Modulation of cathepsin D activity in retinal pigment epithelial cells

Piroska E. RAKOCZY*‡, C. May LAI*, Michael BAINES*, Salvatore DI GRANDI*, J. Helen FITTON† and Ian J. CONSTABLE*

*Molecular Biology and †Biomaterials Research Group, Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, 2 Verdun St., Nedlands 6009, WA, Australia

This project used retinal pigment epithelial (RPE) cells to investigate the effects of up- and down-regulation of cathepsin D expression on the processing of cathepsin D and on the normal phagocytic and digestive function of these cells. RPE cells were transfected with a pHβApr-1-neo vector construct carrying the full-length sequence of the translated region of human cathepsin D in sense and antisense directions. Transfected cells were characterized for the presence and expression of the transgene by PCR amplification using transgene-specific primers. Total aspartic proteinase activity present in transformed RPE cells was measured by an enzyme assay using haemoglobin as substrate. Flow cytometry was used to quantify phagocytosis of fluorescein isothiocyanate-labelled rod outer segments (ROS), and lysosomal digestion of ROS was monitored by immunofluorescence. A 435 bp fragment was present in RPE cells carrying the cathepsin D transgene in sense and antisense orientations after PCR amplification. Expression of both 52 kDa procathepsin D and 34 kDa active cathepsin D was significantly up-regulated in sense cathepsin D-transfected RPE cells and down-regulated in RPE cells transfected with antisense cathepsin D. No other forms of cathepsin D were detected in the transfected cells, suggesting that, if pseudo-cathepsin D exists in RPE cells *in vivo*, it requires the presence of unknown specific regulatory elements. The up- and down-regulation of cathepsin D expression was further confirmed by enzyme assay. Transfected cells retained their phagocytosing ability after ROS challenge and maintained their ability to process ROS. The processing of ROS was significantly slower in RPE cells transfected with antisense than control vector or in sense-cathepsin D-transfected cells. These results demonstrate that cathepsin D is a major proteolytic enzyme participating in the lysosomal digestion of photoreceptor outer segments.

INTRODUCTION

Cathepsin D, a member of the aspartic proteinase family, is a ubiquitous lysosomal enzyme that participates in the maintenance of cells [1] and has been proposed to play an important role in breast cancer metastasis [2,3]. Although the presence of cathepsin D has been demonstrated in numerous cell types and there are many hints as to its function *in vivo*, its role remains to be elucidated. Significantly higher than normal cathepsin D activity is found in retinal pigment epithelial (RPE) cells, suggesting a pivotal role for this enzyme in the normal functioning of these cells [4,5].

A wide range of lysosomal enzymes is present in RPE cells, and these are responsible for the continuous digestion of phagocytosed photoreceptor outer segments [4]. On the basis of enzymic studies, it has been proposed that the most important enzyme involved in the proteolysis of rhodopsin is cathepsin D [6]. Initial enzymic studies showing increased aspartic proteinase activity in RPE cells, around five times that in liver cells [1], have recently been confirmed by Northern-blot analysis demonstrating the up-regulation of cathepsin D transcription [7]. The high level of cathepsin D activity in RPE cells suggests a possible role for it in phagocytosis and digestion of photoreceptor outer segment-derived debris [4]. It has been demonstrated by enzymic assay that inhibition of aspartic proteinase results in a significant decrease in rhodopsin proteolysis [6]. RPE cells provide an excellent model for cathepsin D-related studies. However, in spite of the apparently significant role of cathepsin D in the normal function of RPE cells, at present there is very little known about the control and activation of cathepsin D in these cells and the consequences of any changes that occur in cathepsin D activity.

In this work, DNA technology was used to produce RPE cells with up- and down-regulated cathepsin D activity. These transformed RPE cells were used to monitor the activation of procathepsin D in RPE cells, and changes induced in enzymic activity were also measured. In addition, the significance of this lysosomal enzyme in photoreceptor processing was investigated by studying the effect of modified cathepsin D activity on rod-outer-segment (ROS) phagocytosis and the accumulation of ROS-derived debris.

MATERIALS AND METHODS

Production of constructs carrying cathepsin D in sense and antisense orientations

A 1616 bp *Hin*dIII fragment of a 2038 bp cathepsin D cDNA, isolated from human breast cancer MCF7 cells and cloned into M13mp10 [8], was subcloned into the eukaryotic expression vector pHβApr-1-neo carrying a β-actin promoter [9]. Restriction enzyme analysis (*Eco*RI) was used to confirm the sense and antisense orientation of the human cathepsin D cDNA in the pHβApr-1-neo vector. For transfection work, CsCl-gradient-purified DNA was prepared from both the sense and antisense cathepsin D clones and the vector.

