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Expression of multiple forms of clusterin during light-induced retinal degeneration

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Abstract

Purpose. Clusterin has been associated with active cell death in several different model systems, including animal models of retinal degeneration. Clusterin is also expressed in normal tissues, a finding that leads to the question of how it could then play a cell death-specific role during tissue regression. To address this paradox, we have examined clusterin expression during light-induced retinal damage in rats.

Methods. Normal albino rats were reared in darkness and then exposed to intense visible light to induce retinal degeneration. Clusterin expression was then examined at various times after light treatment. Standard molecular techniques including Northern analysis, immunohistochemistry, and Western analysis were employed.

Results. Northern analysis established that the largest increase in clusterin expression occurs after a decrease in interphotoreceptor retinoid binding protein, IRBP, expression (an indication of a photoreceptor cell dysfunction) and after an increase in heme oxygenase 1, HO-1, expression (an oxidative stress inducible gene), suggesting that induction of clusterin expression is an oxidative stress response. Immunohistochemical analysis with two different clusterin-specific antibodies, anti(SGP-2) and anti(301), localized distinct forms of clusterin to Müller cells and degenerating photoreceptor cells. Western analysis demonstrated degeneration associated isoforms of clusterin in light treated retina that are not present in normal retina.

Conclusion. Clusterin over-expression is characteristic of a retinal degeneration phenotype and we propose that clusterin action may be defined by the nature in which it is modified. We hypothesize that alternate processing leads to retinal degeneration-specific forms of the protein (65, 61, and 50 kDa) that are not present in normal retina.

Keywords: retinal degeneration; light damage; oxidative damage; clusterin; apoptosis

Introduction

Photoreceptor cell damage induced by visible light in rats is a complex process, leading to cell death and blindness, which has been studied as a model system of human retinal dystropies.[1,2] In the rat, rod photoreceptor degeneration by prolonged green light exposure is triggered by excessive bleaching of the photopigment rhodopsin.[1] The extent of retinal damage varies with age, genetics, diet and prior light history of the experimental animals.[2] In the case of albino rats which have been reared in the dark, approximately 80% of the photoreceptor cells are lost over a two week period in response to a 24 h intense light exposure.[3] In general,

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increased periods of light exposure result in more extensive retinal cell damage. The mechanism that leads to light-induced visual cell loss is not completely understood; however, a number of studies have implicated an increase in oxidative-stress as an early event. Induction of heme oxygenase-1 (HO-1) and loss of ascorbic acid (a water soluble antioxidant) from the retina during intense light exposure, together with a reduction in the extent of visual cell damage in animals supplemented with antioxidants prior to light treatment, support this hypothesis.[2,4] The appearance of DNA fragmentation during light-induced retinal damage suggests that photoreceptor cell death occurs by apoptosis.[5,6]

Clusterin (CLU) is a 65–80 kDa glycoprotein that can be cleaved into α- and β-associated dimers prior to secretion.[7] This protein is thought to be multifunctional and has been shown to inhibit complement attack, participate in lipid transport, mediate cell membrane re-modeling, and act as a molecular chaperone.[7–11] The gene coding for clusterin was initially characterized as a testosterone repressed prostatic message (TRPM-2), a gene which is over expressed during prostate regression.[12,13] In addition, clusterin expression is upregulated in neurodegenerative disorders such as Alzheimer's Disease and Parkinson's Disease.[14] Over expression of this gene has also been observed in isolated cases of retinitis pigmentosa and during the time of active photoreceptor cell loss in rodent models of retinal degeneration.[15–17] Taken together, these observations suggest that clusterin plays an important role in active cell death and tissue degeneration. In contrast, clusterin is expressed in a variety of normal tissues and is also present in physiological fluids such as blood.[18–21] A major question is how clusterin could participate in an active death process and yet still be functional in normal tissues. To address this question we have analysed clusterin expression during light-induced retinal degeneration in rats reared in darkness.

Materials and methods

Animals

Weanling male albino Sprague-Dawley rats were obtained from Harlan Inc. (Indianapolis, IN) and kept in darkness for 40 days. At 61 days of age, rats were exposed to intense visible light for up to 24 h. Light exposures were started at 9 AM and performed in green #2092 Plexiglas chambers (Dayton Plastics, Dayton, OH) transmitting 490–580 nm light (green light) with an illuminance of ~1200 lux. The action spectrum of green light overlaps the absorbance spectrum for rhodopsin.[22] Following light exposure, some animals were maintained in the dark for up to 12 days before sacrifice. Rats were sacrificed in carbon dioxide-saturated chambers and retinae were excised and flash frozen in plastic vials on dry ice. They were stored at −80°C until use. For each treatment condition, retinae were obtained and pooled from 2–3 animals. In all cases, animals were cared for in accordance with the guidelines defined by the NIH Guide for the care and use of laboratory animals.

