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# Cystatin C in the anterior segment of rat and mouse eyes

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## ABSTRACT.

**Purpose:** Cystatin C is a mammalian cysteine protease inhibitor. This study describes the localization of cystatin C in the anterior segment of normal rat and mouse eyes. Cysteine proteases play an important role in protein degradation (e.g. of photoreceptor outer segments in the retinal pigment epithelium) and the balance between these proteases and their specific inhibitors is therefore of great interest.

**Methods:** Cells containing cystatin C were identified by immunohistochemistry and quantified by ELISA. Messenger RNA levels were analysed by quantitative real-time polymerase chain reaction.

**Results:** Cystatin C is present at biologically significant levels in the corneal epithelium, endothelium and stromal keratinocytes, lens epithelium, epithelial cells in the ciliary processes, aqueous humour and iris stromal cells. In the rat anterior segment, the highest cystatin C concentrations were found in the ciliary epithelium.

**Conclusions:** Cystatin C is present in several cell types and is probably locally produced. The inhibitor is likely to be an important regulator of cysteine proteases in the retinal pigment epithelium, ciliary epithelium, aqueous humour, lens epithelium and in the corneal endothelium and epithelium.

**Key words:** cystatin C – cysteine protease – cornea – ciliary body – aqueous humour – iris – lens – anterior segment of eye

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## Introduction

Cystatins are protease inhibitors that regulate papain-like cysteine proteases belonging to the C1 enzyme family, such as cathepsins B, H, L and S (Brown & Dziegielewska 1997; Abrahamson et al. 2003). Cystatins regulate protease activity by reversibly binding to their active site clefts in competition with enzyme substrate. Known mammalian cystatins are all composed of at least one 100–120 amino acid residue

domain with conserved sequence motifs (Rawlings & Barrett 1990; Grubb 2000). Of the 12 human cystatins known, cystatin C is the most extensively studied: it shows a wide-spectrum inhibition profile and high-affinity binding to most C1 family cysteine proteases (Abrahamson et al. 2003; Grubb & Löfberg 1982). It is the dominating cysteine protease inhibitor in all body fluids examined and is present in amounts that allow it to control the extracellular activities of cathepsin B

and other C1 family cysteine proteases (Abrahamson et al. 1986).

Cystatin C has been detected in all body fluids examined. The concentration of human cystatin C is markedly higher in cerebrospinal fluid than in blood (Abrahamson et al. 1986). Cystatin C has also been detected in tear fluid (Abrahamson et al. 1986; Reitz et al. 1998). The highest amounts of cystatin C in tissue homogenates from humans, mice and rats are found in brain samples, at levels of 30–300 ng/mg protein (Håkansson et al. 1996). In the rat eye, cystatin C RNA has been studied using *in situ* hybridization (Barka & van der Noen 1994), by which cystatin C RNA was detected in the retina and sclera, but not in the cornea, ciliary body, iris, lens or choroid. We have previously shown that cystatin C is present in high concentrations, at levels of about 100 ng/mg protein, in the rat retina, where it is predominantly localized to the pigment epithelium (Wassélius et al. 2001). To our knowledge, cystatin C has not been studied immunohistochemically or quantitatively in the anterior segment of the eye. In this study we identify and describe the localization of cystatin C in the anterior segment of rat and mouse eyes.

It is usually assumed that the principal biological role of cystatins involves acting as inhibitors of one or more target proteases, but the identification of target proteases of biomedical relevance remains enigmatic in most cases (Grubb 2000). The eye offers highly specialized and extensively examined tissues, a fact that facilitates study of

the functions of their specific components. In this study, we show that different tissues in the eye produce and contain high amounts of cystatin C (retinal pigment epithelium, ciliary epithelium, lens epithelium and corneal epithelium and endothelium).

## Material and Methods

### Animals

Normal adult pigmented PVG rats and C57BL mice were used in the study. The experiments and the animal care were carried out according to the ARVO Convention for Ophthalmological Animal Experimentation and approved by the Swedish Committee for Animal Experimentation Ethics. The animals were killed by carbon dioxide asphyxiation. The anterior chamber was then perforated with a needle and aqueous humour aspirated. After enucleation, the eyes used for histology were perforated with a needle to enable the fixative to penetrate rapidly. Tissues were fixed at 4° for 4 hours in 4% formaldehyde in 0.1 M phosphate buffer at pH 7.4 and further processed for immunohistochemistry. Tissues used for ELISA were rapidly dissected after the animals had been killed and instantly frozen on dry ice and kept at -80°, awaiting further processing. Tissues used for quantitative real-time polymerase chain reaction (QRT-PCR) were similarly dissected and frozen in TRIzol® (Life Technologies, Grand Island, New York, USA) on dry ice.

### Antibodies

A monospecific, polyclonal rabbit anti-serum raised against cystatin C isolated from human urine (Abrahamson et al. 1986) was used. This has previously been shown to give good morphological results (Wassélius et al. 2001).

Secondary antibodies raised against rabbit IgG and conjugated to fluorescein isothiocyanate (FITC) or Texas Red® were obtained from Southern Biotechnology Associates Inc., Birmingham, Alabama, USA and Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA, respectively.

### Immunohistochemistry

Cryostat sectioning (12 µm) and immunolabelling were performed with standard procedures. Labelling control experi-

ments included using different secondary antibodies, omitting the primary antibodies, preabsorbing the primary antibodies with excess recombinant human cystatin C (Abrahamson et al. 1988) and analysis of unlabelled sections for identifying possibly confounding autofluorescence.

