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Regulation of cysteine cathepsin expression by oxidative stress in the retinal pigment epithelium /choroid of the mouse

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Abstract

Cystatin C is the major inhibitor of the cysteine cathepsins. Polymorphisms in the cystatin C gene have recently been associated with the risk of developing Age-related Macular Degeneration (AMD). Oxidative stress is also thought to play a key role in the pathogenesis of AMD. We surveyed the retinal pigment epithelium (RPE) and choroid of the C57BL/6J mouse for the expression of the cysteine cathepsins under normoxic and hyperoxic (75% O₂) conditions. Microarray analysis of RPE/choroid mRNA revealed the expression of cathepsins B and L, as well as cystatin C under all experimental conditions. The microarray results were confirmed by real-time quantitative polymerase chain reaction (PCR). Localization of the mRNA species for cystatin C and cathepsin B, as well as, localization of protein species for cystatin C, cathepsin B and L were performed to evaluate the tissue distribution of these species.

Our results indicate that cystatin C is largely synthesized in the RPE and secreted from the basal side. Cathepsin B is the major cysteine protease in the RPE and choroid. The expression of all mRNAs and proteins was elevated by exposure to oxidative stress.

Keywords

cathepsins; cystatins; retinal pigment epithelium; oxidative stress; mouse

1. Introduction

Polymorphisms in the cystatin C gene have recently been associated with the risk of developing Age-related Macular Degeneration (AMD) and Alzheimer's disease (Finckh, et al., 2000, Lin, et al., 2003, Paraoan, et al., 2003). Because cystatin C is the primary inhibitor of the cysteine proteases (cathepsins B, L, H, and S), both the inhibitor and one or more of the cathepsins are functionally implicated in the pathogenesis of AMD. Many functions have been identified for this class of cathepsins including increasing the invasiveness of endothelial cells (Im, et al., 2005), and participating in TNF stimulated apoptosis (Caruso, et al., 2006) These same processes are also observed in the pathogenesis of AMD.

Oxidative stress has been suggested to be critical to the pathogenesis of several neurodegenerative diseases including AMD. The Age-related Eye Disease (ARED) study demonstrated that management of oxidative stress with vitamin supplements reduced the severity and progression of AMD (ARED, 2001). These same cellular mechanisms are associated with tissue effects of oxidative stress.

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While no adequate model of AMD exists in the mouse, Yamada et al. have developed a chronic oxidative stress model in the C57BL/6J mouse which leads to photoreceptor degeneration after 14 days in a 75% oxygen atmosphere (Yamada, et al., 2001). This model termed Hyperoxia-related Retinal Degeneration (HRRD) is now being studied in several laboratories. HRRD is considered to be a model of chronic oxidative stress. Acute models involving hyperoxia usually are conducted in 95% oxygen, where animal survival is limited to 24 to 72 hours. In our hands, HRRD leads to pathology in the eye alone and not in other tissues of the mouse at 14 days.

The goals of this study are to survey the expression of cystatin C and the cysteine cathepsins in the RPE/choroid of the mouse in the presence and absence of oxidative stress. To conduct this survey, we performed microarray analysis on isolated RNA from the RPE/choroid of mice exposed to room air or 75% oxygen for 14 days. Microarray data were confirmed by quantitative PCR, and further examined by *in situ* hybridization and immunohistochemistry. The results confirm earlier observations on the expression of cathepsin B (Bernstein, et al., 1989, Wasselius, et al., 2003) and cystatin C (Wasselius, et al., 2004, Wasselius, et al., 2001) by RPE cells, as well as, the induction of cystatin C by oxidative stress (Nishio, et al., 2000).

2. Materials and methods

2.1. Animals

Eight-week-old C57BL/6J and C57BL/6J-*Tyr^{c-2J}*/J mice were purchased from The Jackson Laboratory. Experiments and animal care were according to the ARVO Convention for Ophthalmological Animal Experimentation and approved by the UC Davis Committee for Animal Experimentation Ethics.

