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Effect of targeted expression of clusterin in photoreceptor cells on retinal development and differentiation

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SUMMARY

Clusterin expression is increased in tissues undergoing apoptosis, including neurodegenerative retina, but the causal relationships remain to be clarified. To test the hypothesis that overexpression of clusterin could induce apoptosis in neurons, transgenic mice were generated in which rat clusterin transgene was expressed in photoreceptor cells under the transcriptional control of the human interphotoreceptor retinoid-binding protein (IRBP) promoter. Photoreceptor cell death in the resulting transgenic mice was examined by histology and TUNEL techniques. The expression of the clusterin transgene was confirmed by in situ hybridization in the photoreceptor cells, and results in a complex pattern of clusterin protein distribution in the retina. A reduction in apoptotic staining in the transgenic retinas was observed from birth to postnatal day 15. These results suggest that clusterin is not causally involved in apoptotic mechanisms of photoreceptor cell death, but may relate to cytoprotective functions.

Key words: Clusterin, Apoptosis, Retina, Neurodegeneration, Transgenic animal

INTRODUCTION

Clusterin is a generic name given to a heterodimeric, acidic and sulphated glycoprotein of molecular mass 70-80 kDa, originally identified as a cell-aggregating component of ram testis fluid (Blaschuk et al., 1983). Since then, it has been rediscovered independently in more than a dozen laboratories (for reviews see Fritz and Murphy, 1993; Jenne and Tschopp, 1989; May and Finch, 1992). The physiological role of clusterin is unknown, but it has been associated with lipid transport (de Silva et al., 1990), fertilization (Sylvester et al., 1991) and inhibition of complement activity (Jenne and Tschopp, 1992; Murphy et al., 1989). One of the most prominent characteristics of clusterin, and one that has attracted a wide interest, is its altered expression in response to diverse types of cell injury. The first association of clusterin expression with programmed cell death was made when it was identified as the major mRNA species induced during regression of the rat ventral prostate after castration (Montpetit et al., 1986). Since then, clusterin has been regarded as an apoptotic marker because of its high inducibility in damaged tissues (for reviews see Jenne and Tschopp, 1992; May and Finch, 1992; Michel et al., 1992). Overexpression of clusterin was reported in neurodegenerative states, such as brain tumours and epileptic foci (Danik et al., 1991), Alzheimer's and Pick's diseases (Duguid et al., 1989; May et al., 1990; McGeer et al., 1992), scrapie (Duguid et al., 1989), and in retinas affected by retinitis pigmentosa (RP) (Jones et al., 1992). Similarly, upregulation of clusterin was associated with neuronal cell death in experimental models of brain neurodegeneration, which mimic intrinsic neuronal loss and/or deafferentation (Danik et al., 1993; Duguid et al., 1989; May et al., 1990; Michel et al., 1997), and in animal models of retinal degeneration (Agarwal et al., 1996; Jomary et al., 1995, 1999; Wong et al., 1994a,b) in which photoreceptor death occurs by apoptosis (Chang et al., 1993; Lolley et al., 1994; Portera-Cailliau et al., 1994; Tso et al., 1994). In spite of these various findings, the function of clusterin in these situations remains unknown, and whether clusterin is causally involved in neuronal apoptosis or associated with a cytoprotective response remains to be clarified. Evidence in support of a generally cytoprotective role in vitro in non-neuronal cells has been accumulated (French et al., 1994; Humphreys et al., 1997; Schwochau et al., 1988; Sensibar et al., 1995). In an attempt to resolve this question in vivo in neuronal tissue, we generated transgenic mice in which clusterin transgene over-expression was targeted to photoreceptor cells, and the effects of transgene expression on photoreceptor differentiation and maturation were examined.

MATERIALS AND METHODS

Construction of transgenes and generation of transgenic mice

The 1.3 kb-long mouse IRBP promoter was amplified by genomic PCR using the following sense and antisense primers: 5′-CTGCAGCTGCCTACTGAGGCACACAGG-3′ and 5′-CCTTCTCA-GCTGGTGGACAGAAGG-3′, containing *Pvu*II recognition sites. After digestion by *Pvu*II, the amplified fragment was inserted into the dephosphorylated *Sma*I site of the pBSK+ plasmid (Stratagene, La Jolla, CA). A 2 kb-long *Bam*HI cassette was then inserted downstream of the IRBP promoter, containing the full-length rat clusterin cDNA open reading frame (a generous gift from Dr M. Griswold, Washington State University, Pullman, WA), fused to the SV40 t antigen intron and transcription termination sequence. Double restriction digestion of the resulting plasmid with *Cla*I and *Not*I enzymes generated a 3.2 kb fragment containing the whole IRBP-clusterin transgene. Purification of this fragment after electrophoresis on low-melting point agarose gel was carried out using the Geneclean procedure (Bio 101) prior to microinjection into pronuclei of zygotes from superovulated hybrid B6D2F1 females (Iffa Credo, France). Zygote recovery, microinjection and embryo reimplantation in C57Bl/6xCBA females were performed as previously described (Hogan et al., 1986). Transgene positive founders were identified by PCR amplification of genomic DNA extracted from tail samples using specific primers to clusterin cDNA, chosen in two different exons to permit discrimination from the endogenous mouse clusterin, as described by Chatelain and collaborators (1995). Homozygous mice were obtained by crossing founder animals, and transgene copy number was determined by competitive RT-PCR (Chatelain et al., 1995).