### Cystatin C ELISA

Cystatin C concentrations were determined by a double sandwich ELISA as previously described (Håkansson et al. 1996). Recombinant mouse or rat cystatin C was used for appropriate calibration curves. To avoid possible interference with cysteine proteases released at the generation of the homogenates, samples were diluted, so that measurements were obtained at the low end of the calibration curve, with cystatin C concentrations of about 1–5 ng/ml (75–375 pM) in the sample. This is below the equilibrium constant for cystatin C binding to cathepsin B, which is likely to be the predominant confounder in tissues. The majority of cystatin C cysteine protease complexes should thus be dissociated at these concentrations, minimizing interference due to shedding of cystatin C epitopes in cysteine protease complexes. Cystatin C concentration was related to total protein concentration in the homogenates, the latter measured by a dye-binding assay (Bradford 1976).

### Quantitative real-time polymerase chain reaction

Total RNA was prepared from tissue samples essentially as described by Chomczynski & Sacchi (1987), but using TRIzol® in place of guanidinium isothiocyanate. DNA complementary to mRNA (cDNA) was synthesized from 1 µg to 10 µg total RNA by reverse transcriptase (Applied Biosystems, Foster City, California, USA). Regular reverse transcriptase-PCR was performed using AmpliTaq Gold in standard buffer (Applied Biosystems, Foster City, California, USA) and primers at 0.4 µM concentration in an ABI 2400 temperature cycler, by 35 cycles of denaturation (94°, 30 seconds), annealing (58°, 30 seconds) and extension (72°, 30 seconds), with a 5-min preincubation period at 94° before the temperature cycling. The primer pairs used, KH727/KH728 and KH723/KH724, giving specific PCR products

of 454 base pairs (bp) and 152 bp for rat and mouse cystatin C, respectively, have been described previously (Wassélius et al. 2001).

For estimation of cystatin C mRNA levels, quantitative real-time PCR (QRT-PCR) with TaqMan probes and a 7700 Analyser (Applied Biosystems, Foster City, California, USA) were used. For this purpose, cDNA was synthesized with TaqMan reverse transcription reagent (Applied Biosystems) using 50 ng RNA as a template and random hexamers as primers in a total reaction volume of 100 µl. Quantitative real-time-PCR was run with TaqMan Universal Mastermix (Applied Biosystems) in a total reaction volume of 25 µl. The probes were designed by aid of Primer Express 1.5 (Applied Biosystems) and synthesized by DNA Technology A/S, Aarhus, Denmark or Applied Biosystems. The QRT-PCR assay for rat cystatin C relied on primers MA563 (5'-TGC GTA CCA CAG CCG C-3') and MA564 (5'-CCA TCT CCA CAT CCA AAT AAT AGT -3') corresponding to a coding strand sequence in exon 1 (nt 143–158 in the cDNA sequence; GenBank/EMBL acc. X16957; Cole et al. 1989) and a non-coding strand sequence in exon 2 (nt 228–205 in the cDNA) of the rat gene, respectively. The probe (MA565) corresponded to a coding strand sequence spanning over the intron 1 junction (nt 160–190 in the cDNA) and had the sequence 5'-Fam- CCA TAC AGG TGG TGA GAG CTC GTA AGC AGC T-3'-Tamra. The QRT-PCR assay for mouse cystatin C utilized primers MA571 (5'-CAA CAA GGG CAG CAA CGA T-3') and MA572 (5'-TCC AGC CAC GAG CTG CTT -3'), corresponding to a coding strand sequence in exon 1 (nt 186–204 in the cDNA sequence; GenBank/EMBL acc. M59470; Solem et al. 1990) and a non-coding strand sequence encompassing the intron splice site between exons 1 and 2 (nt 261–244 in the cDNA; Huh et al. 1995). The probe (MA573) corresponded to a coding strand sequence in exon 1 (nt 206–227 in the cDNA sequence) and had the sequence 5'-Fam-CGT ACC ACA GCC GCG CCA TAC A-3'-Tamra. As endogenous control, 18S rRNA was amplified with commercially available primers/probes (Eukaryotic 18S rRNA endogenous control; Applied Biosystems). Five, four and 2 µl cDNA were

analysed in the rat cystatin C, mouse cystatin C and 18S rRNA assay, respectively. All QRT-PCR experiments were performed following the guidelines supplied by Applied Biosystems.

To avoid problems with absolute quantification of mRNA, levels were determined relative to a calibrator mRNA. The calibrator for rat assays was a pooled batch of rat liver cDNA and that for mouse assays was a pooled batch of NIH/3T3 cell cDNA; these were included on all assay plates. To generate standard curves, a dilution series of a standard cDNA from rat brain was run in triplicate for the rat assays, and a dilution series of NIH/3T3 cell cDNA was analysed for the mouse assays. Standard curves were plotted from the dilution series and the input amounts of cDNA in samples were calculated from the threshold values ( $C_t$ ) for cystatin C and the endogenous control. The standard curve also allowed calculations to compensate for interassay variations in amplification efficiency. The ratio of input cystatin C cDNA to the input amount of 18S cDNA was calculated. The normalized value was finally divided by the normalized value for the calibrator mRNA sample.

The QRT-PCR results are thus expressed as quotients, and hence linearly related to the tissue concentration of mRNA. We assume they are normally distributed and therefore present the results as mean values  $\pm$  standard error of the mean (SEM).

#### Microscopy and image analysis

The specimens were examined using a Nikon Diaphot 300/Bio-Rad MRC1024 confocal laser scanning microscope and a Nikon Eclipse E800 microscope equipped with an Optronix DEI-750 digital image acquisition system. Images were viewed and processed using Confocal Assistant (©Todd Clark Brelje) and Adobe Photoshop® (Adobe Systems, Mountain View, California, USA).