Lipofectin transfection of RPE cells

Primary human RPE cells from a 7-year-old donor [10] were seeded, after four passages, on to six-well tissue culture plates at a concentration of 10^5 cells in 2 ml of growth medium comprised of Dulbecco's modified Eagle's medium (Australian Biosearch,

Abbreviations used: RPE cells, retinal pigment epithelial cells; FITC, fluorescein isothiocyanate; ROS, rod outer segment(s); RT, reverse transcriptase.

‡ To whom correspondence should be addressed.

Perth, Australia) supplemented with 10% fetal bovine serum. The RPE cells were incubated in a humidified atmosphere of 5% CO_2 until they were 60–80% confluent. They were then washed twice in serum- and antibiotic-free OPTI-MEM (Gibco–BRL, Gaithersburg, MD, U.S.A.). Lipofectin reagents (Gibco–BRL), diluted 1:10 in OPTI-MEM to a final volume of 100 μl, were gently mixed with 5 μg of DNA [antisense or sense cathepsin D and pHβApr-1-neo DNA (vector)] diluted in OPTI-MEM to a final volume of 100 μl and incubated at room temperature for 15 min. An additional 800 μl of OPTI-MEM was then added to the mixture, which was then gently overlaid on to the washed RPE cells in each of the wells. The cells were incubated for 20 h in a humidified atmosphere of 5% CO_2 at 37 °C before the transfection medium was removed and replaced with growth medium. After a further 48 h incubation, the cells were treated with trypsin and subcultured at 1:5 in growth medium and Geneticin (Gibco–BRL) at a final concentration of 600 μg/ml. Successfully transfected cells selected with Geneticin were maintained in growth medium and Geneticin at a final concentration of 300 μg/ml. Confluent transformed cultures were frozen for storage or subcultured for further analysis.

Characterization of transfected cells

Detection of recombinant cathepsin D by PCR

Transfected cells were grown to confluence in 25 cm² tissue culture flasks, washed in PBS, treated with trypsin and then pelleted by centrifugation and resuspended in 2 ml of 10 mM Tris/50 mM EDTA, pH 8. Proteinase K and SDS were added to a final concentration of 0.1 mg/ml and 0.5% respectively. After 2 h incubation at 37 °C, genomic DNA was extracted twice in phenol, once in phenol/chloroform and once in chloroform. The extracted DNA, precipitated using ethanol, was spooled and dried before being resuspended in 0.5 ml of 10 mM Tris/1 mM EDTA, pH 8. The DNA was quantified spectrophotometrically at 260/280 nm.

The following oligonucleotide primers were used for the specific amplification of the recombinant cathepsin D in sense or antisense orientation. The forward primer 5′-TCCCCGGGCGAGCT-CGAATT-3′ was designed to prime the polylinker region of the M13mp10 vector that was present in the 1616 bp *Eco*RI fragment used for subcloning into pHβAPr-1-neo. The reverse primer was 5′-TACTTGTGGTGGATCCAGCA-3′, corresponding to nucleotide positions 400–419 of the cathepsin D coding sequence [8]. PCR amplification was performed in a 25 μl reaction volume containing 1 μg of genomic DNA, 2 mM $MgCl_2$, 50 ng of each primer, 0.2 mM each of the four deoxyribonucleoside triphosphates, 50 mM KCl, 10 mM Tris/HCl, pH 9, 0.1% Triton X-100 and 1 unit of *Taq* polymerase (Promega, Madison, WI, U.S.A.). The pHβAPr-1-neo-cathepsin D DNA present in the transfected human RPE cells was amplified as follows: 1 cycle of denaturing at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 2 min followed by 30 cycles of annealing at 62 °C for 1 min, extension at 72 °C for 1 min and 1 cycle of denaturing at 94 °C for 1 min, annealing at 62 °C for 1 min with a final extension at 72 °C for 10 min using a GeneAmp PCR system 2400 thermocycler (Perkin–Elmer, Foster City, CA, U.S.A.).

Detection of recombinant cathepsin D expression by reverse transcriptase (RT)-PCR

Total RNA was isolated from confluent monolayers of transfected human RPE cells (25 cm² tissue culture flasks) using a RNAzol B kit (Tel-Test Inc, Friendswood, TX, U.S.A.) according to the protocol supplied. From the total RNA, 1 μg was reverse-transcribed using the GeneAmp Thermostable Reverse Transcriptase RNA PCR kit from Perkin–Elmer. Reverse transcription was performed at 60 °C and the subsequent PCR amplification was carried out using the conditions described above for the DNA PCR.