Transgene expression

RNA extraction and northern blot analysis

Total RNA was extracted from frozen tissues using the RNAXEL system (Laboratoires Eurobio, Les Ulis, France) according to the manufacturer's instructions. For northern blot analysis, 5 μg samples from pooled tissues (from a minimum of six animals) were denatured, electrophoresed in 1.3% agarose gels and blotted onto nylon membranes (Amersham Int., UK). Cloned inserts from plasmids were labelled with [α-^{32}P]dCTP using the Rediprime kit (Amersham Int., UK) and hybridized to the blots in the presence of 50% formamide at 42°C overnight, followed by stringent washing and autoradiography. The probes were stripped off between separate hybridizations. A control probe for RNA loading was a cDNA for rat glyceraldehyde phosphate-3-dehydrogenase (GAPDH; a gift of Dr B. O'Hara, Stanford University, USA).

RT-PCR analysis

Total retinal RNA was prepared as described above and reverse-transcribed into cDNA using random hexamers as primers. Detection of clusterin transgene expression was performed by PCR amplification using a sense IRBP promoter primer (A) 5′-AGACCTTCTGTCCACCAGG-3′, in combination with one of three different anti-sense mouse clusterin primers: (B) 5′-CTGGAC-GGCGTTCTGAATCTCC-3′, (C) 5′-TCTCCAGCAGGGAGTCG-ATGCG-3′, or (D) 5′-TCATTCCATGCGGCTTTTC-3′. As a control, amplification was performed using sense and anti-sense β-actin primers: 5′-GACAGGATGCAGAAGGAGAT-3′ and 5′-TTGCTGATCCACATCTGCTG-3′, respectively. PCR was performed in the presence of 200 μM dNTP, 0.5 μM oligonucleotides, 0.5 units of Taq DNA polymerase (Appligene, France), and amplification reactions were carried out using 30 cycles as follows: 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. The PCR fragments were separated by electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide. The absence of contaminating DNA was verified by PCR on non-reverse-transcribed RNAs. β-actin was used as an internal standard. Rat and mouse clusterin are 95% homologous at cDNA level and the clusterin-specific oligonucleotides were designed using regions of identity between the two species.

In situ hybridization

To localize clusterin gene expression, linearized clusterin cDNA plasmids were used to generate sense and antisense [^{35}S]UTP-radiolabelled (Amersham Int., UK) cRNA probes (approx. 10^4