2.2. Hyperoxia exposure

Eight 8-week-old C57BL/6J-*Tyr^{c-2J}*/J mice were allowed to acclimate to the environment of the animal room for two weeks before starting the hyperoxia experiment. Thus animals were 10 weeks old at the start of the experiment. Four mice were housed in an air-tight chamber for 14 days in an atmosphere of 75% oxygen. This gas mixture was supplied at a constant flow of 2 l/hr. The temperature, oxygen content, and humidity were regulated for the entire length of the experiment. Normal controls (n= 4) were housed in standard mouse cage supplied with room air (normoxia). Animals were fed a standard Purina rodent diet (Lab Diet-5001) *ad libitum*, and water was provided by Napa Nectar (Systems Engineering Lab, Napa, CA). Animal health was assessed daily. Animal cages were cleaned weekly, thus exposing the hyperoxia animals to normoxic conditions for up to 30 min.

2.3. Tissue Processing

Mice were sacrificed by carbon dioxide asphyxiation before the eyes were enucleated. One eye was immediately fixed overnight at 4°C with 4% paraformaldehyde in PBS (10 mM sodium phosphate, pH 7.5, 0.9% saline) and embedded in paraffin. Six μ m sections were mounted onto slides (SuperFrost Plus; Fisher Scientific, Fairlawn, NJ) for immunohistochemistry and *in situ* hybridization studies.

The other eye was placed into a Petri dish containing PBS buffer and kept on ice. Eyes were dissected in an RNase-free environment using a stereo zoom microscope (UNITRON ZSB, Japan). To aid with dissection, the optic nerve stump was glued to the bottom of a 35mm Petri dish using a drop of Superglue (Henkel Consumer Adhesives, Inc, Avon, OH). After removal of the anterior segment from the posterior pole using surgical scissors, the lens was removed. The retina was then peeled from the RPE/choroid, cut free from the optic nerve, and placed in 300 μ l of RNAlater buffer (Ambion, Inc. Austin, TX). Then the RPE/choroid was peeled from the sclera as a sheet and placed in 300 μ l of RNAlater buffer. Tissue samples in RNAlater

buffer were placed at 4°C for a few hours and stored at -20°C for further analysis at a later time. RNA was purified as described in Section 2.8.

2.4. Antibodies

The following goat anti-mouse primary polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA): cathepsin B (cat. No. sc-6490), cathepsin L (cat. No. sc-6501), and cystatin C (cat. No. sc-16989). The secondary antibody, biotinylated rabbit anti-goat IgG (cat. No. BA-5000), was purchased from Vector Laboratories (Burlingame, CA.). A total IgG fraction from non-immune goat serum (Zymed, cat. No. 02-6202 South San Francisco, CA) was used as a control.

2.5. Immunohistochemistry

Paraffin-embedded tissue sections were utilized for immunohistochemical analysis as follows. Paraffin was removed from the sections using xylene, and rehydrated through graded alcohols, followed by a brief rinse in PBS. Tissue sections were subjected to antigen retrieval by boiling the slides for 10 min in a Coplin jar containing 10 mM sodium citrate buffer, pH 6.0; the slides were then kept at room temperature for 20 min. Slides were rinsed in PBS for 3 min and then blocked in 5% normal rabbit serum (VECTOR Laboratories) in PBS for 30 min. After aspirating off the blocking solution, the slides were covered with the appropriate primary antibody (6 µg/ml anti-cathepsin B, 2 µg/ml anti-cathepsin L, 2 µg/ml anti-cystatin C antibody) diluted in 5% normal rabbit serum in PBS, and incubated overnight at 4°C in a humidity chamber. Slides were washed three times for 3 min each with PBS, incubated for 30 min with 7.5 µg/ml of biotinylated rabbit anti-goat IgG diluted in PBS, washed two times for 3 min each in PBS, incubated for 30 min in avidin-biotin complex (Vector Laboratories, Burlingame, CA), and washed two times for 3 min each in PBS. Labeling was visualized by incubating for 10 min with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) substrate, which generates an alcohol-insoluble dark blue/purple stain, and in Nitro Blue Tetrazolium (NBT), which enhances the color of the BCIP (Vector Laboratories, Burlingame, CA). The reaction was stopped by washing in PBS for 5 min. The tissue was counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA) for 10 min, washed in tap water, dehydrated, cleared, and coverslipped using VectaMount permanent mounting media (Vector Laboratories, Burlingame, CA). Immunohistochemistry control experiments included omission of primary antibody and the use of non-immune total IgG at the corresponding primary antibody concentration. Slides were viewed and photographed using a light microscope (Nikon Eclipse E800). Digitized images were captured through the digital camera using Q capture software (Q imaging RETIGA 1300I, Canada).