Western-blot analysis

Transfected RPE cells were grown to confluence in 75 cm² cell culture flasks as described above. The cells were washed and treated with 0.25% trypsin. The harvested cells were then prepared for SDS/PAGE by heating at 100 °C for 5 min in reducing sample buffer. The same number of cells (10^6) was loaded per gel track; electrophoresis and blotting were carried out as described previously [11]. After transfer, the blots were incubated for 30 min with 5% dried skimmed-milk powder dissolved in PBS (blocking buffer) and overnight with 200 μl of rabbit anti-cathepsin D serum diluted in 100 ml of blocking buffer, with continuous agitation. Blots were then washed for 30 min with five changes of blocking buffer and then incubated for 4 h with radioiodinated donkey anti-rabbit immunoglobulin (2×10^7 c.p.m./100 ml of blocking buffer). After washing with five changes of PBS over 30 min, the blots were dried and exposed to X-ray film (Cronex X-ray film; Dupont–NEN, Boston, MA, U.S.A.) for 72 h.

Measurement of aspartic proteinase activity

Cathepsin D activity was assayed using haemoglobin as substrate with modifications to the method of Boulton et al. [12]. Approx. 2×10^6 cells were extracted with 500 μl of 0.1 M sodium acetate buffer, pH 3.3, containing 0.2% Triton X-100 for 30 min at room temperature. The cell debris was pelleted, and the supernatant collected and diluted 1:10 in the same buffer. Substrate (50 μl of 2% haemoglobin in 0.1 M sodium acetate buffer, pH 3.3) was added to each 50 μl sample ($n = 6$) of cell lysate. After incubation for up to 20 h at 37 °C, the reaction was stopped with 50 μl of ice-cold 10% trichloroacetic acid. The samples were stored at 4 °C for 20 h before centrifugation at 250 ***g*** for 10 min. The released peptides from the digested haemoglobin (contained in the supernatant) were measured in a standard bicinchoninic acid assay (Pierce Chemicals, Rockford, IL, U.S.A.) which is detergent-stable. The resulting bicinchoninic acid complex was colorimetrically assessed at 570 nm using a Dias Plate Reader (Dynatech Medical, Guernsey, Channel Islands, U.K.). Standard curves were established using cathepsin D (Sigma Chemical Company, St. Louis, MO, U.S.A.). Peak enzyme activity with regard to haemoglobin substrate in 0.1 M sodium acetate buffer was established at pH 3.3. Standard curves were run at the same time as the cell lysates to ensure uniformity of incubation time and substrate concentration.

Activity of each sample ($n = 6$) was determined relative to the standard curve and normalized against the protein content of the cell lysate. Protein concentrations of each cell lysate ($n = 4$) were also assessed using the bicinchoninic acid method against a BSA standard. For each sample, standard deviations were calculated. Standard curves were plotted and sample activities were calculated from the regression curve using Cricket Graph (Computer Associates International, Islandia, NY, U.S.A.). One unit of cathepsin D activity was defined as the amount that produces an increase in A_{280} of 1.0 per ml at pH 3.3 and 37 °C measured as trichloroacetate-soluble products using haemoglobin as substrate. Experiments as described above were repeated twice using independent cultures of transformed RPE cells.

To check the specificity of the assay for aspartic proteinases, pepstatin (Sigma) was added in excess (1.5 μg/50 μl of cell lysate) half an hour before incubation with haemoglobin substrate, and the remaining enzyme activity was measured as described above.

Quantification of ROS phagocytosis by FACS

The phagocytosing capacity of transfected cells was measured by flow cytometry using fluorescein isothiocyanate (FITC)-labelled ROS as described by Kennedy et al. [13]. Briefly, transfected RPE cells were cultured in 24-well tissue-culture plates in medium containing 300 μg/ml Geneticin. For each transfected cell type, triplicate wells were used. Samples were challenged with 1 ml of growth medium containing 10^7 FITC-labelled bovine ROS for 6 h. After incubation, unchallenged controls and challenged samples were washed three times in PBS and harvested by trypsin treatment, washed and resuspended in 0.15 M NaCl to be measured by FACS [14].