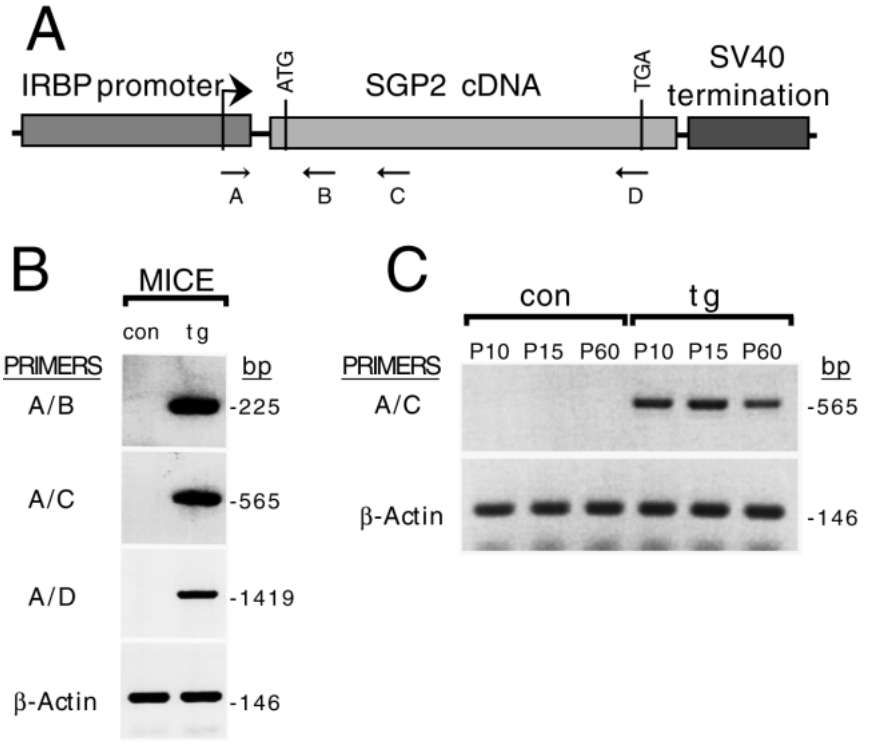


Fig. 1. (A) Schematic representation of the 3.2 kb fragment of the IRBP-clusterin transgene. Arrows indicate the position of the oligonucleotides used in RT-PCR and their orientation. (B) Comparative expression of the transgene in control (con) and in transgenic (tg) retinas. RT-PCRs were performed with different pairs of oligonucleotides. β-actin was used for standardization. (C) Comparative expression of the transgene in control (con) and in transgenic (tg) retinas by RT-PCR at postnatal (P) ages 10 days, 15 days and 60 days.

cpm/ml) in vitro (Jomary et al., 1995). Tissue sections (10 μm thick) were processed for in situ hybridization (Jomary et al., 1995) and dipped in Kodak NTB2 emulsion (IBI Ltd, UK). 2-3 weeks later the slides were developed and stained with Toluidine Blue.

For the detection of the transgene expression, sense and antisense oligonucleotides corresponding to the junction between the human IRBP promoter and rat clusterin sequences (5′-CTGTCCACCAGG-GGGGATCCCCGGACTCCA-3′) were labelled at the 3′ end with deoxyadenosine [α-^{35}S-thio]triphosphate (Amersham Int., UK) using terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim Ltd, UK) to a specific activity of about 10^8 cpm/μg. Frozen retinal sections were hybridized following a protocol previously described (Giorgi et al., 1992), and processed for autoradiography as described above.

Immunocytochemistry

Enucleated eyes were fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer overnight, incubated in 30% sucrose, embedded and frozen as previously described (Jomary et al., 1993b). Cryostat sections (10 μm) were processed for immunocytochemistry using the classical immunofluorescence technique (Robinson et al., 1990). The primary antiserum was a sheep anti-rat clusterin antibody (Quidel, USA) at 1:100 dilution, and the secondary antibody an FITC-coupled anti-sheep IgG used at 1:200 (Sigma-Aldrich Co. Ltd., Poole, UK).

Retinal morphology

Enucleated eyes were fixed in 3.5% glutaraldehyde and processed for light and electron microscopy as described by Marshall and collaborators (1975).

TUNEL procedure

Visualization of DNA fragmentation in situ was performed using the TUNEL method (Gavrieli et al., 1992). Frozen retinal sections (10 μm) were processed using the TUNEL procedures previously described (Michel et al., 1997). Briefly, the sections were treated with proteinase K (20 μg/ml), then incubated in 2% H_2O_2 to block endogenous peroxidase. Sections were then incubated with terminal deoxynucleotidyl transferase (TdT) (Promega) at 300 units/ml and biotinylated dUTP (Amersham, UK) at 6 nM for 1 hour at 37°C. After washing in 300 mM sodium chloride and 30 mM sodium citrate solution, the staining was developed with a horseradish peroxidase-coupled ABC system (Vector Laboratories, Peterborough, UK). Negative slide controls were processed for TUNEL with the omission of TdT from the incubation with biotinylated dUTP.

Quantitative analysis of TUNEL-positive nuclei in the different layers of the retina was performed by counting a minimum of three different regions per section from the optic disk to the ora serrata, in four animals per time point for control and transgenic strains. The data were subjected to statistical analysis using Student's *t*-test.

RESULTS

Transgenic mice production

The clusterin transgene construct consists of the complete rat clusterin cDNA ligated 3′ to the human IRBP promoter and 5′ to the SV40 transcription termination sequence (Fig. 1A). Transgenic founders and offspring were identified by PCR analysis of tail DNA. Southern blot analysis (not shown) of F_1 mice revealed the existence of only one integration site. F_1 mice, heterozygous for the transgene, were then cross-bred together and around 1/4 of newborns obtained were homozygous for the construct IRBP-clusterin. Homozygous mice were cross-bred for subsequent generations. Transgene copy number per diploid genome was determined by competitive PCR and was estimated to be around 40 copies (not shown) (Chatelain et al., 1995). All mice were viable and showed no apparent phenotypical difference from the wild type.