## Results

### Localization of cystatin C immunoreactivity

There were no significant morphological differences between rat and mouse anterior segments. The descriptions below are therefore valid for both.

#### Cornea and conjunctiva

Cystatin C immunoreactivity was present in the cytoplasm of most basal cells of the entire corneal epithelium, leaving the nuclei unlabelled. Occasionally, cystatin C labelled cells were also seen in the middle and outer layers of the epithelium (Fig. 1A). There was a definite interindividual variation in the proportion of labelled cells in the basal layer, ranging from about half the population up to the entire layer. The labelling disappeared at the limbus, and there was no significant labelling in the conjunctival epithelium (Fig. 1D). There was no observable difference between the central and peripheral parts of the cornea. In the stroma, most or perhaps even all keratocytes were labelled, as detectable with double labelling with the nuclear stain DAPI (not illustrated). The entire cytoplasm of the endothelial cells was also strongly labelled throughout the cornea, leaving the nuclei unlabelled as in the epithelium (Fig. 1A). There was no detectable cystatin C immunoreactivity in Bowman's membrane or Descemet's membrane.

The conjunctival epithelial cells were not significantly labelled and the transition between the labelled corneal epithelium and the unlabelled conjunctival epithelium was usually easily detectable (Fig. 1D).

#### Iris

Weak cystatin C labelling was localized to cytoplasmic spots in randomly distributed cells throughout the entire iris stroma (Fig. 1C). Cystatin C labelling was also evident in the muscle layer of small arteries and arterioles (Fig. 1D). There was usually no or at most only weak labelling of the dilator, the pigmented epithelium, and the sphincter without consistent regional differences (Fig. 1C, D).

#### Ciliary body

Cystatin C immunoreactivity was present in the ciliary processes, where it was predominantly localized to the cytoplasm of the distal layer of epithelial cells lining them (Fig. 1D, E). Only weak specific immunoreactivity was seen in the pigmented, proximal layer of epithelial cells or in more proximal parts of the ciliary processes. Occasionally, cystatin C immunofluorescent granules appeared in leucocytes.

There was some weak cystatin C labelling in the filtering meshwork of the chamber angle (Fig. 1D), but otherwise there was no definite specific immunolabelling in the ciliary body.

#### Lens

Cystatin C immunolabelling was localized to the epithelium of the lens (Fig. 2). The labelling was present in the form of subcellular cytoplasmic granules usually concentrated to the innermost part of the cell (lowermost in the illustrations). It was present in all cells covering the anterior surface of the lens, except at the germinative zone, where the labelling waned (Fig. 2B, C). The cell nuclei were unstained. There was no significant staining in the lens fibres, not even in the parts abutting the posterior capsule.