Immunofluorescence analysis of ROS-challenged transfected cells with a bovine ROS polyclonal antibody

Transfected RPE cells were seeded on to chamber slides and challenged with ROS for 6 h as described above. After challenge, the cells were washed with PBS and further incubated in growth medium. At 0, 72 and 144 h after washing, the transfected RPE cells were washed again in PBS, fixed in methanol at -20 °C for 30 s and permeabilized by incubating with 47.5% ethanol in water for 15 min at room temperature. Cells were rehydrated in PBS/0.1% BSA at room temperature for 30 min and then incubated at room temperature for 30 min with 10% normal goat serum in PBS containing 0.1% BSA. The goat serum was then drained off and replaced with the primary antibody overnight at 4 °C. The cells were then washed three times in PBS containing 0.1% BSA followed by incubation with secondary antibodies (anti-rabbit IgG TRITC conjugate; Sigma) at room temperature for 1 h. Slides were examined at 570 nm excitation wavelength and photographed with the Olympus equipment as described above except that a 2 s exposure time was used for all samples.

RESULTS

Cloning cathepsin D into pHβApr-1-neo vector

After restriction enzyme digestion, the 1616 bp *Hin*dIII fragment containing the full-length cathepsin D cDNA with some untranslated region was recovered from the 2038 bp cathepsin D cloned in M13mp10CatD and subcloned into a *Hin*dIII-linearized pHβApr-1-neo vector (Figure 1). The orientation of the cathepsin D inserts was analysed by *Eco*RI digestion. Clones carrying the cathepsin D fragment in sense and antisense orientation presented different restriction patterns with bands appearing at 4.3 and 7.3 and 5.7 and 5.9 kb respectively (results not shown).

Characterization of transfected RPE cells

Detection of recombinant cathepsin D transgenes

Initial selection of transfected cells was performed by using antibiotic pressure [9]. To confirm the presence of recombinant cathepsin D, a PCR primer pair was selected to amplify the transgenes specifically and not endogenous cathepsin D. PCR amplification of DNA isolated from transfected cells carrying the transgene in a sense or antisense orientation demonstrated the presence of a 435 bp fragment (Figure 2, lanes 3 and 4). Amplification of genomic DNA from native RPE cells or vector-transfected cells did not result in the appearance of a signal (Figure 2, lanes 1 and 2).

Expression of recombinant cathepsin D mRNA in sense and antisense direction

RNA transcripts of recombinant cathepsin D were detected by reverse transcriptase (RT)-PCR using the same primer pair as above. Both sense- and antisense-transfected cells in the presence of RT produced fragments 435 bp long (Figure 3, lanes 5 and 7). No recombinant transcript was detected in vector-transfected cells (Figure 3, lane 1). Likewise no signal was detected in native RPE cells and sense and antisense cathepsin D-infected cells in the absence of RT (Figure 3, lanes 2, 6 and 8).

Analysis of cathepsin D expression by Western-blot analysis

Western-blot analysis of pHβApr-1-neo vector-transfected RPE cells revealed the presence predominantly of a 34 kDa form of cathepsin D (Figure 4, lane 1). In contrast, cells transfected with sense cathepsin D demonstrated the presence of two forms of cathepsin D at 34 and 52 kDa, both of which appeared to be up-regulated (Figure 4, lanes 2 and 3). No other forms of cathepsin D were detected at any time. The up-regulation of cathepsin D expression in sense cathepsin D-transfected cells was further confirmed by the presence of the full-length 52 kDa form in the supernatant of these cells (Figure 4, lane 6). No cathepsin D was detected in the supernatant of vector-, antisense-transfected or native RPE cells (results not shown). The most dramatic change was observed in antisense cathepsin D-transfected cells. In these cells, using the conditions described for the vector- and sense cathepsin D-transfected cells, neither form of cathepsin D was detectable (Figure 4, lanes 4 and 5).

Measurement of aspartic proteinase activity by enzyme assay

To check the specificity of the assay, pepstatin (a known aspartic proteinase inhibitor) was added to the cell lysates before incubation with substrate. No residual aspartic proteinase activity was detected in any pepstatin-treated cell lysate examined, demonstrating that the assay was specific for aspartic proteinases. There was a dramatic difference in aspartic proteinase activity between the control vector-transfected and sense and antisense cathepsin D-transfected RPE cells. The mean enzyme activities from each of six samples from two experiments were 252.7 and 261.4 units/mg of protein in vector-transfected, 19.8 and 50.1 units/mg of protein in antisense cathepsin D-transfected and 404.5 and 369.0 units/mg of protein in sense cathepsin D-transfected RPE cells.