2.6. cDNA clones and riboprobes

2.6.1. Cathepsin B—A cRNA probe was synthesized from cathepsin B cDNA (Ctsb) cloned in pCMV-SPORT6 vector (ATCC MGC-6211). We identified a specific region of Ctsb for an *in situ* hybridization (ISH) probe based on two criteria: (1) homology to the region of the human CSTB gene that was used in the literature (Niedergethmann, et al., 2004), and (2) the lack of cross-reactivity with any other mouse gene, as determined by an extensive BLAST search against mouse genome specific sequences using Vector NTI software. Oligonucleotides complementary to regions of Ctsb 195–215 bps (5'GCTGTCGGATGACCTGATTA 3') and 657–677 bps (5'GGAGGGATGGTGTATGGTAA3') (QIAGEN, Alameda, CA) were used in a PCR reaction (Platinum® PCR SuperMix High Fidelity Kit, Invitrogen, CA; cat. No. 12532-016 with Taq DNA polymerase).

The PCR product of 483 bp was visualized on a 1.2% agarose gel. The amplified region of Ctsb (195 bps- 677 bps) was subcloned into the TA cloning site of the pCRII vector

(Invitrogen). We chose this vector because it enabled us to synthesize both antisense and sense riboprobes from SP6 or T7 promoters flanking the TA-vector cloning site.

The pCRII-CathB was transformed into Top10F'competent *E. coli* cells (TA Cloning® Dual kit, Invitrogen, CA, cat. No. K2060-01). Large plasmid prep was performed using the QIAfilter plasmid Midi Kit (QIAGEN). To determine the size of the construct, the pCRII-CathB plasmid was linearized with XhoI and HindIII separately. The orientation of the fragment within the vector was determined using double digests of BglII/HindIII and XhoI/BglII.

Linearized plasmid was used in a SP6 *in vitro* transcription reaction to synthesize sense (XhoI digested), and antisense (HindIII digested) probe with T7 RNA polymerase. RNA probes were labeled with digoxigenin-UTP by an *in vitro* transcription reaction [DIG RNA Labeling Kit (SP6/T7), cat. No. 11175025910 Roche, CA]. The digoxigenin (DIG)-labeled antisense and sense RNA probes were treated with DNaseI, followed by LiCl/Ethanol precipitation. The efficiency of DIG-labeling of the RNA probes was tested by following the procedure from the DIG-RNA Labeling Kit. The probe concentrations were determined using a 2100 BioAnalyzer and the RNA 6000 Nano Assay LabChip (Agilent, CA).

2.6.2. Cystatin C—Digoxigenin (DIG)-labeled antisense and sense RNA probes for Cst3, the gene coding for cystatin C, were synthesized from a cDNA clone (mi12b04.y1) from a mouse retinal cDNA library made in pSPORT1 vector. In this library cDNA inserts were cloned into NotI/SalI sites of the vector (NEIBank cDNA library) (Wistow, et al., 2002). Linearized plasmid was used in an *in vitro* transcription reaction to synthesize a sense (BamHI digestion) riboprobe with T7 RNA polymerase and an antisense (EcoRI digestion) riboprobe with SP6 RNA polymerase. The length of the RNA probe was 563 bp (105 to 668 nt). Labeling of the cRNA probes was done as described for cathepsin B using the DIG RNA Labeling Kit (SP6/T7, Roche, CA). The efficiency of DIG-labeling of the RNA probes was determined by following the procedure from the DIG RNA Labeling Kit (Roche). The probe concentration was determined using a 2100 BioAnalyzer (Agilent, CA)

2.7. In Situ Hybridization (ISH)

2.7.1. Hybridization—All buffers and salt solutions were prepared with RNase-free chemicals dissolved in molecular biology grade water (purchased from CAMBREX Bioscience, Inc., Rockland, ME); solutions were either autoclaved or sterile-filtered.