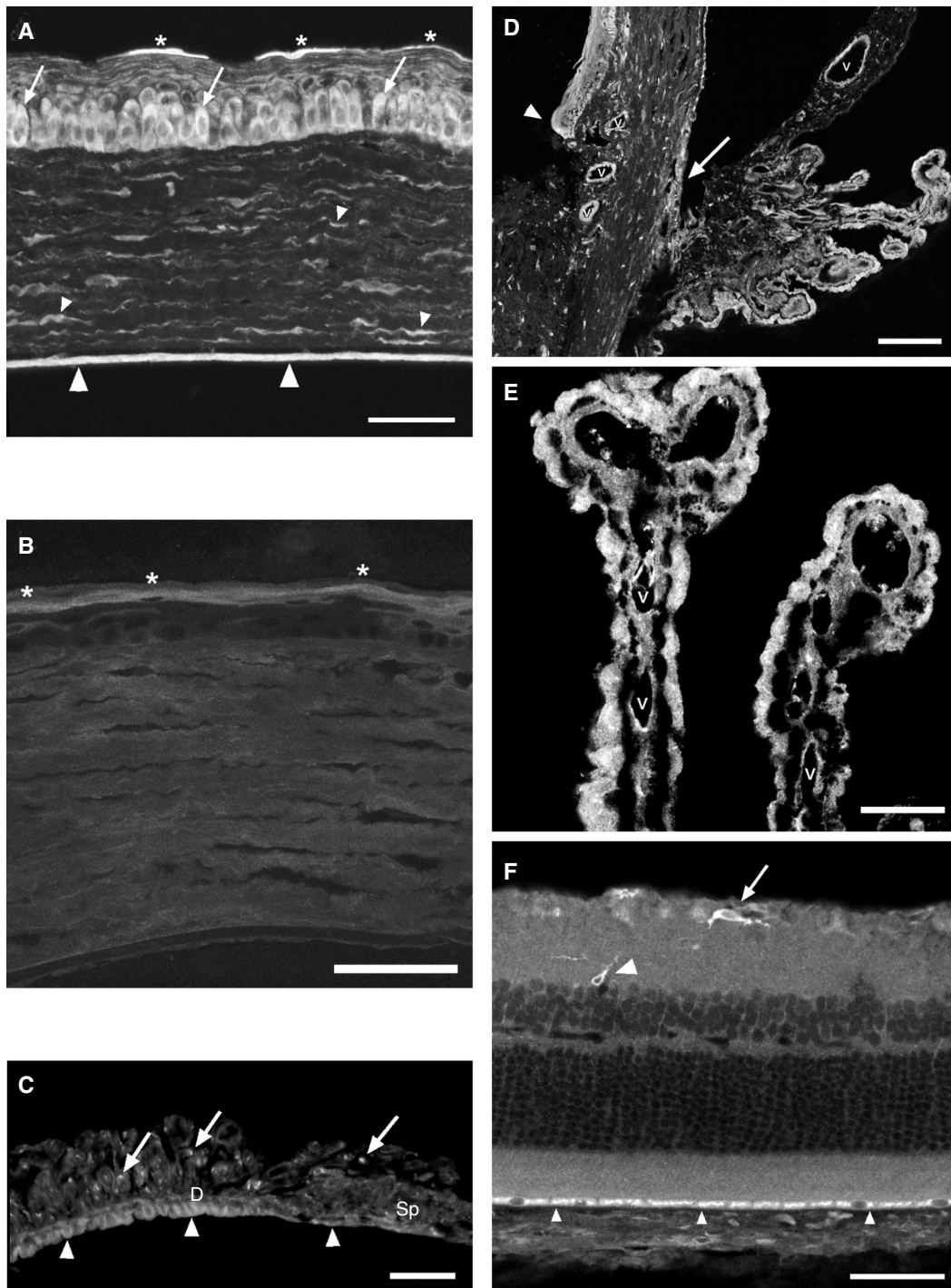
#### Retina

As we have reported previously (Wasélius et al. 2001), cystatin C in the retina is expressed in all retinal pigment epithelium cells, where it is localized to the cytoplasm, leaving the nuclei unstained (Fig. 1F). Cystatin C antibodies also labelled ganglion cells to a varying extent, ranging from occasional labelled cells up to most of the ganglion cells. The labelling of the ganglion cells was mainly localized to the cell soma and proximal parts of the axon. Occasional amacrine, bipolar, horizontal and cone photoreceptor cells were also labelled by the cystatin C antibody. The entire cytoplasm of these cells appeared to be labelled, allowing us to identify them as belonging to various different classes.

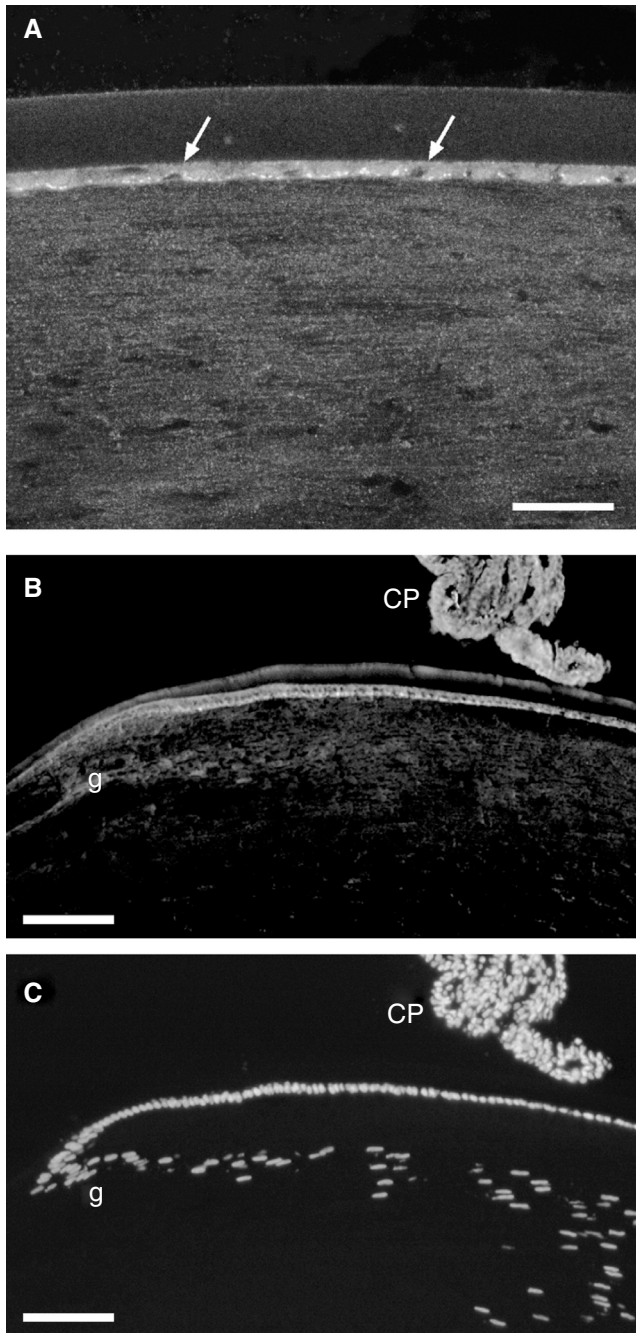
### Quantification of cystatin C

The levels of cystatin C in different parts of the eyes of mice and rats were determined using a specific ELISA (Fig. 3). Because of their small size, mouse eye tissues were analysed as pooled anterior segments, lens and retina. For comparison, rat eyes were analysed as pooled samples of cornea, ciliary body, iris, lens and retina.

In the mouse (Fig. 3A), the lens showed much less cystatin C than the retina and the anterior segment. The same pattern was observed in the pooled rat tissues (not illustrated, but see further below). A series of other rat organs (spleen, kidney, liver and brain) were also analysed in parallel, to allow