Measurement of the phagocytosing capacity of transfected RPE cells

In order to assess the effect of any variation in cathepsin D activity on processing, it was important to establish whether the transformed cells had retained their phagocytosing capacity. After trypsin treatment, RPE cells were detached from the plastic surface of the culture flasks and all surface-bound outer segment particles were also removed [15]. The amount of internalized outer-segment-derived particles was then measured by a FACS analyser. After 6 h of challenge with FITC-labelled ROS, the mean fluorescence readings above background of vector-transfected, antisense and sense cathepsin D-transfected cells

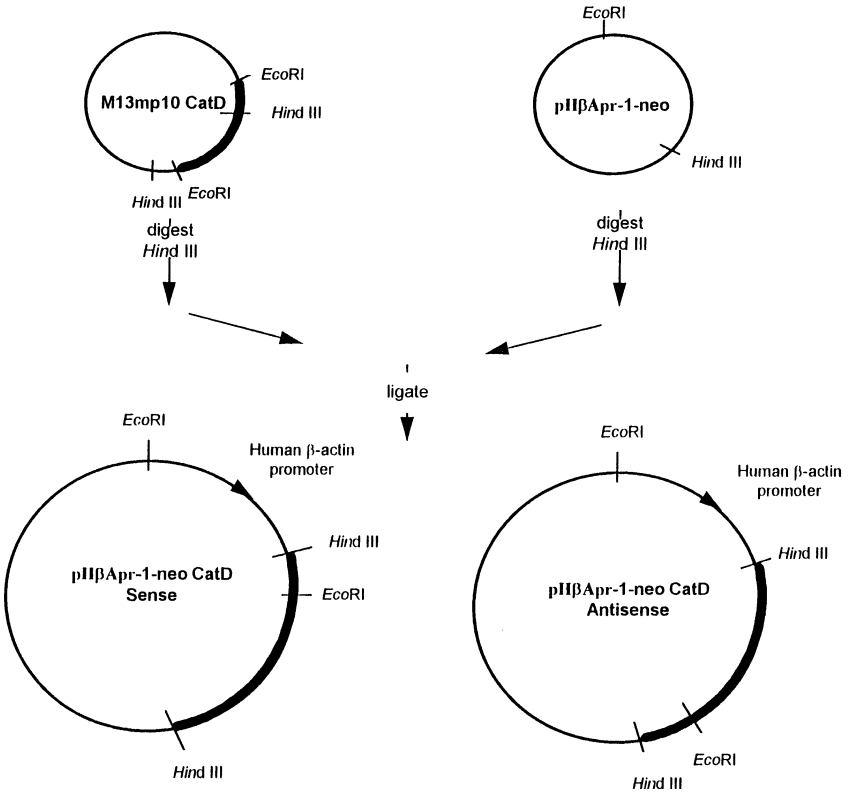

Figure 1 Flow chart of cathepsin D subcloning into pHβApr-1-neo vector

Constructs carrying cathepsin D insert in sense or antisense direction gave different sized restriction enzyme fragments after *Eco*RI digestion.

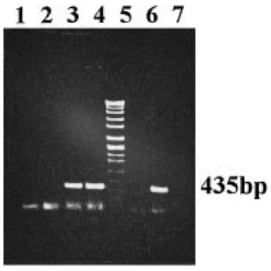

Figure 2 PCR amplification of RPE cells using a primer pair specific for recombinant cathepsin D in transfected cells

Lane 1, native RPE cells; lane 2, RPE cells transfected with the vector; lane 3, RPE cells transfected with sense cathepsin D; lane 4, RPE cells transfected with antisense cathepsin D; lane 5, molecular-mass marker SPPI *Eco*RI (Progen, Darra, Queensland, Australia); lane 6, negative control; lane 7, positive control (pHβApr-1-neo-Cat D).

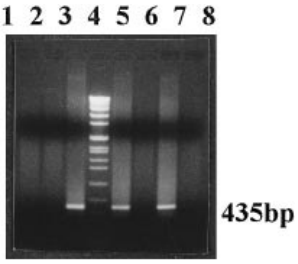

Figure 3 Detection of recombinant cathepsin D mRNA expression using RT-PCR

Lane 1, pHβApr-1-neo-vector-transfected cells; lane 2, native RPE cells in the presence of RT; lane 3, positive control pHβApr-1-neo-Cat D; lane 4, molecular-mass marker SPPI *Eco*RI; lane 5, sense-cathepsin D-transfected cells with RT; lane 6, sense-cathepsin D-transfected cells without RT; lane 7, antisense-cathepsin D-transfected cells with RT; lane 8, antisense-cathepsin D-transfected cells without RT.

demonstrated the presence of significant amounts of ROS particles in RPE cells (Table 1).