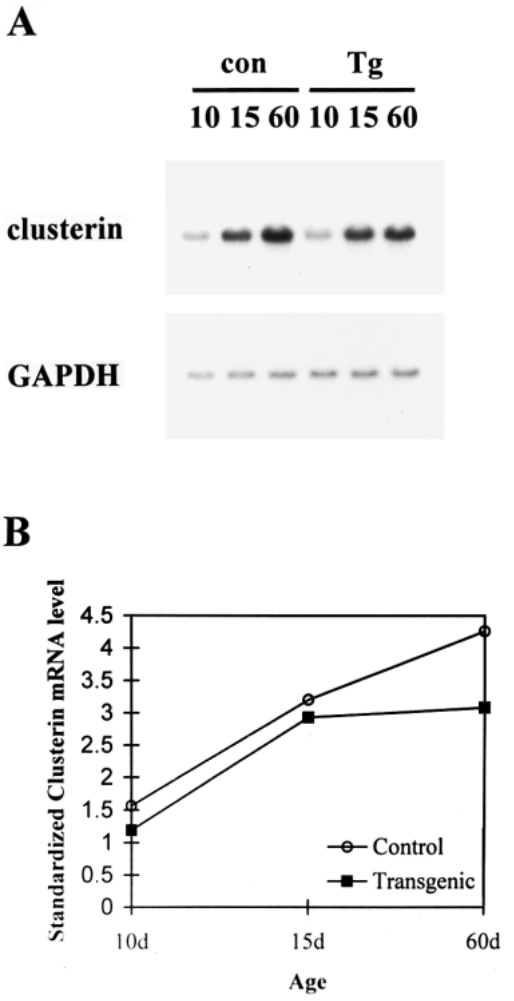


Fig. 2. (A) Northern blot analysis of gene expression in control (con) and transgenic (Tg) mouse retina. (Top) probed with clusterin cDNA; (bottom) probed with GAPDH cDNA. (B) Comparison of clusterin mRNA levels in control and transgenic retinas standardized densitometrically to GAPDH levels (calculated as ratio of peak optical density values) from the autoradiographs in A. Numbers in A indicate postnatal age in days.

Clusterin transgene expression analysis

RT-PCR

When RT-PCR was performed using a specific sense IRBP promoter primer in combination with different anti-sense clusterin cDNA primers, expression was detected only in transgenic (adult) retinas, showing that the IRBP regulatory sequences were active in the genomic context surrounding the transgene insertion locus (Fig. 1B,C). Furthermore, the transgene was expressed in retinas of mice at every age tested (Fig. 1C). The expression of the transgene was specific to the retina since no PCR amplification was seen in other organs such as brain, liver, ovary, testis, lung, heart, spleen and kidney (not shown).

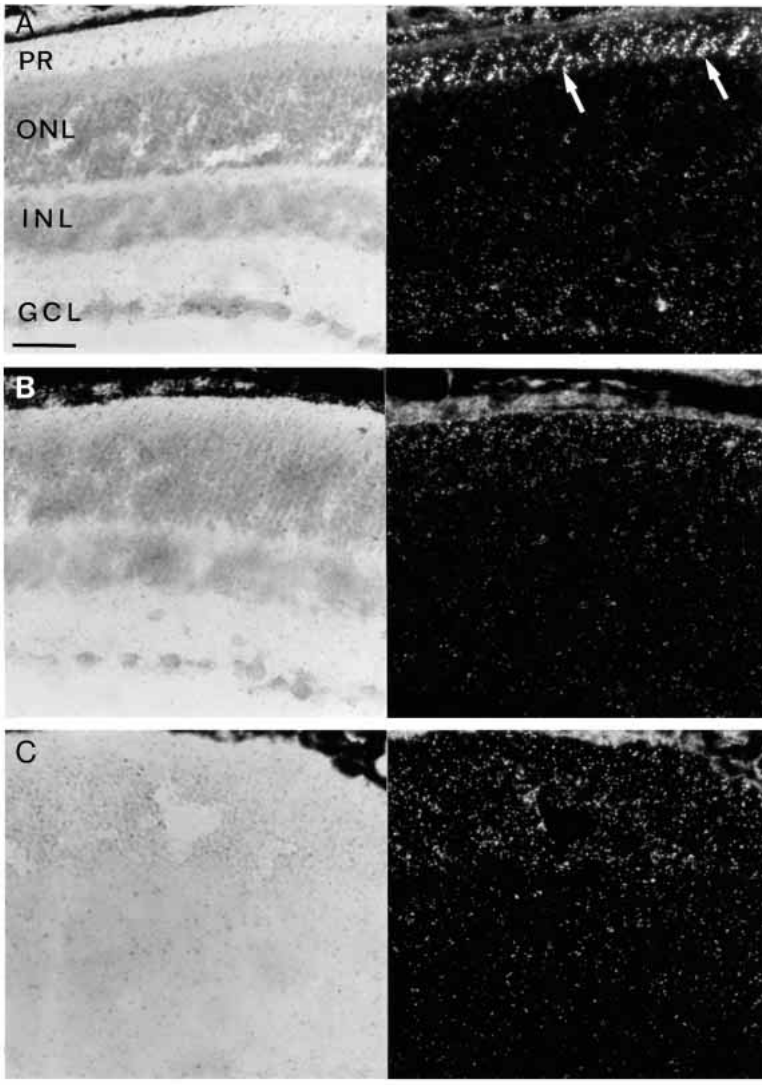


Fig. 3. In situ hybridization autoradiographs of clusterin transgene mRNA expression in retinal sections (10 μm) at postnatal day 15. (A,C) Transgenic retinas, (B) C57Bl/6 retina. Hybridized with antisense (A,B) and sense (C) ^{35}S-labelled oligoprobes. Dark-field (right panels) and bright-field (left panels) for the same sections are shown. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors. Arrows show the in situ hybridization signal located at the photoreceptor inner segments. Bar, 100 μm.