**Fig. 1.** Cystatin C immunoreactivity in rat ocular tissues. (A, B, D–F) Confocal fluorescence micrographs; (C) fluorescence micrograph. (A) Cystatin C immunoreactivity in the rat cornea, where it is prominent in the basal layers of the epithelium (arrows) as well as in the endothelium (large arrowheads). Most or possibly all stromal keratocytes (small arrowheads) show cystatin C immunofluorescence. The strong fluorescence at the surface (asterisks) is an artefact. Scale bar: 50  $\mu$ m. (B) A similar section in a control experiment where the primary antibody has been omitted. Weak immunofluorescence is seen at the surface (asterisks) and that immunoreactivity is therefore regarded as unspecific. Scale bar: 50  $\mu$ m. (C) Cystatin C immunofluorescence in the rat iris. Weak cytoplasmic dots appear in randomly distributed stromal cells (arrows). The immunofluorescence of the pigment epithelium (arrowheads) and the smooth muscles is only marginally above autofluorescence. Sp = sphincter; D = dilator muscle. Scale bar 50  $\mu$ m. (D) Montage showing cystatin C immunoreactivity in the ciliary body region of a rat eye. The epithelium of the ciliary processes is prominently labelled, as is the corneal endothelium and smooth muscles in vessel walls of the iris (v) and limbal conjunctiva (v). The junction between the corneal epithelium and labelled basal cells and the conjunctival epithelium without specific labelling is in the upper left corner (arrowhead). There is also some weak labelling in the trabecular meshwork of the chamber angle (arrow). Scale bar 100  $\mu$ m. (E) Cystatin C immunoreactivity of ciliary processes in the rat eye. There is specific fluorescence only in the superficial cells. The fluorescence on the inside of the vessel walls (v) is not specific. Scale bar 30  $\mu$ m. (F) Cystatin C immunoreactivity of the rat retina labelling the retinal pigment epithelium (small arrowheads), neurons in the ganglion cell layer (arrow) and inner nuclear layer (arrowhead). Scale bar: 50  $\mu$ m.



**Fig. 2.** (A) Cystatin immunoreactivity in the epithelial cells of the anterior part of the rat lens; cystatin C labelling. The topmost layer is the elastic lens capsule with the epithelial cells (arrows) underneath. The core fibres show only background fluorescence. Confocal fluorescence micrograph; scale bar 30  $\mu$ m. (B, C) DAPI nuclear staining and double staining of cystatin C immunoreactivity of the rat ciliary processes (CP) and lens close to the germinative zone (g) of the same section as in (A). There is marked cystatin C labelling in the ciliary processes and the lens epithelium except near and in the germinative zone. The lens fibres show only weak background fluorescence. Fluorescence micrographs; scale bars 100  $\mu$ m.

comparison with earlier results. Brain tissue contained high amounts of cystatin C, while spleen, kidney and liver tissue contained low levels (not illustrated).

Our cystatin C ELISA was found to be sufficiently sensitive for measurements on tissue samples from single rats, which we therefore ran (Fig. 3B).

The retina showed high levels, as reported earlier (Wassélius et al. 2001), whereas the lens, spleen and liver showed very low levels. The cornea, iris and ciliary body contained intermediate amounts. These results agreed well with the results from the pooled tissue samples analysed previously.

Aqueous humour pooled from rat eyes (five samples, each pooled from two eyes) contained  $1570 \pm 130$  ng cystatin C/ml (mean  $\pm$  SEM).

#### Quantification of cystatin C mRNA

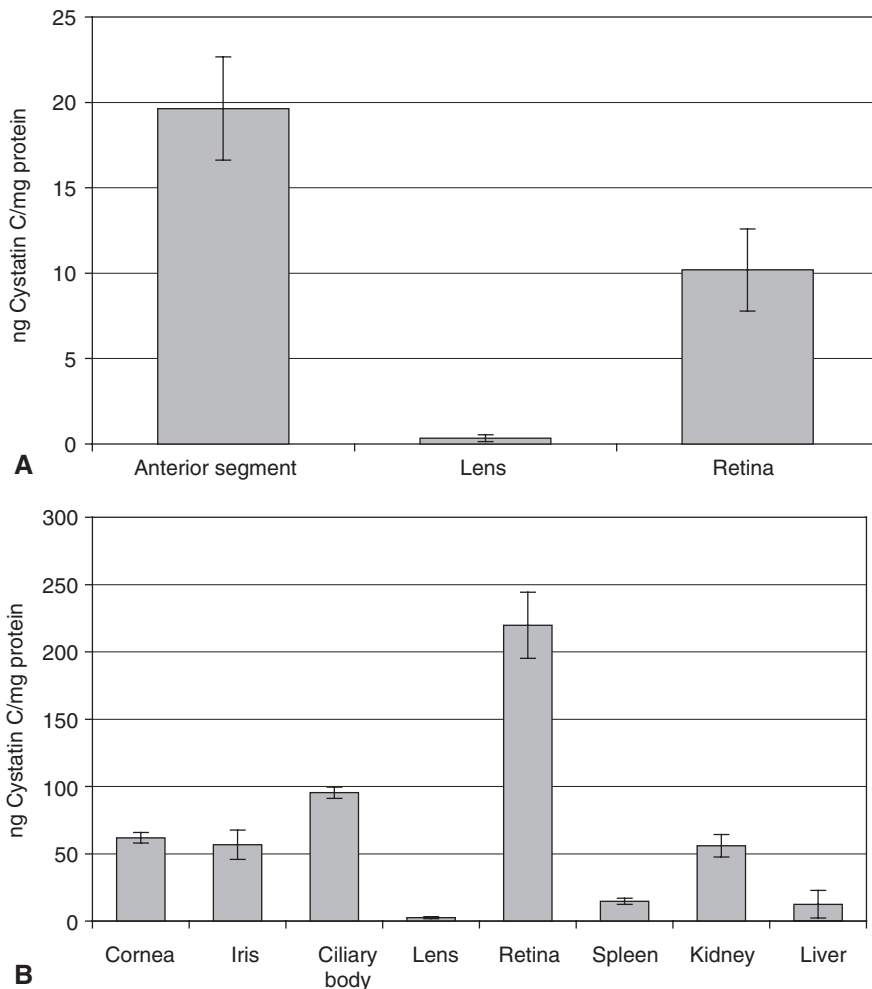
Regular reverse transcriptase-PCR for cystatin C detected mRNA in all samples analysed from different parts of mouse and rat eyes (not illustrated). To determine the relative amounts of cystatin C encoding mRNA in the different samples, specific QRT-PCR assays were set up. The results from these assays were compared to the QRT-PCR measurement of 18S rRNA and finally related to the sample with the lowest level of cystatin C mRNA (lens tissue in mice and liver tissue in rats), in order to illustrate the relative amounts in different tissues (Fig. 4).