Hybridization with DIG-labeled sense riboprobes served as a control for nonspecific hybridization. ISH was performed according to Braissant and Wahli (Braissant and Wahli, 1998) with slight modifications (Rheinhardt and Finkbeiner, 2001). Briefly, ISH was performed on paraffin sections using DIG-labeled riboprobes as follows: paraffin was removed from slides using xylene, cleared in 100% ethanol, and rehydrated through graded alcohols to PBS. Slides were incubated in 20% glacial acetic acid at 4°C for 5 min, washed for 5 min in 2X SSC, incubated in 0.2 M HCl for 10 min and then washed for 5 min in PBS at room temperature.

Slides were then incubated for 10 min at 37°C with freshly prepared Proteinase K (Sigma P-2308) (10µg/ml) dissolved in 100 mM Tris (pH 7.6), 50 mM EDTA. Next, slides were washed for 5 min in PBS. To stop Proteinase K activity, the slides were incubated in a solution containing 200 mM Tris-HCl (pH 7.6), 100 mM glycine, for 15 min at room temperature, followed by a 5 min wash in PBS. Slides were fixed in 4% paraformaldehyde (PFA) prepared in 1X PBS buffer, pH 7.4, for 30 min, washed with PBS for 5 min, equilibrated for 2 min in 0.1 M triethanolamine HCl (TEA), pH 8.0, and incubated 10 min in 0.25% acetic anhydride diluted in 0.1 M TEA at room temperature. Slides were then rinsed in 2X SSC. Prehybridization was carried out at 50°C for both cathepsin B and cystatin C for 4 hours in prehybridization

mixture (50% formamide, 5X SSC, 1 x Denhardt's, 10% dextran sulfate, 0.1 mg/ml yeast tRNA, and 100µg/ml salmon sperm DNA).

DIG-RNA probes were freshly diluted in hybridization buffer with following concentration for each probe: cathepsin B (4 ng/µl) and cystatin C (1 ng/µl) and then denatured at 80°C for 5 min, and quenched on ice for 5 min. This hybridization mix was added (70 µl/section) directly to sections after removal of the pre-hybridization buffer. In a hybridization oven (Fisher Scientific, Los Angeles, CA), slides were incubated for 15 h in a humid chamber containing paper towels moistened with 50% formamide and 5X SSC. The temperature for pre-hybridization, hybridization and post-hybridization washes was adjusted according to the GC content of each probe and was selected for high stringency: 50°C was used for both cathepsin B and cystatin C.

2.7.2. Posthybridization Washes—The next day, slides were washed in 4X SSC at room temperature for 2 min to remove excess probe, followed by three 5 min high-stringency washes at the hybridization temperature (50°C), with agitation in: 2X SSC/50% formamide, 2X SSC, and 1X SSC. Cystatin C slides were treated with RNase by equilibrating in STE for 1 min, then incubating with 40 µg/ml RNase A (Fermentas Inc. Hanover, MD) in STE for 30 min at 37°C. After RNase treatment, slides were washed for 5 min at the hybridization temperature in 2X SSC/50% formamide, followed by a 5 min wash in 1X SSC and 0.5X SSC at room temperature, all with agitation. Cathepsin B slides were not treated with RNase A, but washed in 1X SSC at room temperature for 5 min with agitation.

2.7.3. Immunodetection—Slides were rinsed in Buffer 1 (0.1 M Tris/0.15 M NaCl, pH 7.5) for 1 min at room temperature. Nonspecific binding was blocked in freshly prepared Blocking Buffer containing 0.5% DIG blocking reagent (10% blocking reagent, Roche, cat. No. 109617 and 1X maleic acid buffer, Roche cat. No. 1585762) made in Buffer 1 for 30 min at room temperature. The slides 1:500) in 0.5% DIG blocking reagent in were incubated with anti-DIG-F_{ab}-AP conjugate (diluted Buffer 1 at room temperature for 1 h. Slides were washed in Buffer 1 for 5 min and in Buffer 3 (100 mM Tris-HCl at pH 9.5, 100 mM NaCl) for 10 min.