DNA probes

The rat TRPM-2 cDNA (for the detection of clusterin mRNA) and mouse IRBP cDNA probes were obtained directly from available plasmids.[23,24] A HO-1 DNA probe was generated by PCR as previously described.[4] DNA probes were radiolabeled with [α-^{32}P] dCTP by oligonucleotide labeling to a specific activity of not less than 10^8 cpm/μg.

RNA isolation and northern analysis

Total RNA was isolated using RNAzol (Tel-Test, Inc., Friendswood, TX) following the manufacturer's protocol. RNA was either kept as a pellet in ethanol or dissolved in DEPC-treated water and kept frozen at −80°C until use.

Total RNA was electrophoresed in 1.0 or 1.2% agarose gels in a formaldehyde running buffer system.[25] Northern transfer onto Genescreen Plus nylon membrane (Dupont, NEN, Boston, MA) was carried out by passive blotting. Prehybridization and hybridization were carried out in Hybrisol II (ONCOR, Gaithersburg, MD). Probes were added at a concentration of 2×10^6 cpm per ml of hybridization mix and incubated with the membrane at 65°C for 16 h. The membranes were washed twice in 2 × SSC at 65°C for 15 min, once in 2 × SSC, 0.1% SDS at 65°C for 30 min and once in 0.1 × SSC, 0.1% SDS at 65°C for 10 min. Autoradiography was carried out at −80°C using Kodak X-OMAT film between two intensifying screens. The expression profiles were verified by multiple Northern blot analysis. The intensity of bands on autoradiographs and negatives was quantified using the Kodak Digital Science 1D image analysis system (Eastman Kodak Co., Rochester, NY). In the case of Northern blots, we accounted for possible differences in band intensities due to differences in RNA loading by using the relative intensity of the 18S ribosomal band in each sample to normalize the respective probe value.[26] Statistical analysis was performed using Quatro Pro Version 7.0 software (Corel, Ottawa, Canada).

Antibodies

A clusterin polyclonal antibody, anti(SGP-2), was kindly provided by Dr. M. Griswold (Washington State University, Pullman, WA). A second clusterin antibody, ant(301), was generated using four peptides derived from the α subunit of rat clusterin (residues 1–205 of the proprotein). The peptides included residues 1–17 (EQEFSDNELQELSTOGSC), residues 32–48 (KHIKTLIEKTNAERKS), residues 52–72 (LEEAKKKKEGALDDTRDSEC), and residues 133–148 (NGDRIDSLLESDRQQSC) (J. Lakins, personal communication). The specificity of each antibody for clusterin has been verified by western blot analysis and competition assays.

Immunohistochemistry

Enucleated eyes were fixed in 4% paraformaldehyde in Tyrode's buffer overnight before embedding in diethylene glycol distearate (DGD) wax.[27] Sections (2 μm) were cut

using glass knives and placed on chrom-alum/gelatine-coated slides. Prior to immunohistochemistry, the DGD wax was removed from the slides with xylene (2 × 15 min) and the tissue section was rehydrated in an alcohol series and rinsed in phosphate buffered saline, PBS, (3 × 5 min). To reduce non-specific binding, sections were incubated with normal goat serum (1 : 200, Vector, Burlingame, CA) for 30 min. Primary polyclonal antibodies, anti(SGP-2) or anti(301) were diluted 1 : 300 in PBS containing 1% bovine serum albumin. Sections were incubated with primary antibody overnight at room temperature. Slides were then rinsed in PBS and the reaction was visualized using a Vectastain ABC kit (Vector, Burlingame, CA) and developed with diaminobenzidine. Control sections were treated according to the above scheme with the omission of the primary antibodies.

Western analysis of retinal extracts in PBS

Retinal extracts were prepared in PBS (supplemented with 10^{-9} M EDTA, pH 7.0, 7 µg/ml phenylmethanesulfonyl flouride (Sigma P7626), 0.7 µg/ml leupeptin (Sigma L2884), and 0.5 µg/ml pepstatin A (Sigma P4265)) using standard protocols. Equal amounts of protein (10 µg) were prepared under non-reducing conditions and loaded in each lane of a 10% SDS-polyacrylamide (PAGE) gel. Electrophoresis was performed according to Laemmli.[28] Following electrophoresis, proteins were electrophoretically transferred to nitrocellulose. The blot was washed with 0.1% Tween 20 in PBS, blocked with 3% gelatin at 37°C for 1 hr and then incubated for 2 hr at 24°C with the primary antibody. Alkaline phosphatase conjugated to a goat anti-mouse IgG (Sigma, St Louis, MO) was used with chromogen for visualization.