ROS processing by transfected RPE cells

In this work a polyclonal antibody raised against bovine ROS was used to label the undigested ROS particles with rhodamine. As demonstrated in Figures 5(A)–5(C), after a 6 h challenge with ROS, a significant number of ROS-derived particles was present in the transfected cells. There was heterogeneity of phagocytic activity within the cell populations after the 6 h challenge and 6 days after the challenge. All necessary controls including non-immune serum and unchallenged RPE cells lacked a fluorescent signal. After 6 h of challenge, RPE cells were rinsed with PBS to remove excess unbound ROS and incubated for a further 3 days and 6 days. There was no significant difference between vector- and cathepsin D-transfected RPE cells after 3 days of incubation. After 6 days of incubation no signal could be detected in vector-transfected and sense cathepsin D-transfected RPE cells (results not shown). However, in antisense cathepsin D-transfected cells, the signal remained strong, demonstrating

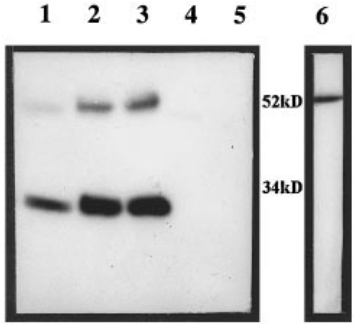

Figure 4 Western-blot analysis of RPE cells using a cathepsin D polyclonal antibody

Lane 1, RPE cells transfected with pHβApr-1-neo vector; lanes 2 and 3, RPE cells transfected with sense cathepsin D; lanes 4 and 5, RPE cells transfected with antisense cathepsin D; lane 6, medium of RPE cells transfected with sense cathepsin D. kD, kDa.

Table 1 Phagocytosis of FITC-labelled ROS by transfected RPE cells after 6 h of challenge

Sample		Fluorescence intensity: Raw data	Fluorescence intensity: Mean ± S.D.
Vector	No ROS	13.9, 12.8, 13.3	13.3 ± 0.6
Vector	+ FITC-labelled ROS	424.9, 486.5, 435.9	499.1 ± 32.9
Antisense cathepsin D	No ROS	16.6, 20.4, 16.5	17.8 ± 2.2
Antisense cathepsin D	+ FITC-labelled ROS	379.0, 357.7, 374.5	370.4 ± 11.2
Sense cathepsin D	No ROS	21.3, 18.8, 21.4	20.5 ± 1.5
Sense cathepsin D	+ FITC-labelled ROS	263.7, 272.3, 269.5	265.5 ± 4.4

the presence of a considerable amount of undigested debris (Figure 5D).

DISCUSSION

In order to elucidate the role of up-regulated cathepsin D activity in RPE cells, it is necessary to manipulate cathepsin D levels in these cells. Recently, *in situ* expression of sense and antisense genes has been used to regulate the production of specifically targeted genes [9,16,17]. This technique provides stable transient but long-term expression of the transgene sequence. Expression is regulated by a specific promoter and is independent of the genomic sequence. The method eliminates the use of complicated protective groups and usually provides high specificity. One of the disadvantages of this technology is that transformed cells are selected by antibiotic suppression, thus preventing direct comparison of native and transfected cells. However, transfection

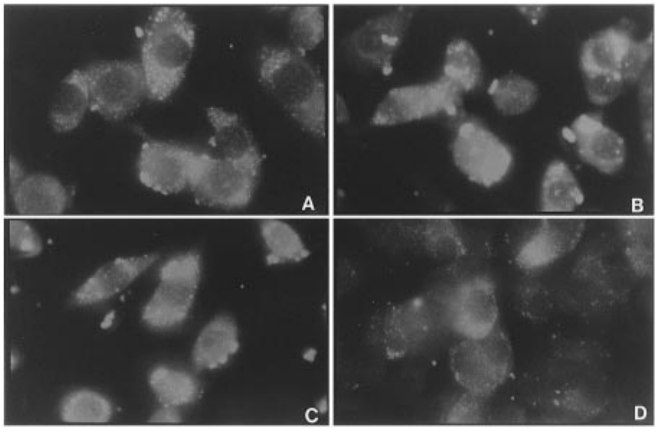

Figure 5 Immunofluorescence detection of ingested ROS using a polyclonal antibody raised against bovine ROS

RPE cells transfected with vector pHβApr-1-neo (**A**), sense cathepsin D (**B**) and antisense cathepsin D (**C**) were challenged with bovine ROS for 6 h. Residual ROS particles in antisense cathepsin D-transfected cells at 6 days after the challenge (**D**) remain visible.

with the vector can generate the necessary control cells carrying the antibiotic-resistance [18]. Although some alteration in the natural behaviour of these cells cannot be excluded, insertion of the vector does not appear to affect untargeted cellular functions. The approach has provided some spectacular results in the study of the effects of up- and down-regulation of a wide range of genes on cellular function. In this work we have produced pHβApr-1-neo vector-transfected and pHβApr-1-neo-sense and antisense cathepsin D-transfected RPE cells. The transfected cells retained epithelial-like morphology, although the typical cobblestone appearance was less marked.