In mouse eyes (Fig. 4A), the lens contained significantly less cystatin C mRNA than the anterior segment and the retina.

In rat eyes (Fig. 4B), the highest amounts of cystatin C mRNA were found in the ciliary body. The retina and iris also showed relatively high amounts, whereas the lens and cornea contained relatively low amounts of cystatin C mRNA.

## Discussion

Little has previously been published about the presence and localization of cystatin C in the eye, and nothing about its possible functions. Significant levels of cystatin C have been demonstrated in most cell lines kept *in vitro* (Grubb 2000). In native tissues, cystatin C has been found with immunohistochemical methods in neuroendocrine cells (Möller et al. 1985), the Sertoli cells of the testes (Esnard et al. 1992), certain neurons in the brain (Yasuhara et al. 1993), bone tissue (Yamaza et al. 2001), and retinal pigment epithelial cells (Wassélius et al. 2001). It has generally been difficult to identify the target enzymes for cystatins in different tissues (Grubb 2000), and the cystatins may well turn out to have a large and varied spectrum of regulatory functions, different in different tissues. Our quantitative results on the protein level could indicate that cystatin C has such functions in the ocular tissues, because the cystatin C content, as measured by ELISA on tissue homogenates, is generally high.



**Fig. 3.** Cystatin C protein levels in ocular tissues. (A) Cystatin C in mouse ocular tissues, showing averages of triple ELISA measurements on three tissue samples, each containing material from 19 eyes (10 different mice), with the SEM indicated on the bars. The lens shows markedly low levels; the retina and the pool of anterior segment tissues show higher levels. (B) Cystatin C in rat tissues, showing averages of triple ELISA measurements on individual samples from four rat eyes with the SEM indicated on the bars. As expected, the liver and spleen show low levels. The lens shows very low levels and the retina shows the highest levels.

However, it should be stressed that the quantitative data should be interpreted with caution, as our immunohistochemical results show that the proportion of immunoreactive cells varies between different ocular tissues.

In the anterior segment, we found cystatin C immunoreactivity in several kinds of cells, mainly of epithelial types, and, using a specific ELISA, we verified its presence in the tissues in biologically significant concentrations. In addition, we detected cystatin C mRNA in the tissues, also at biologically significant levels. It can be concluded that cystatin C is not only present in certain ocular cells, but is also produced locally.

In the posterior segment of the eye, cystatin C is predominantly localized to the pigment epithelial cells, but it is also

present in the neuroretina in occasional cells of almost all known types, mostly ganglion cells (Fig. 1F). Our previous studies also showed cystatin C and its mRNA to be present in significant amounts in the neuroretina (Wassélius et al. 2001), and we have confirmed these observations in this study.

#### Cornea and conjunctiva

In the cornea, cystatin C is localized to the basal layers of epithelial cells, to endothelial cells and to stromal keratocytes (Fig. 1A). The level of cystatin C in the rat cornea, measured by ELISA (Fig. 3B), is intermediary. The amount of cystatin C mRNA according to QRT-PCR is also significant (Fig. 4B), suggesting local production of cystatin

C in the cornea. Corneal epithelial and endothelial cells show high cystatin C immunolabelling, and cystatin C might therefore be used as a marker for corneal epithelial and endothelial cells.

Cathepsin L2 (also known as cathepsin V) has recently been observed to appear in particularly high concentrations in the corneal epithelium (Adachi et al. 1998; Kinoshita et al. 2001). Its function has not been established, but the correlation with a high content of cystatin C in the corneal epithelium is noteworthy, as cystatin C has a high affinity for cathepsin L and L2 (Abrahamson et al. 1986, 2000 unpublished).

There is a hydrophobic leader sequence in the precystatin C sequence, predicting that cystatin C should in most cases be a secreted protein. There are significant concentrations of cystatin C in tear fluid (Abrahamson et al. 1986; Reitz et al. 1998), the origin of which has not been established. Our observations suggest that the corneal epithelium may be one of several possible sources. The bulbar conjunctival epithelium seems to be an unlikely source, judging from its lack of cystatin C.