Total clusterin mRNA expression

Northern blot analysis

Retinal RNA samples from control and transgenic animals were analysed by northern blot hybridization using clusterin and GAPDH cDNA probes. Densitometric analysis of the resulting autoradiographs was performed and the expression of clusterin mRNA standardized to that of GAPDH (Fig. 2). Clusterin mRNA levels were similar in control and transgenic animals at postnatal day 10 (P10), and showed similarly increased levels at P15. A further increase in clusterin mRNA expression was seen in control at P60, but in transgenic retina the level was approximately 30% lower at this time-point.

Evaluation of IRBP mRNA levels revealed no difference between the control and the transgenic animals (data not shown).

Clusterin transgene expression in the retina

In situ hybridization

To identify the localization of the transgenic component of clusterin mRNA expression, we employed in situ hybridization of ^{35}S-labelled oligo-probes specific to the junction sequences between IRBP promoter and clusterin cDNA on retinal tissue sections (Fig. 3). Specific hybridization was detected at the photoreceptor level in transgenic retinas (Fig. 3A), but not in the control retinas at P15 (Fig. 3B).

Total clusterin mRNA expression

In situ hybridization

Using a probe capable of detecting both endogenous and transgenic clusterin mRNA, expression was first detected at P10, predominantly in the retinal pigment epithelium (RPE) cells and, at a lower level, in the inner nuclear and ganglion cell layers in both control and transgenic animals (Fig. 4B,F). In the case of the RPE, careful examination permitted discrimination of the autoradiographic signal from light scatter due to the pigment granules. From P15 to maturity, the labelling pattern was comparable in control retina, but was surprisingly almost absent in the neuronal retina of transgenic animals (Fig. 4G,H).

Clusterin protein expression

Immunofluorescence detection of clusterin expression in control and transgenic retina is shown in Fig. 5. In retinas of newborn mice, immunoreactivity was associated with the well-

demarcated multiple cell layer of presumptive ganglion cells (Fig. 5A,B). By P8, immunolabelling was also detected in the inner and outer plexiform layers lying on either side of the inner nuclear layer (INL) (Fig. 5C,D). At these two earliest time points, the pattern and intensity of immunoreactivity was similar in control and transgenic retinas. At P10, when the photoreceptor outer segments have begun to differentiate, the labelling was similar to that at P8, except that there was additional immunoreactivity at the outer segments in the transgenic strain only (data not shown). By P15, differences had even more clearly emerged. While in the control there was still strong immunoreactivity in the inner retina, and faintly detectable staining at the photoreceptor segments (Fig. 5E), in transgenic mice, the immunolabelling of the inner retina was substantially reduced, with only moderate staining of the ganglion cell layer (GCL), but with a markedly increased labelling of the photoreceptor segments (Fig. 5F). At maturity, the distribution was now more similar in control and transgenic retinas (Fig. 5G,H). In control retinas, the ganglion cells were strongly immunoreactive and there was increased labelling of the photoreceptors compared with that at P15 (Fig. 5G). While the overall pattern of immunoreactivity was similar in transgenic retina, the intensity of labelling in the inner retina was noticeably reduced (Fig. 5H). Control sections lacking primary antibody showed only non-specific fluorescence at the retinal vasculature (data not shown).

Morphological studies

To assess whether the transgenic expression of clusterin has an effect on retinal differentiation and maturation, histomorphological characteristics were studied by light and electron microscopy of retinal sections (Fig. 6). No morphological differences were observed between the control and transgenic animals. The length of the combined photoreceptor inner and outer segments, which decreased toward the periphery, was similar in comparable regions of both retinas. Careful observation of a sample population of photoreceptor nuclei revealed no detectable abnormalities.

Localization and quantification of apoptotic nuclei

To examine if the pathway leading to DNA fragmentation accompanying apoptosis was induced by expression of the transgene, we stained both control and transgenic retinas using the TUNEL procedure, which detects free 3′OH DNA ends. As shown in Fig. 7, DNA fragmentation was detected in both control and transgenic retinas at various time points (P10, P15, P60). At P10 and P15, on average more labelled nuclei were observed in the non-transgenic retinas (Fig. 7A-D,G-I). By statistical evaluation of counted positive nuclei, at P10 this difference was significant only in the ONL ($P<0.05$; Fig. 7G-I). However, at P15, all nuclear layers showed a significant reduction in apoptotic labelling in the transgenic retinas compared with controls (Fig. 7G,H: $P<0.001$; Fig. 7I: $P<0.01$).