The production of the active form of cathepsin D involves several post-transcriptional modification steps [2,19]. Cathepsin D is expressed in a full-length inactive form (52 kDa), which is then subjected to a series of activation steps in the lysosomal compartments to produce a 34 kDa active form of the enzyme [2,20,21]. Unlike other aspartic proteinases, cathepsin D is usually not secreted from normal cells. The presence of secreted cathepsin D in tumours has recently been used as a hallmark of metastasis [2,21]. It has been demonstrated previously that some RPE cells express not only the 34 kDa active form but also the high-molecular-mass forms, presumably pseudo- and prepro-cathepsin D [11]. Therefore it was expected that, with the up-regulation of cathepsin D expression, inactive forms of the enzyme would also accumulate in the transfected RPE cells. Indeed the up-regulation of cathepsin D expression in sense-cathepsin D-transfected cells resulted in a significant increase in both the 52 and 34 kDa forms of cathepsin D. No other forms of cathepsin D were detected in the transfected cells, suggesting that, if pseudo-cathepsin D exists in RPE cells *in vivo*, it requires the presence of unknown specific regulatory elements. Excess unprocessed cathepsin D present in sense cathepsin D-transfected RPE cells was secreted and readily detectable in the medium. Although cathepsin D is ubiquitously expressed in retinal cells under normal circumstances, it is not present in the photoreceptor outer segment layer [22]. However, it has recently been demonstrated that cathepsin D is secreted into the photoreceptor outer segment layer in the Royal College of Surgeons (RCS) strain of rat [23]. RCS rats suffer from defective phagocytosis of the ever-growing outer segments, resulting in ac-

cumulation of undigested debris [24,25]. It is unclear whether the secretion of cathepsin D is a specific characteristic of RPE cells, but it is interesting to note that transfection of NIH 3T3 fibroblast cells with the same construct did not induce cathepsin D secretion (P. E. Rakoczy, unpublished work).

Strong down-regulation of cathepsin D activity was observed in RPE cells carrying the transgene in an antisense orientation. Expression of antisense cathepsin D mRNA resulted in the complete inhibition of cathepsin D translation, as demonstrated by the lack of signal in the Western-blot analysis and a significant decrease in aspartic proteinase activity. Some residual activity was detected by the enzyme assay. This may be due to the presence of aspartic proteinases other than cathepsin D in RPE cells or it may just reflect the different sensitivities of the two techniques. Nevertheless, both techniques used in this study demonstrated that transfection of cells is a suitable method for controlling cathepsin D activity in RPE cells.

One of the most distinctive characteristics of RPE cells is their high phagocytosing capability [26], and this ability is retained by cultured RPE cells [27,28]. In spite of intense research over the last decade, the mechanism of photoreceptor outer segment uptake remains unknown. A receptor-mediated process has been proposed by several researchers [7,29]. The phagocytosing capacity of transformed RPE cells produced in this work was comparable with that of other untransformed human RPE cells. The variation seen between the vector-transfected, sense- and antisense cathepsin D-transfected cells fell within the normal distribution observed for other human RPE cells [13], suggesting that there were no significant changes in the expression of surface proteins involved in phagocytic uptake.

In this work, we used the presence of opsin, visualized by immunofluorescence, to monitor the digestion of phagocytosed ROS. The disappearance of ROS-derived fluorescence signal from vector-transfected and sense cathepsin D-transfected cells demonstrated that lysosomal processing remained unimpaired in these cells. In spite of the severalfold increase in cathepsin D activity in sense cathepsin D-transfected cells, no apparent acceleration was observed in lysosomal digestion of rhodopsin. This observation suggests that the cathepsin D-mediated proteolysis is preceded by other digestive steps. In antisense cathepsin D-transfected RPE cells, which have a reduced amount of enzymically active cathepsin D, lysosomal digestion of ROS was significantly impaired. A strong fluorescence signal was present even 6 days after the challenge (Figure 5D), in contrast with the lack of fluorescence noted in sense- and vector-transfected cells. These results demonstrate for the first time that cathepsin D is indeed a major enzyme responsible for the proteolysis of the ever-growing photoreceptors, and a decrease in the activity of this enzyme may lead to ROS-derived debris accumulation in RPE cells.