#### Ciliary body

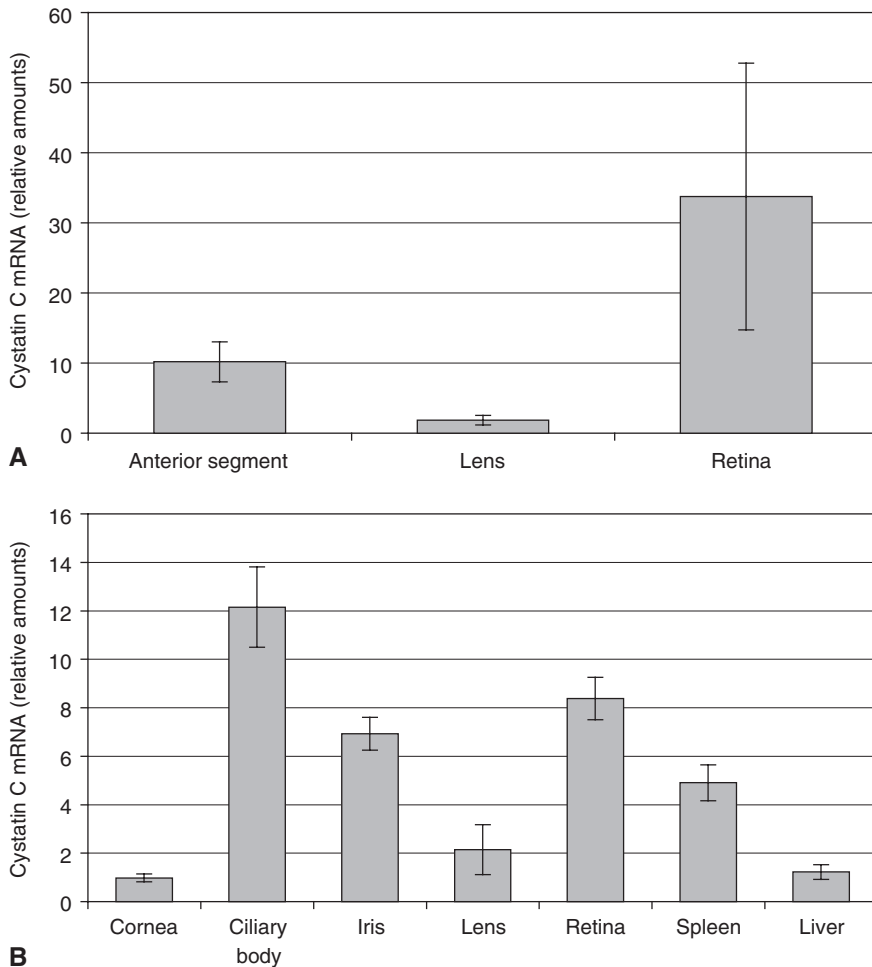
Our results indicate a high level of production of cystatin C in the ciliary body, where cystatin C is predominantly localized in the epithelial cells lining the ciliary processes. This is as expected if cystatin C is to be secreted to the aqueous humour.

Cathepsin B has been reported to be present in the ciliary epithelium, although its precise localization and function is not known (Hayasaka et al. 1983). It could well be a target enzyme for the cystatin C localized to the ciliary processes, which would then regulate cathepsin B activity in an intracellular compartment or provide protection against the activity of misrouted cathepsin B.

#### Aqueous humour

The cystatin C concentration in aqueous humour (1500 ng/ml, or about 115 nM) is about 400 times higher than the equilibrium constant for dissociation of the cystatin C–cathepsin B complex, which is 0.29 nM, and >400 times higher than the dissociation constant for cathepsin L and other cysteine





**Fig. 4.** QRT-PCR on anterior segment tissue in mice (A) and rats (B). Averages from triplet measurements from three mouse eyes and four rat eyes with the SEM indicated on the bars. All values are relative amounts. In the mouse, the lens shows markedly low values. In the rat, the liver, as expected, shows low levels. The cornea and lens show markedly low values, while the retina and ciliary body show the highest levels.

proteases with papain-like properties (Abrahamson et al. 1986). Hence, essentially all cysteine proteases liberated into the aqueous humour will rapidly be bound by their inhibitor, cystatin C (Bieth 1980, 1995; Abrahamson et al. 1986). We have here shown that the ciliary processes can be expected to produce cystatin C that is released into the aqueous humour, and they are therefore a likely source of the cystatin C detected in the aqueous humour, although other tissues may also contribute. It is possible that the cystatin C serves to protect the surrounding tissues from leaking cysteine proteases in the normal eye and in eyes suffering from acute uveitis. A similar function has been postulated for cystatin C released into cerebrospinal fluid (Catala 1998).

**Chamber angle**

The gene for cystatin C has been found to be up-regulated in the trabecular meshwork of patients suffering from glaucoma (Gonzalez et al. 2000), and in this study we observed cystatin C immunolabelling in the trabecular meshwork of the chamber angle (Fig. 1D). However, the murine and human trabecular meshworks are not exact anatomical equivalents, and the significance of the observation is therefore not clear.

**Iris**

The high content of cystatin C and its mRNA in the iris contrast with the sparse immunohistochemical findings. It is not easy to separate the iris from the ciliary body and processes at dissec-

tion, and the significance of the high titres measured is therefore uncertain.

**Lens**

The low levels of cystatin C and cystatin C mRNA, measured by ELISA and QRT-PCR, respectively, are obviously due to the large volume of lens fibres that do not contain cystatin C. Judging from the immunohistochemical observations, the true concentration in the epithelial cells is likely to be high, but it is not readily assayed.

The absence of cystatin C in the germinative zone suggests that the ability to store cystatin C is an acquired feature.

Cystatin C is an efficient inhibitor of cathepsin B (Abrahamson et al. 1986), which is known to be present in the lens (Gorthy & Azari 1987; Eisenhauer et al. 1988), although its specific localization has not to our knowledge been published. However, pilot experiments have suggested that cathepsin B in the lens is found exclusively in its epithelium (Wassélius et al. 2003), and it is thus a possible target enzyme for cystatin C.

**Conclusion**

Cystatin C is ubiquitously expressed and present in various mammalian tissues. The inhibitor is therefore likely to play a role in many different complex biological processes involving cysteine proteases. We have here shown that it is present in several ocular cell types, mainly of epithelial character, in mouse and rat eyes. It is probably produced in the same cell types.