Slides were incubated with the substrate 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), and Nitro Blue Tetrazolium (NBT) in buffer 3 (Vector Laboratories, cat. No. SP-5400, Burlingame, CA) in a humid chamber at room temperature in the dark. The reaction was continued for up to 4 hr for cystatin C and 1 hr for cathepsin B, and then stopped by rinsing slides in PBS. Next slides were rinsed in tap water, counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA), and observed using a Nikon Eclipse E800 microscope. Images were converted to a gray scale and sharpened, and brightness adjusted using Adobe Photoshop v 7.01(Adobe, San Jose, CA).

2.8. Isolation of RNA for microarray analysis and real time quantitative TaqMan PCR

Tissue stored in RNAlater buffer at −20°C was removed from the RNAlater and homogenized using a 22 gauge needle and a QIAshredder spin column. Total RNA was isolated using the QIAGEN RNeasy Kit (QIAGEN, Valencia, CA). While C57BL/6J mice were used for microarray experiments, all other experiments, including TaqMan analysis used C57BL/6J-*Tyr^{c-2J}*/J animals. The typical yield of total RNA was 0.4µg/RPE and 4µg/retina. The purity of total RNA was initially determined from the A_{260/280} ratio using a spectrophotometer. Total RNA quality and concentration were determined by running a sample on the Agilent 2100 BioAnalyzer using the RNA 6000 Nano Assay LabChip.

2.9. Probe preparation for microarray analysis

Probes for the Affymetrix GeneChip analysis were made following the manufacturer's protocols. Since the starting total RNA material of the RPE layer of mouse retinal tissue was limiting, we followed the two-cycle cDNA synthesis protocol for 0.100 µg of RNA. The cRNA probes were hybridized to Affymetrix mouse GeneChip MOE 430A according to standard Affymetrix protocols. Hybridized arrays were then scanned by an Agilent GeneArray Scanner. Data were analyzed using Affymetrix GCOS and GREX software. Raw data were normalized based on 100 housekeeping genes present on the MOE 430A GeneChip, and scaled to the median Signal value of 500. GCOS algorithm was used to evaluate the abundance of each transcript represented on the array and to label it as either present (P), absent (A), or marginal (M). The comparative level of analysis was done with Affymetrix GREX software that generated Signal Log Ratios and significance estimates. Values of SigLogRatio larger than or equal to 1 were considered up-regulated (I). Values of SigLogRatio smaller than or equal to -1 were considered down-regulated (D). Values between -1 and 1 were considered as no change (NC).

2.10. Real time quantitative TaqMan PCR systems

For each target gene, a pre-developed TaqMan PCR assay (Assay-on-Demand) was purchased from Applied Biosystems (AB, Foster City, CA). These assays contain 900 nM of primers and 200 nM of 6-FAM (6-carboxyfluorescein) labeled with TaqMan MGB probes. The MGB probes spanned an exon-exon boundary to prevent detection of genomic DNA background.

2.10.1. Nucleic acid preparation—Starting from the total RNA isolations (as described in Section 2.8), genomic DNA was digested using RNase-free DNase (Invitrogen, Carlsbad, CA) for 15 min at 37°C. DNase was inactivated by heat, and the absence of genomic DNA confirmed using a mouse-specific GAPDH TaqMan assay.

2.10.2. RT-reaction and real-time TaqMan PCR—Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Life Technologies), 600 ng of random hexadeoxyribonucleotide (pd(N)₆) primers (random hexamer primer), 10 U of RNaseOut (RNase inhibitor), and 1 mM of dNTPs (all Invitrogen, Carlsbad, CA) in a final volume of 40 µl. The reverse transcription reaction proceeded for 120 min at 50°C. After the addition of 60 µl of water, the reaction was terminated by heating for 5 min at 95°C and cooling on ice.