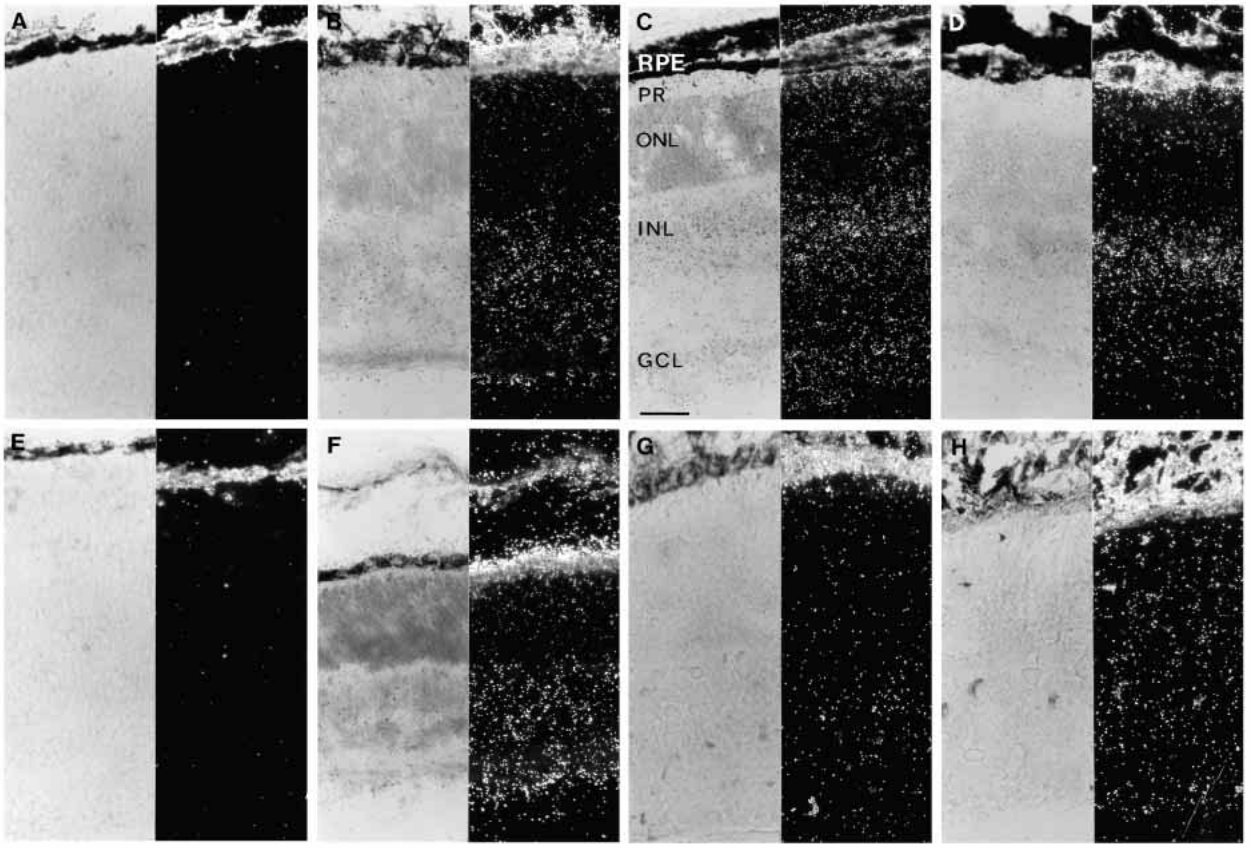


Fig. 4. Light micrographs comparing clusterin in situ hybridization in the developing control (A-D) and transgenic mice (E-H). Bright-field view (left panels) and dark-field view (right panels) of the same field are shown for different postnatal stages: newborn (A,E); 10 days (B,F); 15 days (C,G); 60 days (D,H). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium. Bar, 100 μm.