Western analysis of retinal extracts made in TRIzol

Retinal lysates were also made using TRIzol, an organic based protein extraction method, which requires the eventual protein pellet to be dissolved in 1% SDS (Gibco, BRL, Gaithersburg, MD). In this method no additional protease inhibitors are required. Equal amounts of protein were prepared under reducing conditions and separated on 12.5% polyacrylamide gels[28] and transferred to nitrocellulose membrane. Western analysis was performed using standard protocols. Briefly, membranes were washed twice for 15 min in 10 mM PBS with 0.35% Tween 20 (pH 7.5) and blocked with 1% heat-denatured casein. Membranes were then incubated overnight at 4°C with primary antibody diluted 1/1000 in blocking solution. Membranes were washed twice for 15 min in 10 mM PBS with 0.35% Tween-20, twice in blocking solution for 15 min each, and then incubated in secondary antibody (biotinylated anti-rabbit Ig diluted 1/400 in blocking solution (Amersham, Oakville, Canada)) for 2 h at room temperature. The antibody conjugate was detected by chemiluminescence (Boehringer Manheim, Indianapolis, IN). In order to verify the specificity of the antibodies to rat retinal clusterin, the antibody was competed (pre-absorbed) with purified recombinant rat clusterin prior to reaction with the blot.

Results

Alteration of clusterin mRNA levels occurs down-stream of an oxidative-stress event

Changes in clusterin mRNA levels were compared in parallel with the expression profiles of interphotoreceptor retinoid binding protein, IRBP, and heme oxygenase-1, HO-1 (Figs. 1 & 2). Within the retina, IRBP is a photoreceptor-specific protein which plays a role in retinoid transport between retinal photoreceptors and pigment epithelial cells.[29] IRBP was used in the current study as a marker of normal photoreceptor cell function. HO-1 is an oxidative-stress inducible gene and was used as a marker for the presence of oxidative-stress.[30] Functionally, HO-1 activity degrades heme, a prooxidant, to biliverdin, which in turn is converted to bilirubin, an antioxidant.[31] To determine the relative kinetics of clusterin mRNA expression, two different extended

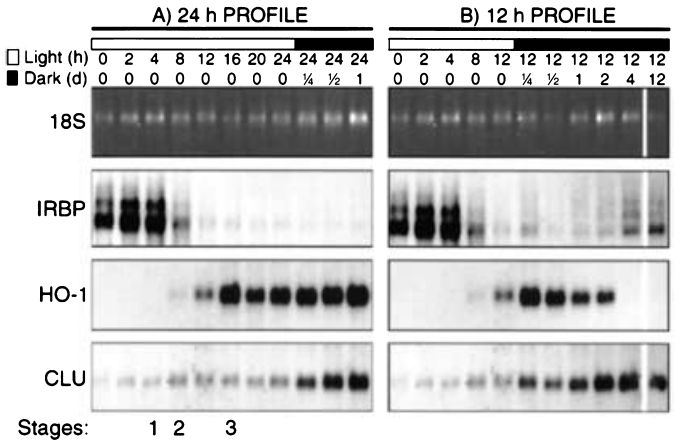

Figure 1. Time Courses: Representative Northern blot analysis of retinal clusterin mRNA levels. Two different time courses were analyzed by Northern analysis for IRBP, HO-1, and CLU mRNA levels. Lanes marked by an empty box represent an increasing light exposure treatment profile (Light) measured in hours (h). Lanes marked by a filled box represent a dark recovery period time course following a fixed light exposure (Dark) measured in days (d). In the top leaf is the photographic negative of the ethidium bromide stained gel showing the 18S RNA before transfer of the RNA to the membrane. Approximately 3 µg of total RNA is loaded per lane. The same blot was probed, stripped, and re-probed with the various markers indicated. The resulting autoradiographs from probing with the different markers are shown. The profile of gene expression allows us to divide the degeneration process into specific stages. Stage 1 is the step just prior to a marked decrease in normal photoreceptor cell function (as deduced from changes in IRBP mRNA levels). Stage 2 represents the stage at which the marked decrease in IRBP mRNA levels occurs. Stage 3 represents the stage at which the maximum state of oxidative stress is reached (as deduced from the HO-1 mRNA levels).