Each PCR reaction contained 20x Assay-on-Demand primer and probes for the respective TaqMan system, and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphate, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 12 µl. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI). ABI's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals were collected during the annealing temperature and C_t values extracted with a threshold of 0.04 and baseline values of 3–15. For stronger signals, the baseline was adjusted manually to 3–10.

2.10.3. Housekeeping gene validation experiment—In order to determine the most stably transcribed housekeeping gene, a housekeeping gene validation experiment was run on a representative number of samples from all tissue types. Four commonly used housekeeping genes were used for this experiment: a TaqMan PCR system specific for mouse Gapdh (glyceraldehyde 3-phosphate dehydrogenase), Actb (beta actin), Hprt1 (hypoxanthine phosphoribosyltransferase-1), and Tfr2 (transferrin receptor 2; CD71). Gapdh was found to

show the lowest standard deviation in all tissues and was therefore transcribed most stably. Hprt1 C_t values served to normalize against the target gene C_t values.

2.10.4. Relative quantitation of gene transcription—Final quantitation was done using the comparative C_t method (User Bulletin #2, Applied Biosystems) and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA (i.e. lowest target gene transcription). In brief, the housekeeping gene, Gapdh, was used to normalize the C_t values of the target genes (ΔC_t). The ΔC_t was calibrated against the weakest signal within each target gene. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta C_t}$. Therefore, all gene transcription is expressed as an n-fold difference relative to the calibrator.

3. Results

3.1. mRNA Quantification

In order to perform a complete analysis of all cysteine cathepsins that might be inhibited by cystatin C, we first elected to examine levels of gene expression using the Affymetrix mouse GeneChip MOE 430A. This microarray has a large collection of cathepsins (20 cathepsin genes), and the total number of probes is 25 (some genes have multiple probe sets).

Next we isolated RNA from the RPE/choroid of six animals, three of which were exposed to hyperoxia for 14 days (75% O_2 , experimental) and three to normoxia (control). This RNA was labeled by standard methods (see Methods 2.9.) and quantified by microarray analysis. Fig. 1 presents the data obtained from these experiments. The most abundant cysteine cathepsin in our samples was cathepsin B. This gene clearly had elevated expression in hyperoxia and although the difference did not reach statistical significant at the level $p < 0.05$, the value was close. Next in abundance was the mRNA for cathepsin L, which shows elevated expression under hyperoxia, though again the difference was not statistically significant at the level $p < 0.05$. Finally, cathepsin S expression was measured, and the expression of this gene was significantly upregulated in hyperoxia ($p < 0.01$). In addition to the cathepsins, we quantified the expression of cystatin C in the RPE/choroid under normoxic and hyperoxic conditions (Fig. 1). Cystatin C was clearly upregulated by hyperoxia, although the change in expression did not reach statistically significant value ($p = 0.06$).

Other cysteine cathepsins have been reported to utilize cystatin C as an inhibitor (cathepsin H, for example) (Brzin, et al., 1984), but none of these showed significant expression in the RPE/choroid when compared with cathepsins B, L, and S, as shown above.

For an independent measure of the changes in gene expression between the control and experimental groups, we performed real-time quantitative TaqMan PCR. The results of this analysis are shown in Fig. 2 for cathepsins B and L, and cystatin C. These data show that cathepsins B and L and cystatin C are induced under hyperoxic conditions. Note that comparison of hyperoxic and normoxic values for cathepsins B and L and cystatin C are all significantly different ($p < 0.005$) when measured by TaqMan-PCR. These results confirm our finding by microarray studies. Cathepsin S was omitted from further studies, as the expression level of mRNA was low.

3.2. In situ hybridization

The data in Fig. 1 and Fig. 2 represent average values for whole RPE/choroid complexes. To examine the distribution of the individual mRNAs under control and experimental conditions, we performed *in situ* hybridization studies for cathepsin B and cystatin C.

Results for cathepsin B are shown in Fig. 3. These images confirm that cathepsin B is expressed in the RPE, and that it is also expressed in the mouse choroid in, as yet, unidentified cells. The comparison between panels B and D suggests that the overall expression of cathepsin B is increased in the experimental group, with perhaps slightly more expression in the choroid. (Note: Expression of cathepsin B is higher in RPE cells under normoxic conditions. Under hyperoxic conditions, the level of cathepsin B is higher in the choroid compared to RPE cells.)

