range of effects in immune cells. Pre-treatment of human neutrophils with

chicken cystatin (and the broad inhibitor E-64) was found to inhibit chemotaxis induced by C5a, but not by formyl-methionine leucine phenylalanine (fMLP) or interleukin 8 (IL-8) (Barna and Kew 1995).

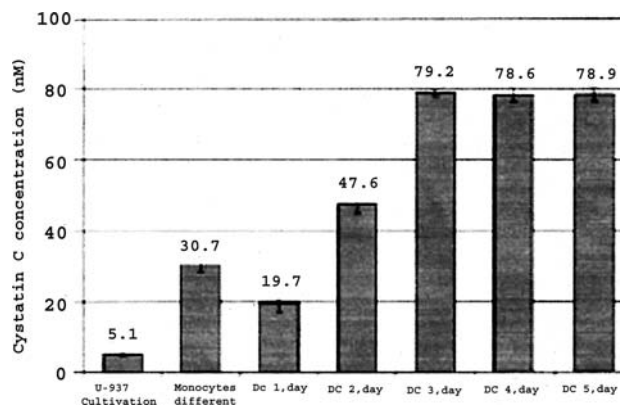
Inhibition had a sharp optimum around 10 µM, which is above normal physiological levels of cystatin C, but may reflect the use of a non-human protein. Cystatins also induce tumor necrosis factor alpha and interleukin 10 synthesis, and they stimulate nitric oxide production by interferon gamma activated murine macrophages.

In turn, nitric oxide has inhibitory activity on cysteine proteases, especially those from parasitic protozoa. Cystatins isolated from parasitic nematodes also have immunomodulatory activities that are distinguishable from those induced by lipopolysaccharide-like molecules from endosymbiotic bacteria.

Cystatin C secretion by monocytes and macrophages is itself decreased by IFN and LPS. Significantly mice given a lethal dose of *L. donovani* were completely cured by a combination of chicken cystatin and a suboptimal dose of IFN-gamma.

Cysteine proteases have essential functions in antigen presenting cells. To clarify, the role of CyC in human Dendritic Cells (DC) (Tina et al. 2005) first sought to determine whether CyC content, localization and secretion change in response to interleukin (IL)-4 which enables differentiation of monocytes to immature DC, and to maturation of these DC (Fig. 1).

Cystatin C is a potent, reversible inhibitor in vitro of the human lysosomal cysteine proteases e.g., Cat (S) (Ki = 8 pM), Cat L (Ki = 8 pM) and Cat H (Ki = 220 pM). These protease are all located along the endocytic pathway of dendritic cell and involved in the controlled proteolysis associated with the degradation of antigen to antigenic peptides (Pluger et al. 2002).



**Fig. 1** Secreted CyC determined by CyC-specific ELISA secreted CyC, was determined in culture media after 5 days of promonocyte U-937 cultivation, after 5 days of monocyte differentiation to immature DC, and for 5 days of DC maturation with TNF- $\alpha$ . Figure adopted from Tina et al. (2005)

Besides these specific tasks, they are also necessary for the intracellular catabolism of other proteins in late endosomes and lysosomes (Turk et al. 2000). To determine whether CyC can have an impact on Cat S, Cat L and Cat H in human DC, their content, intracellular localization and secretion have been defined and compared during the differentiation and maturation of DC *in vitro*. It was shown that in immature DC, CyC content was much higher than in monocytes and promonocyte U-937 cells. The low content of CyC in monocytes and promonocyte U-937 cells was not the result of increased CyC secretion, which was quite low compared with that in immature DC. High CyC secretion in immature DC accords with their strong endocytic activity but was not observed for promonocyte U-937 cells (Tina et al. 2005). or for monocytes (Repnik et al. 2003). Therefore the low CyC content in promonocyte U-937 cells and monocytes resulted from lower CyC expression.

It has thus been demonstrated that the content, localization and secretion of CyC are differentiation and maturation dependent. Changes in secretion may constitute an additional mechanism of DC for controlling their intracellular CyC content (Tina et al. 2005).

### Recent Advances

In the last decade enormous progress has been achieved in the field of cystatin and their target enzymes. Various programs like the random centroid optimization computer program for genetics (RCG) was applied to double site mutation for the entire sequence of human cystatin C (HCC) with 120 residues for improving its protease inhibitory activity. The RCG program selected two sites simultaneously and amino acid residues to replace the sites selected in the sequence in order to find the best papain-inhibitory activity and stability of the protease inhibitors (Masahiro et al. 2002).

### Conclusion

Despite these impressive advances in the field of cystatin engineering there remain a great many unanswered questions. A major challenge that still remains for the genetic engineers is perhaps the creation of ‘SUPER CYSTATIN’ that might enhance cysteine proteinase inhibitory activity. Such molecules may be used to inhibit enzymes of bacterial or viral origin and minimize microbial growth and subsequent tissue degradation. Although much work needs to be done, these new findings may open doors to slow, halt or reverse disease progression and forestall their devastating symptoms. To realize the full diagnostic potential of

cystatin more research needs to be performed on the interaction of all cystatin species found in the biological fluids, as well as the regulatory mechanisms that control their production. Further studies are required to better understand protease biology and facilitate clinical translation. In particular precise role of endogenous inhibitors still needs to be deciphered.

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