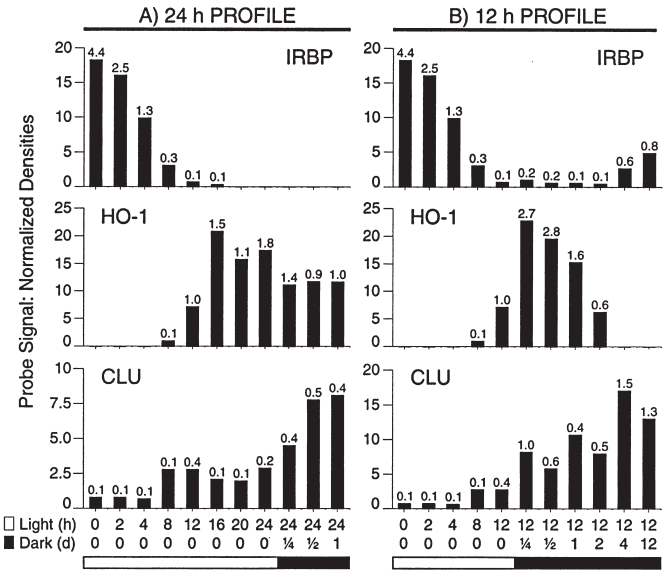

Figure 2. Graphical analysis of Northern profiles. The expression profiles were verified by multiple Northern blot analysis. The intensity of bands on autoradiographs and negatives was quantified using the Kodak Digital Science 1D image analysis system (Eastman Kodak Co., Rochester, NY). We accounted for possible differences in band intensities due to differences in RNA loading by using the relative intensity of the 18S ribosomal band in each sample to normalize the respective probe value.[26] Statistical analysis was performed using Quatro Pro Version 7.0 software (Corel, Ottawa, Canada). Standard deviations are indicated above each bar. The sample size used for each treatment condition is a minimum of 3.

time courses were examined (Fig.1). First, to determine the duration of light exposure required to alter gene expression, animals were exposed to light for increasing lengths of time. A second series of experiments were performed to determine the effect of dark recovery on gene expression after a fixed light exposure. Two distinct recovery treatment profiles are shown in Figure 1, one representing the long term effects of a 12 h light exposure and the second representing the long term effects of a 24 h light exposure. A longer light exposure results in a more severe immediate degeneration phenotype than a shorter light exposure.[1] We have performed multiple scans of a series of Northern profiles in order to determine the relative levels of IRBP, HO-1 and clusterin mRNA at each specific treatment point examined (Fig. 2).

In dark-reared animals, the effects of light treatment were pronounced. IRBP mRNA levels decreased markedly after a 4–8 h light exposure (Figs. 1 & 2). HO-1 mRNA levels were initially detectable after an 8 h light exposure. HO-1 levels increased thereafter and remained high throughout the dark recovery period following a 24 h light exposure. After a 12 h light exposure, HO-1 mRNA levels were high for up to 2 days, after which levels declined to undetectable levels (Figs. 1B & 2B). After an initial 12 or 24 h light exposure, clusterin mRNA levels increased at a much slower rate than that observed for HO-1, but they remained high throughout the remainder of each recovery period examined. The initial increase in clusterin mRNA levels also occured after 8 h of light exposure (Fig. 2). The peak in clusterin mRNA levels coincided with a noticeable decrease in HO-1 levels during the recovery period (Fig. 2). There is an apparent partial recovery of IRBP mRNA expression which coincides with a sharp decrease in HO-1 mRNA levels 4 days after a 12 h light exposure. During this recovery, clusterin mRNA levels remained elevated even after HO-1 mRNA levels have returned to background levels.

Immunohistochemical localization of clusterin in light damaged retinas

A) anti(SGP-2) immunoreactivity

Anti-(SGP-2) reactivity was not detected on normal retina sections (Fig. 3A). After a 12 h light exposure, anti(SGP-2) stained a number of elements in the inner nuclear layer (INL, Fig. 3B, arrows). After a 24 h light exposure and a 24 h dark recovery period, INL staining was retained along with the appearance of heavy anti(SGP-2) staining of inner and outer segments of photoreceptor cells (Fig. 3C). In addition, weak anti(SGP-2) staining was evident in the outer nuclear layer (ONL).

B) anti(301) immunoreactivity

The anti(301) staining pattern was different from the pattern seen with anti(SGP-2). In controls, anti(301) staining results in immunoreactivity in all retinal layers (Fig. 3D). Anti(301) staining was absent from the inner and outer segment regions of the photoreceptor cells and confined to the region between photoreceptor cells in the ONL. There was intense anti(301) staining in the inner plexiform and outer plexiform layers as well as in the inner and outer limiting membranes. Collectively, the anti(301) immunostaining pattern suggests that the clusterin recognized by anti(301) may be located within the Müller cells. The changes observed during the time course of light treatment were subtle, with a slight increase in anti(301) staining as the duration of light increased (Fig. 3E). The primary change was a redistribution of anti(301) staining towards the photoreceptor cell layer as the duration of light exposure was increased. Animals allowed a 24 h dark recovery period after a 24 h light exposure showed a further re-distribution of anti(301) immunoreactivity, with an even staining of the ONL and a weak but uniform staining of the inner and outer segments of photoreceptor cells (Fig. 3F). The uniform pattern of immunostaining by anti(301) suggests that it may recognize a conformation of clusterin unique to the intracellular milieu.