Finally, the *in situ* hybridization studies on cystatin C are presented in Fig. 4. In these images, it is clear that the RPE is the major cell type expressing cystatin C mRNA under both normoxic and hyperoxic conditions. It is also clear that there is an enhancement of expression in the experimental group as opposed to the control group.

Staining was highly variable within the RPE for all mRNAs.

3.3. Immunohistochemistry

As a follow-up to our *in situ* hybridization studies, we performed immunohistochemistry to evaluate the distribution of the proteins for cathepsins B and L and cystatin C. Once again it is clear that both the RPE and unknown elements of the choroid stained for cathepsin B (Fig. 5). Controls exhibited no background staining. In the comparison between normoxic and hyperoxic animals, cathepsin B seems to be highly expressed in both the RPE and choroid.

Fig. 6 shows immunohistochemistry images of cathepsin L. These results suggest that under hyperoxic conditions, more cathepsin L is localized in the RPE and choroid when compared with the normoxic tissue. Hyperoxia increases the expression of cathepsin L in the RPE and choroidal cells.

Finally, we evaluated the immunohistochemical localization of cystatin C. Under normoxic conditions, nearly all of the protein is localized in the choroid and very little appears to be localized in the RPE (Fig. 7). This also appears to be the case for samples from animals treated with hyperoxia.

4. Discussion

In this study we have reported the overall expression levels of the most abundant cysteine cathepsins in the RPE/choroid, as well as, the expression levels of their inhibitor, cystatin C. For the most abundant of these cathepsins, we studied the expression of mRNA and protein under high levels of induced oxidative stress in order to evaluate how pathological conditions might alter the expression patterns. Our findings indicated that cathepsins B, L, and S were the most prevalent members of this class. We also demonstrated an abundant expression of cystatin C.

Previous localization studies have primarily focused on cathepsin B and cystatin C. A study of the whole rat eye revealed localization of cystatin C in the RPE (Wasselius et al., 2004), whereas an earlier study found no evidence of cystatin C in the RPE (Barka and van der Noen, 1994). Previous studies on the bovine eye demonstrated the presence of cathepsin B in the RPE (Frohlich and Klessen, 2001). In the same year, cystatin C was localized in the RPE of the rat, mouse, and human eye (Wasselius et al., 2001).

The present study reveals that cathepsin B is the major cysteine cathepsin in the RPE/choroid and is localized to the RPE, confirming these earlier studies. We have also demonstrated the expression of cystatin C at the mRNA level in the RPE, but did not identify the protein in the RPE. This is presumably due to the secretion of the cystatin C protein by the RPE at its basal

surface. Cystatin C was previously shown to be a secreted cathepsin inhibitor in a variety of cell types (Paraoan, et al., 2001).

In order to test the effect of oxidative stress, we employed exposure to 75% oxygen for two weeks, as first described by Yamada (Yamada et al., 2001). In this original study, it was demonstrated that over a two week period initial photoreceptor degeneration begins to develop in the inferior hemisphere of the retina.

The effects of oxygen treatment were in all cases to up-regulate the genes that were initially found to be expressed in the RPE/choroid. It has been previously reported that cystatin C expression is up-regulated by oxidative stress (Nishio et al., 2000).

The biological function of cystatin C polymorphism associated with the risk of AMD has also been investigated. A follow-up study in cultures of RPE showed that the change in amino acid sequence of the protein generated by this polymorphism alters the trafficking of cystatin C from normal secretion through the Golgi complex to accumulation within the cell (Paraoan, et al., 2004). This observation strengthens the notion that cystatin C, and thus the secreted cysteine cathepsins B and L, may have an important role in the extracellular environment of Bruch's membrane.