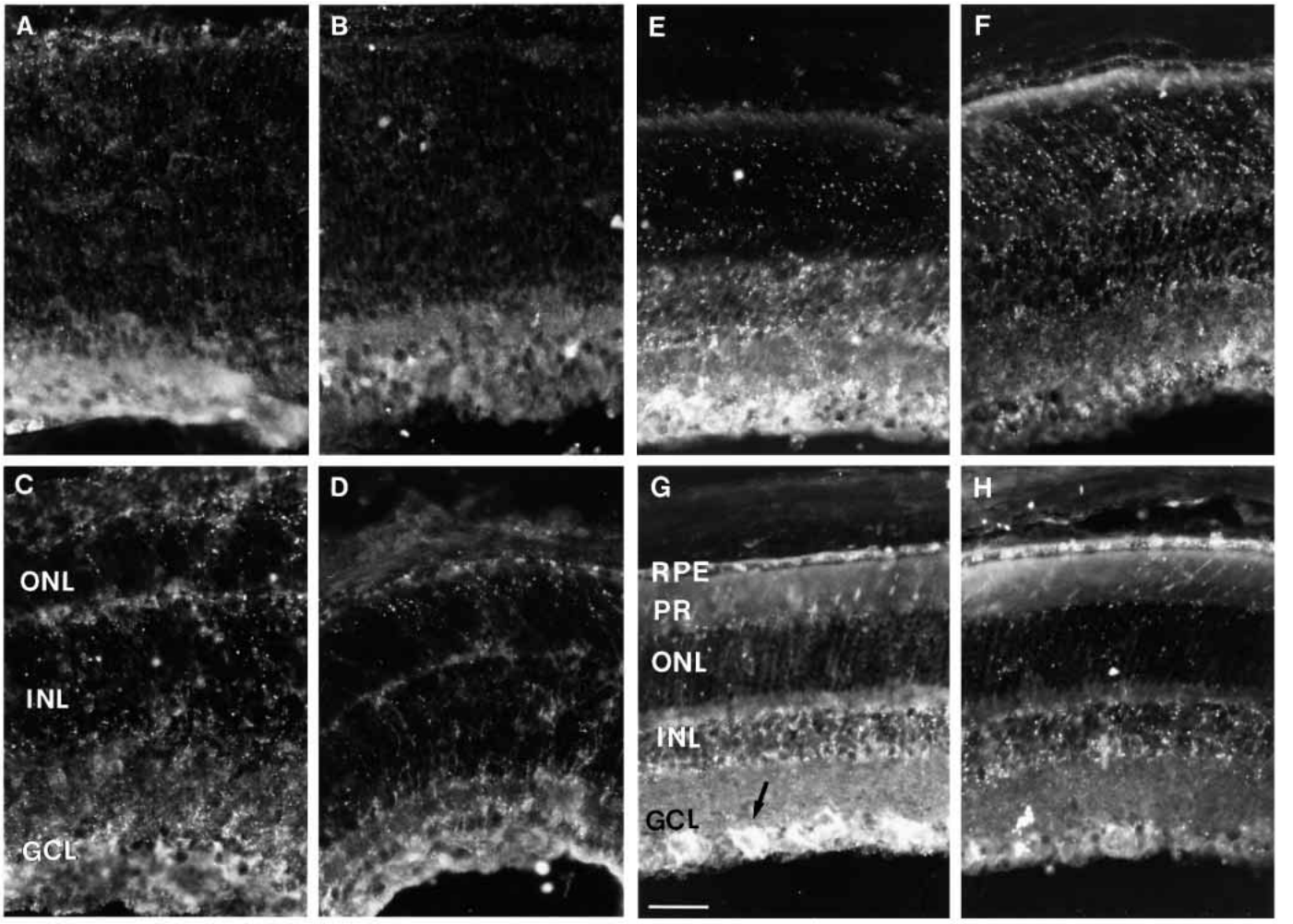


Fig. 5. Immunofluorescence detection of clusterin expression in control (A,C,E,G) and transgenic (B,D,F,H) mouse retina during postnatal development. Newborn (A,B); 8 days (C,D); 15 days (E,F); 60 days (G,H). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium. Arrow shows the immunolabelling of the ganglion cells (G). Bar, 100 μm.

In contrast, at maturity (P60), the TUNEL reactivity of control and transgenic retinas was similar (Fig. 7E-I). No specific staining was detected in control slides lacking TdT (data not shown).

DISCUSSION

The aim of the current study was to determine whether or not clusterin is causally involved in neuronal apoptosis, since both pro-apoptotic and anti-apoptotic functions have been proposed for this protein. To test the hypothesis in vivo, we generated transgenic mice overexpressing clusterin in photoreceptor (PR) cells. Among the different genes associated with retinal cell death, clusterin has been shown to be upregulated in inherited retinal degenerative states (Agarwal et al., 1996; Jomary et al., 1993a,b, 1995; Jones et al., 1992; Wong, 1994; Wong et al., 1994a,b), and in retinal degeneration (rd) and retinal degeneration slow (rds) mouse models of retinitis pigmentosa (Agarwal et al., 1996; Jomary et al., 1995; Wong et al., 1994a). Therefore, if clusterin is implicated in cell death, its transgenically directed expression in PRs would likely affect their differentiation and maturation, potentially resulting in retinal dysgenesis or degeneration by apoptosis.