Clusterin protein levels increase during light-induced damage

To confirm the immunohistochemical data, retinal lysates made in PBS were analyzed by Western blotting using the

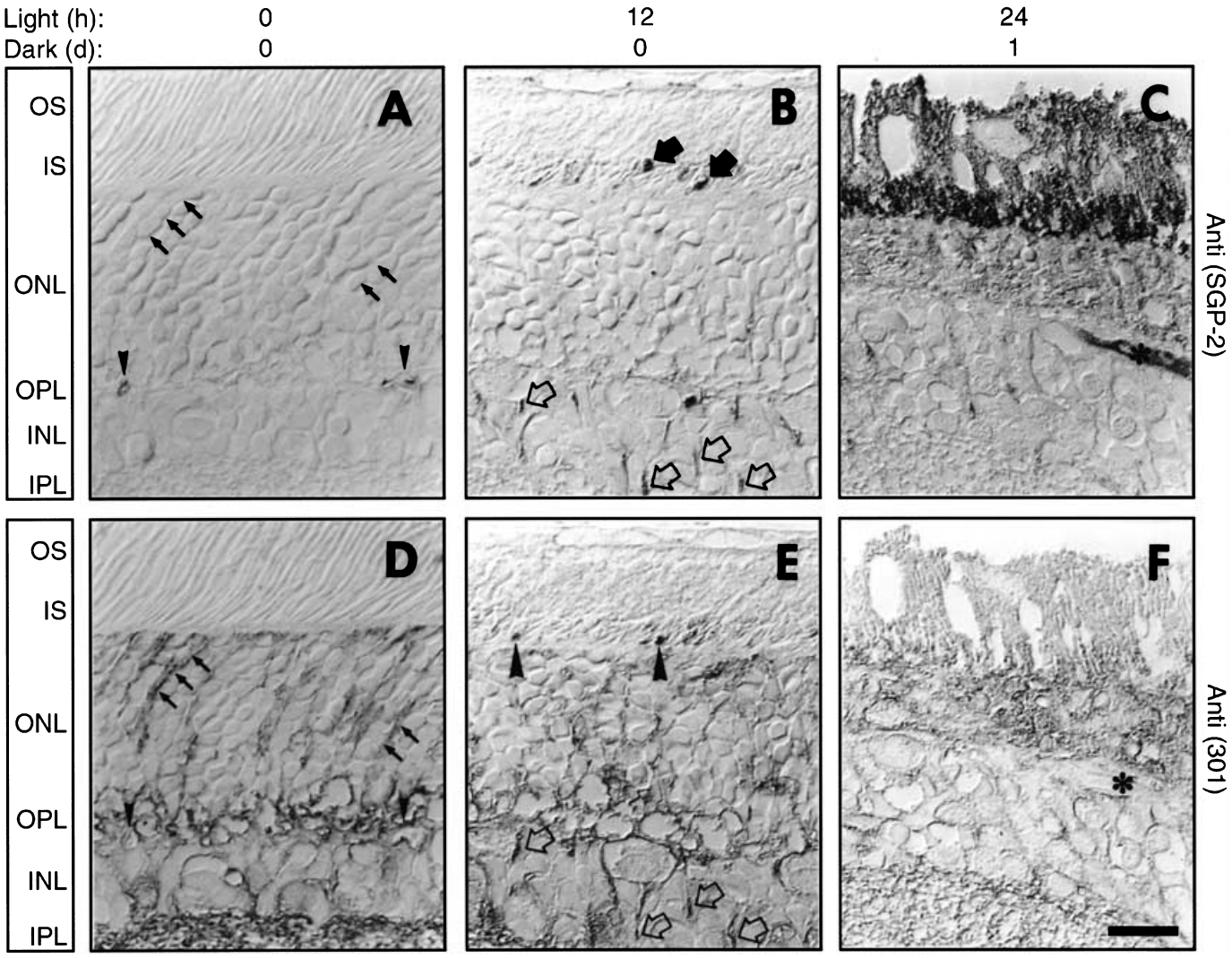

Figure 3. Immunohistochemical detection of clusterin in retinas of light damaged rats. Anti(SGP-2) and anti(301) produce different immunostaining profiles. Immunodetection of clusterin on 2 µm retinal sections of untreated (A, D) rats and those treated with intense visible green light (Light) for 12 hours, h (B, E), and 24 h followed by a 1 day (d) dark recovery period, dark, (C, F) are shown. A–C have been reacted with anti(SGP-2) as the primary antibody. D–F have been reacted with anti(301) as the primary antibody. In A and D, thin arrows point to regions in the outer nuclear layer of untreated animals which are labeled by anti(301) but not anti(SGP-2). In panels B and E, filled in arrow heads point to sporadic regions at the level of the outer limiting membrane that have reacted to the respective primary antibody, unfilled arrows indicate regions in the inner nuclear layer that have cross reacted with the respective primary antibody. The majority of immunostaining after a 12 h exposure is in the INL. In panels C and F the * marks a region in which a blood vessel runs through the section. In this case anti(SGP-2) detects the blood vessel but anti(301) does not. At this level there is a marked anti(SGP-2) staining of the inner and outer segment regions of the photoreceptors and weaker immunodetection by anti(301) antibody to the outer nuclear region of the retina. The various of retinal layers have been labeled in the above panels (OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer). The scale indicated in Panel F applies to all panels and indicates a distance of ~29 µM.