It is also interesting to consider the reported roles for these cathepsins relative to theories of the pathogenesis of AMD. Cathepsin B is now thought to be capable of releasing endostatin from collagen XVIII (Ferreras, et al., 2000, Zatterstrom, et al., 2000) and the presence of this collagen has also been demonstrated in Bruch's membrane in rat (Marneros, et al., 2004). There is also a study by Bhutto et al. demonstrating that endostatin declines in AMD, yet collagen XVIII (non-endostatin portion) is similar in level (Bhutto, et al., 2004). The loss of endostatin would remove anti-angiogenic activity possibly leading to choroidal neovascularization.

In general, cysteine cathepsins have enzymatic activity pH optima that are closer to neutral than to the relatively acidic pH of the lysosome (Kirschke, et al., 1995). This observation strengthens the notion that these cathepsins play some extracellular role in matrix turnover.

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Fig. 1.

Affymetrix GeneChip Expression Analysis of cathepsins B, L, S and cystatin C, in the RPE/choroid of C57BL/6J mice after 14 days of exposure to hyperoxic (75% oxygen) or normoxic conditions (21% oxygen). Gene expression of cathepsins B, L, S and cystatin C increased under oxidative stress (solid columns). Asterisks denotes a statistically significant difference between the normoxic and hyperoxic group ($p < 0.05$).

Fig. 2.

Cathepsin B, cathepsin L and cystatin C mRNA, measured using real-time quantitative TaqMan PCR on the RPE/choroid of C57BL/6J-*Tyr^{c-2J}*/J mice, after 14 days of exposure to hyperoxic or normoxic conditions. Each value represents an average of three measurements from three eyes. Cathepsin B, L and cystatin C mRNA levels were significantly elevated under oxidative stress (solid columns). Asterisks denotes a statistically significant difference between the normoxic and hyperoxic groups ($p < 0.005$).

Fig. 3. Cathepsin B mRNA expression in the RPE/choroid of the C57BL/6J-*Tyr^{c-2J}*/J mouse, after 14 days of exposure to hyperoxic or normoxic conditions (control). (A) Staining of normoxic tissue with the sense riboprobe. (B) Staining of normoxic tissue with the anti-sense riboprobe. (C) Staining of hyperoxic tissue with the sense riboprobe. (D) Staining of hyperoxic tissue with the anti-sense riboprobe. Scale bar = 10 μM.

Fig. 4. Cystatin C mRNA expression in the RPE/choroid of the C57BL/6J-*Tyr^{c-2J}*/J mouse, after 14 days of exposure to hyperoxic or normoxic conditions (control). (A) Staining of normoxic tissue with the sense riboprobe. (B) Staining of normoxic tissue with the anti-sense riboprobe. (C) Staining of hyperoxic tissue with the sense riboprobe. (D) Staining of hyperoxic tissue with the anti-sense riboprobe. Scale bar = 10 μM.

Fig. 5. Cathepsin B immunoreactivity in the RPE/choroid of the C57BL/6J-*Tyr^{c-2J}*/J mouse, after 14 days of exposure to hyperoxic or normoxic conditions (control). (A) Staining of normoxic tissue with normal IgG. (B) Staining of normoxic tissue with anti-cathepsin B antibody. (C) Staining of hyperoxic tissue with normal IgG. (D) Staining of hyperoxic tissue with anti-cathepsin B antibody. Scale bar = 10 μ M.

Fig. 6. Cathepsin L immunoreactivity in the RPE/choroid of the C57BL/6J-*Tyr^{c-2J}*/J mouse, after 14 days of exposure to hyperoxic or normoxic conditions (control). (A) Staining of normoxic tissue with normal IgG. (B) Staining of normoxic tissue with anti-cathepsin L antibody. (C) Staining of hyperoxic tissue with normal IgG. (D) Staining of hyperoxic tissue with anti-cathepsin L antibody. Scale bar = 10 μ M.

Fig. 7. Cystatin C immunoreactivity in the RPE/choroid of the C57BL/6J-*Tyr^{c-2J}*/J mouse, after 14 days of exposure to hyperoxic or normoxic conditions (control). (A) Staining of normoxic tissue with normal IgG. (B) Staining of normoxic tissue with anti-cystatin C antibody. (C) Staining of hyperoxic tissue with normal IgG. Scale bar = 10 μ M.