Received 23 December 1996/24 February 1997; accepted 24 February 1997

We thank Professor Michael Garlepp (Department of Medicine, University of Western Australia) for the pHβApr-1-neo vector, Professor Henri Rochefort (Faculty of Medicine, University of Montpellier, Montpellier, France) for the human cathepsin D clone, and Professor Michael Hall (Jule Steine Eye Institute, University of California, Los Angeles, CA, U.S.A.) for providing the bovine ROS-derived polyclonal antibody and the relevant protocol.

REFERENCES

1 Yamamoto, K. (1995) in Aspartic Proteinases; Structure, Function, Biology, and Biomedical Implications, (Takahashi, K., ed.), pp. 223–229, Plenum Press, New York
2 Rochefort, H. (1990) Cancer Biol. **1**, 153–160
3 Rochefort, H., Capony, F. and Garcia, M. (1990) Cancer Metastasis Rev. **9**, 321–331
4 Zimmerman, W. F., Godchaux, III, W. and Belkin, M. (1983) Exp. Eye Res. **36**, 151–158
5 Alder, J and Martin, K. J. (1983) Curr. Eye Res. **2**, 359–365
6 Regan, C. M., De Grip, W. J., Daemen, F. J. M. and Bonting, S. L. (1980) Exp. Eye Res. **30**, 183–191
7 Cavaney, D. M., Rakoczy, P. E. and Constable, I. J. (1996) Aust. N. Z. J. Ophthalmol. **24**, 75–78
8 Augereau, P., Garcia, M., Mattei, M. G., Cavailles, V., Depadova, F., Derocq, D., Capony, F., Ferrara, G. B. and Rochefort, H. (1988) Mol. Endocrinol. **2**, 186–192
9 Gunning, P., Leavitt, J., Muscat, G., Ng, S. and Kedes, L. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4831–4835
10 Rakoczy, P., Kennedy, C., Thompson-Wallis, D., Mann, K. and Constable, I. (1992) Biol. Cell. **76**, 49–54
11 Rakoczy, P. E., Baines, M., Kennedy, C. and Constable, I. J. (1996) Exp. Eye Res. **63**, 159–167
12 Boulton, M., Moriarty, P., Jarvis-Evans, J. and Marcyniuk, B. (1994) Br. J. Ophthalmol. **78**, 125–129
13 Kennedy, C. J., Rakoczy, P. E. and Constable, I. J. (1996) Curr. Eye Res. **15**, 998–1002
14 Rakoczy, P. E., Mann, K., Cavaney, D. M., Roberston, T., Papadimitreou, J. and Constable, I. J. (1994) Invest. Ophthalmol. Vis. Sci. **35**, 4100–4108
15 Kennedy, C., Rakoczy, P., Robertson, T. A., Papadimitriou, J. H. and Constable, I. (1994) Exp. Cell Res. **210**, 209–214
16 Saleh, M., Stacker, S. A. and Wilks, A. F. (1996) Cancer Res. **56**, 393–401
17 Weinander, R., Mosialou, E., DeJong, J., Tu, C. D., Dypbukt, J., Bergman, T., Barnes, H. J., Hoog, J. and Morgenstern, R. (1995) Biochem. J. **311**, 861–866
18 Lin, C., Ng, S., Gunning, P., Kedes, L. and Leavitt, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6995–6999
19 Richo, G. and Connor, G. E. (1991) in Structure and Function of the Aspartic Proteinases, (Dunn, B. M., ed.), pp. 289–296, Plenum Press, New York
20 Richo, G. and Conner, G. E. (1994) J. Biol. Chem. **269**, 14806–14812
21 Liaudet, E., Derocq, D., Rochefort, H. and Garcia, M. (1995) Cell Growth Differ. **6**, 1045–1052
22 Yamada, T., Hara, S. and Tamai, M. (1990) Invest. Ophthalmol. Vis. Sci. **31**, 1217–1223
23 El-Hifnawi, E., Kuhnel, W., El-Hifnawi, A. and Laqua, H. (1994) Ann. Anat. **176**, 505–513
24 Mullen, R. J. and La Vail, M. M. (1976) Science **192**, 799–801
25 Porrello, K., Yasmura, D. and LaVail, M. M. (1986) Exp. Eye Res. **43**, 413–429
26 Young, R. W. (1967) J. Cell Biol. **33**, 61–72
27 Feeney, L. and Mixon, R. N. (1976) Exp. Eye Res. **22**, 533–548
28 Hall, M. O. and Abrams, T. (1987) Exp. Eye Res. **45**, 907–922
29 Crossman, M., Boulton, M., Marshall, J. and Mellerio, J. (1989) Biochem. Soc. Trans. **302**, 1989–1990