anti(SGP-2) and the anti(301) antibodies (Figs. 4A, B). In both cases the protein profile parallels the clusterin mRNA profile by showing undetectable levels of protein in normal retinae and an increase in the amount of clusterin protein with increasing durations of light exposure. In addition, clusterin protein levels remain high 1 day after a 24 h light exposure. Under non-reducing conditions a single band migrating at approximately 65 kDa was detected, under reducing conditions the uncleaved form of the clusterin migrated in the area of 75–80 kDa (data not shown), which is in agreement with published values.[32] In addition examination of non-reduced and reduced proteins in these extracts revealed that the predominate form of clusterin in the retina is as an uncleaved high molecular weight protein.

Whereas the Western profile for anti(SGP-2) is consistent with its immunohistochemical profile, results using the

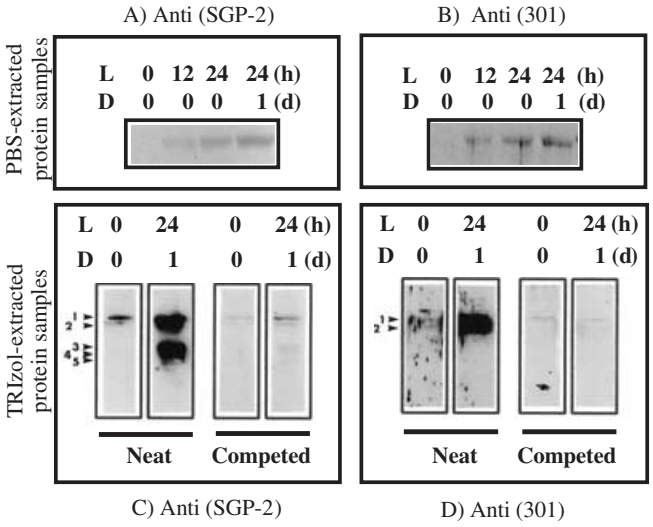

Figure 4. Western blot analysis. Retinal extracts were prepared from dark reared animals treated as defined in material and methods (L: light treatment in hours (h); D: dark recovery period in days (d)). 10 µg of protein was loaded per lane. Panel A and B represent retinal lysates made in phosphate buffered saline (PBS) and electrophoresed under non-reducing conditions. The band shown in panel A and B is a uniform 65 kDa in size which is consistent with the size of intact clusterin in other tissues under these treatment conditions. In panel C and D, retinal protein extracts have been solubilized in 1 % SDS after TRIzol extraction and samples have been electrophoresed under reducing conditions. Band 1 = 79 kDa; Band 2 = 76 kDa; Band 3 = 65 kDa; Band 4 = 61 kDa; Band 5 = 50 kDa. Bands 3–5 represent retinal degeneration associated forms of clusterin. Bands 1 and 2 are consistent with the sizes of intact clusterin reported for other tissues under these treatment conditions. Bands 3, 4, and 5 represent specific forms of clusterin specific that appear after light treatment. Pre-absorption of each antibody with purified recombinant clusterin protein prior to immunostaining effectively competed detection of bands 1–5 (panels C and D), thus verifying the specificity of the antibodies used and demonstrating that the proteins detected in bands 1–5 are different forms of clusterin.