**References**

Abrahamson M, Alvarez-Fernandez M, Nathanson CM (2003): Cystatins. *Biochem Soc Trans* in press.  
 Abrahamson M, Barrett AJ, Salvesen G, Grubb AO (1986): Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. *J Biol Chem* **261**: 11282–11289.  
 Abrahamson M, Dalbøge H, Olafsson I, Carlsen S, Grubb AO (1988): Efficient production of native, biologically active human cystatin C by *Escherichia coli*. *FEBS Lett* **236**: 14–18.  
 Adachi W, Kawamoto S, Ohno I, Nishida K, Kinoshita S, Matsubara K, Okubo K (1998): Isolation and characterization of human cathepsin V: a major proteinase in corneal



- epithelium. *Invest Ophthalmol Vis Sci* **39**: 1789–1796.
- Barka T & van der Noen H (1994): Expression of the cysteine proteinase inhibitor cystatin C mRNA in rat eye. *Anat Rec* **239**: 343–348.
- Bieth JG (1980): Pathophysiological interpretation of kinetic constants of protease inhibitors. *Bull Eur Physiopathol Respir* **16**: 183–197.
- Bieth JG (1995): Theoretical and practical aspects of proteinase inhibition kinetics. *Meth Enzymol* **248**: 59–84.
- Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
- Brown WM & Dziegielewska KM (1997): Friends and relations of the cystatin superfamily – new members and their evolution. *Protein Sci* **6**: 5–12.
- Catala M (1998): Embryonic and fetal development of structures associated with the cerebrospinal fluid in man and other species. Part I. The ventricular system, meninges and choroid plexuses. *Arch Anat Cytol Pathol* **46**: 153–169.
- Chomczynski P & Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159.
- Cole T, Dickson PW, Esnard F, Averill S, Risbridger GP, Gauthier F, Schreiber G (1989): The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. *Eur J Biochem* **186**: 35–42.
- Eisenhauer DA, Berger JJ, Peltier CZ, Taylor A (1988): Protease activities in cultured beef lens epithelial cells peak and then decline upon progressive passage. *Exp Eye Res* **46**: 579–590.
- Esnard A, Esnard F, Guillou F, Gauthier F (1992): Production of the cysteine proteinase inhibitor cystatin C by rat Sertoli cells. *FEBS Lett* **300**: 131–135.
- Gonzalez P, Epstein DL, Borrás T (2000): Genes upregulated in the human trabecular meshwork in response to elevated intraocular pressure. *Invest Ophthalmol Vis Sci* **41**: 352–361.
- Gorthy WC & Azari P (1987): Biochemical and histochemical evidence for lysosomal proteases in rodent lenses. *Exp Eye Res* **44**: 747–753.
- Grubb AO (2000): Cystatin C-properties and use as diagnostic marker. *Adv Clin Chem* **35**: 63–99.
- Grubb AO & Löfberg H (1982): Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. *Proc Natl Acad Sci U S A* **79**: 3024–3027.
- Håkansson K, Huh C, Grubb A, Karlsson S, Abrahamson M (1996): Mouse and rat cystatin C: *Escherichia coli* production, characterization and tissue distribution. *Comp Biochem Physiol B Biochem Mol Biol* **114**: 303–311.
- Hayasaka S, Shiono T, Hara S, Fukuyo T (1983): Partial purification of cathepsin B in the bovine ciliary body and iris. *Invest Ophthalmol Vis Sci* **24**: 682–686.
- Huh C, Nagle JW, Kozak CA, Abrahamson M, Karlsson S (1995): Structural organization, expression and chromosomal mapping of the mouse cystatin C gene (Cst3). *Gene* **52**: 221–226.
- Kinoshita S, Adachi W, Sotozono C, Nishida K, Yokoi N, Quantock AJ, Okubo K (2001): Characteristics of the human ocular surface epithelium. *Prog Retin Eye Res* **20**: 639–673.
- Möller CA, Löfberg H, Grubb AO, Olsson SO, Davies ME, Barrett AJ (1985): Distribution of cystatin C (gamma-trace), an inhibitor of lysosomal cysteine proteinases, in the anterior lobe of simian and human pituitary glands. *Neuroendocrinology* **41**: 400–404.
- Rawlings ND & Barrett AJ (1990): Evolution of proteins of the cystatin superfamily. *J Mol Evol* **30**: 60–71.
- Reitz C, Breipohl W, Augustin A, Bours J (1998): Analysis of tear proteins by one- and two-dimensional thin-layer isoelectric focusing, sodium dodecyl sulfate electrophoresis and lectin blotting. Detection of a new component: cystatin C. *Graefes Arch Clin Exp Ophthalmol* **236**: 894–899.
- Solem M, Rawson C, Lindburg K, Barnes D (1990): Transforming growth factor beta regulates cystatin C in serum-free mouse embryo (SFME) cells. *Biochem Biophys Res Comm* **172**: 945–951.
- Wassélius J, Håkansson K, Johansson K, Abrahamson M, Ehinger B (2001): Identification and localization of retinal cystatin C. *Invest Ophthalmol Vis Sci* **42**: 1901–1906.
- Wassélius J, Wallin H, Abrahamson M & Ehinger B (2003): Cathepsin B in the rat eye. *Graefes Arch Ophthalmol* (in press).
- Yamaza T, Tsuji Y, Goto T, Kido MA, Nishijima K, Moroi R, Akamine A, Tanaka T (2001): Comparison in localization between cystatin C and cathepsin K in osteoclasts and other cells in mouse tibia epiphysis by immunolight and immunoelectron microscopy. *Bone* **29**: 42–53.
- Yasuhara O, Hanai K, Ohkubo I, Sasaki M, McGeer PL, Kimura H (1993): Expression of cystatin C in rat, monkey and human brains. *Brain Res* **628**: 85–92.

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