anti(301) antibody were unexpected (Fig. 4B). The immunohistochemical staining of retinal cells in normal retina by anti(301) (Fig. 3D) indicates that clusterin protein is present. The absence of clusterin detection by anti(301) in normal retina on Western analysis suggests that the clusterin that is detected by anti(301) is not easily soluble under the conditions used. In order to test for this possibility we used a more stringent method (protein extraction in TRIzol and solubilized in 1% SDS, conditions which should extract soluble and not readily soluble proteins from tissues) to extract proteins from retina for Western analysis and performed a competition assay to verify antibody specificity to clusterin (Figs. 4C & D). In normal dark-reared retinae there were detectable high molecular weight (HMW) clusterin bands (79 and 76 kDa) by both antibodies upon Western analysis under reducing conditions. Since little clusterin was detected in retinal lysates made in PBS from untreated retinal tissue, the finding of amply detectable clusterin levels in TRIzol-derived samples suggest that, at this level, there are two distinct isoforms of clusterin in the normal retina, with different solubilities. Interestingly, the lack of clusterin immuno-reactivity by anti(SGP-2) on normal retinal sections suggests that the insoluble form of clusterin, *in vivo*, may have a conformation that hides the specific epitopes detected by this particular antibody. The levels of 79 and 76 kDa protein that is detected by anti(SGP-2) increase in the retina after light treatment. Western analysis, under reducing conditions, of light treated and untreated retina with anti(301) also revealed an increase in the 79 kDa and 76 kDa bands after light exposure (Fig. 4D). Preabsorption of each antibody with recombinant clusterin protein effectively competed out detection of these bands upon subsequent Western analysis, indicating that both the 79 kDa and 76 kDa bands detected by anti(SGP-2) and anti(301) represent different forms of clusterin (Figs. 4C & D).

Western analysis of TRIzol-derived light treated retina samples with anti(SGP-2) identified increased levels of the 79 kDa and 76 kDA bands as well as additional bands of 65, 61, and 50 kDa that were not detected in normal rat retina (Fig. 4C). These additional bands represent retinal degeneration enriched forms of the protein. These additional bands are not detected at any time with anti(301). Preabsorption of anti(SGP-2) with recombinant protein effectively competed out detection of these new bands upon subsequent Western analysis (Fig. 4C), indicating that they also represent variants of the clusterin protein.

Discussion

Intense light exposure leads to a series of damaging reactions within the retina resulting in photoreceptor cell loss.[1,6] Among these critical events in this cascade is the generation of an oxidative-stress, photoreceptor cell dysfunction and, ultimately, photoreceptor cell death. These changes are reflected in a progressive change in gene expression over the time course of retinal degeneration. Because photoreceptor cell loss resulting from constant light exposure becomes fully manifest several days after the initial light insult,[33] our results indicate that an alteration of clusterin expression is part of the cascade of events that occurs between the initial light insult and the final manifestation of retinal cell death. First, there is a decrease in IRBP mRNA levels, indicative of photoreceptor cell dysfunction. This is followed by the appearance of HO-1 mRNA, indicating that the retina is undergoing oxidative-stress. The highest level of HO-1 mRNA, and thus the period of maximum oxidative stress, occurs after a 16 h light exposure. Induction of clusterin expression also initiates just after the decrease in IRBP mRNA levels is observed. Clusterin mRNA levels, however, continue to increase even after HO-1 mRNA levels have decreased to background, indicating that clusterin is expressed even after the light induced oxidative-stress subsides. This suggests that the major alteration of clusterin expression maybe subsequent to the

primary target(s) of oxidative-stress. The increase in clusterin mRNA levels after intense light exposure is consistent with the time frame of actual photoreceptor cell death. Pretreatment of rats with the antioxidant, dimethylthiourea, delays the increase in clusterin mRNA levels for as long as 12 h during light exposure[6] and is known to also substantially decrease the expression of HO-1.[4] Mechanistically, this reinforces the possibility that clusterin expression is induced in response to secondary effects of light-induced oxidative-stress in the retina and may therefore represent a general response to retinal degeneration.

Results of the immunohistochemical analysis indicate that retinal clusterin protein levels increase after light exposure. In normal rat retina, the immunostaining by anti(301) of regions in the ONL (but which are clearly not photoreceptor cells), the ganglion cell layer, INL, OPL, inner plexiform layer and inner and outer limiting membranes (ILM, OLM) suggests that there may be stores of clusterin in cells within the retina, perhaps the Müller glial cells. These are the only cells known to traverse the entire retina. The *in situ* localization of clusterin mRNA to the INL and ganglion cell layer is also consistent with a Müller cell localization.[34,35] A number of cell types including megakaryocytes[36] and adrenal medullary cells[37] have been reported to maintain intracellular stores of clusterin.

Western analysis of both PBS and TRIzol-extracted protein lysates revealed that two distinct forms of clusterin, with respect to solubility, exist in the normal retina (soluble and insoluble clusterin). During light induced retinal degeneration, the soluble form, as detected by both anti(SGP-2) and anti(301), increases with increasing light damage (Fig. 4A). Likewise a comparison of the 79 and 76 kDa band in the TRIzol-extracted samples in light-exposed and unexposed retinas also suggests that the insoluble fraction of high molecular weight clusterin is induced after light treatment. Western analysis of TRIzol-extracted retinal samples from light treated animals allowed the identification of degeneration associated clusterin forms (65, 61, 50 kDa) by anti(SGP-2) that are not readily detectible in normal retina. These degeneration associated forms of clusterin are not detected by anti(301).

The current understanding of clusterin biogenesis is that it is initially synthesized as a 50 kDa holoprotein which is processed to a high mannose intermediate form of 58–65 kDa (under reducing conditions) and subsequently modified into a complex carbohydrate form of 70–80 kDa.[38,39] Prior to secretion, the protein is often proteolytically cleaved to an α and a β subunit which dimerize. The 79 and 76 kDa clusterin forms are likely to represent the complex carbohydrate form of retinal clusterin. The 50 kDa band detected in light treated retina may represent the holoprotein and the 65 and 61 kDa forms may represent intermediate forms en route to generating the HMW species. This suggests an induction of new clusterin protein synthesis during the degeneration process. This possibility however would not explain why these subsequent bands are detectable with anti(SGP-2) and not anti(301) under denaturing conditions. Alternatively, the degeneration-associated form of clusterin may reflect alternatively cleaved forms of the intact HMW clusterin species. Anti(301) was raised using 4 specific peptides taken from the α-subunit region of the rat clusterin sequence. In our preliminary characterization of anti(301) we found that the antisera was most reactive against the two peptides that are located just before the first cysteine rich motif (data not shown). Whether the degeneration associated products represent a specific modification at this end of the protein or a more general modification due to protein degradation remains to be determined. Regardless of the mechanism, an alteration in protein structure could mediate a change in its biological function.

Our observations suggest that clusterin exists in a number of different forms in the rat retina, as defined by solubility and/or molecular size. With respect to the degeneration associated forms, the specific detection of these variants by anti(SGP-2) on Western analysis of TRIzol-extracted proteins correlates well with the detection of clusterin by the same antibody on the inner and outer segment regions of degenerating photoreceptor cells by immunohistochemistry. In chromaffin granules, membrane associated forms of clusterin behave as integral membrane proteins and require high concentrations of detergent to affect solubilization.[40] We suspect that the degeneration associated forms of clusterin seen on Westerns of TRIzol-extracted samples, represent membrane associated forms of the protein.

A number of cellular functions have been attributed to clusterin. Among them is the possibility that it plays a role in removal of membrane catabolites, lipid transport, and membrane remodeling.[9,10] Rod photoreceptor cell outer segment membranes, are particularly lipid rich and are highly susceptible to oxidation *in vitro*.[41] An increase in lipid oxidation has been shown to occur upon prolonged light exposure *in vivo*.[42] Binding of clusterin may therefore represent an attempt to minimize the effects of peroxidized membrane lipids.[43] Alternatively clusterin may bind to unstable proteins and attempt to stabilize their structure in the same way that heat shock proteins do under stress conditions.[11] Clusterin is also thought to stabilize cell membranes at diverse fluid-tissue interfaces, thus the necessity of clusterin expression might be dictated by the extracellular milieu of the tissue in question.[44] The presence of clusterin in the inner segments of the degenerating photoreceptors therefore, could be an attempt to maintain the integrity of the outer limiting membrane, the barrier between the neural retina and the interphotoreceptor matrix. In either case, the role of clusterin in the degenerating retina might be to promote general retinal survival. In contrast, clusterin, in some systems, can exist in an alternative form that functions intracellularly as a nuclear factor that mediates the induction of the active cell death process.[45] In our study we did not observe a nuclear localization of clusterin protein with the antibodies that we used. In a recent study, astrocyte secreted clusterin was found to be taken up and accumulate in dying neurons following

hypoxia-ischemia induced injury where it mediates a caspase independent pathway of cell death.[46] This is an intriguing observation and leads us to consider the possibility that Müller cells might be secreting clusterin that is taken up by dying photoreceptor cells in the face of light-induced retinal degeneration.

We have observed a consistent up-regulation in clusterin expression during the degenerative phase of retinal cell loss in a number of rodent models of retinal degeneration (rd, Rds, and vitiligo mice) and with light-induced retinal damage in rats.[16,17] It would appear, therefore, that the over expression of clusterin represents a conserved cellular event that occurs in the face of a neurodegenerative insult to the retina. The observation of degeneration/death associated forms of clusterin provides a plausible explanation of how one protein could play an active death-specific role during tissue degeneration/regression as well as other "non-death" roles in normal tissues.

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