To develop transgenic mice to test the effect of clusterin on photoreceptor cell death, we needed to use a promoter of a photoreceptor-specific gene to drive clusterin expression. Many of the promoters for photoreceptor-specific genes have not been well characterized, or else the genes themselves are expressed only in fully differentiated photoreceptor cells but not in retinoblasts. Of those promoters most suited to our objectives, the human IRBP promoter has several advantages: (1) it has been well characterized (Liou et al., 1991, 1994) and shown to direct reporter gene expression specifically to the photoreceptor cells in transgenic mice (Liou et al., 1990; Yokoyama et al., 1992); (2) IRBP mRNA is present in PR cells of the retina, prevalently in rod cells; (3) the protein is detectable as early as embryonic day 17 (Carter-Dawson et al., 1986) before the rods start to be generated from embryonic day 13 (Carter-Dawson and LaVail, 1979) and before opsin mRNA appears by post-natal day 5 (Bowes et al., 1988); (4) IRBP expression peaks at postnatal day 14, and stays approximately constant during adult stage (van Veen et al., 1988). As with other retinally expressed genes (Blackshaw and Snyder, 1997),

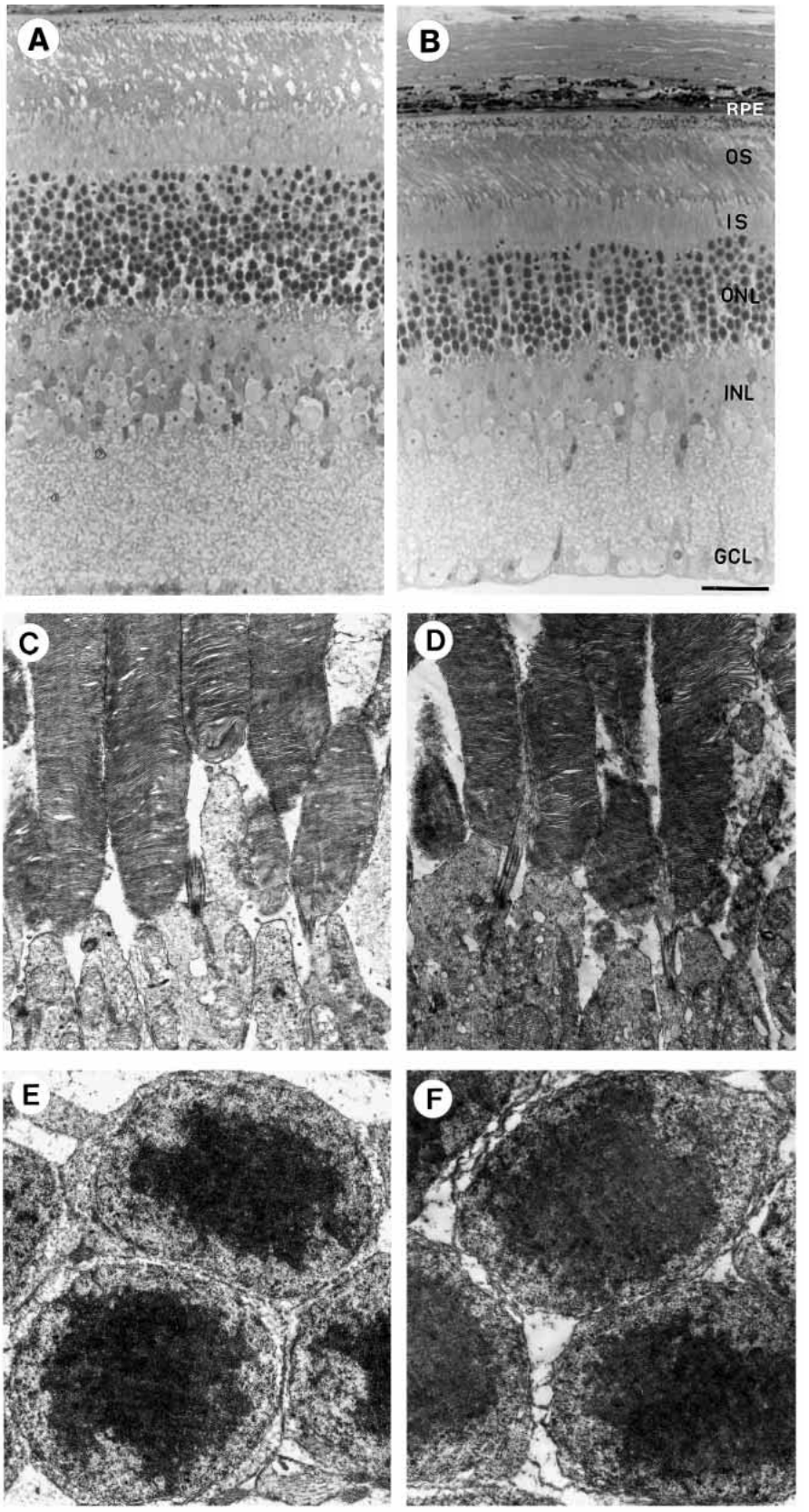
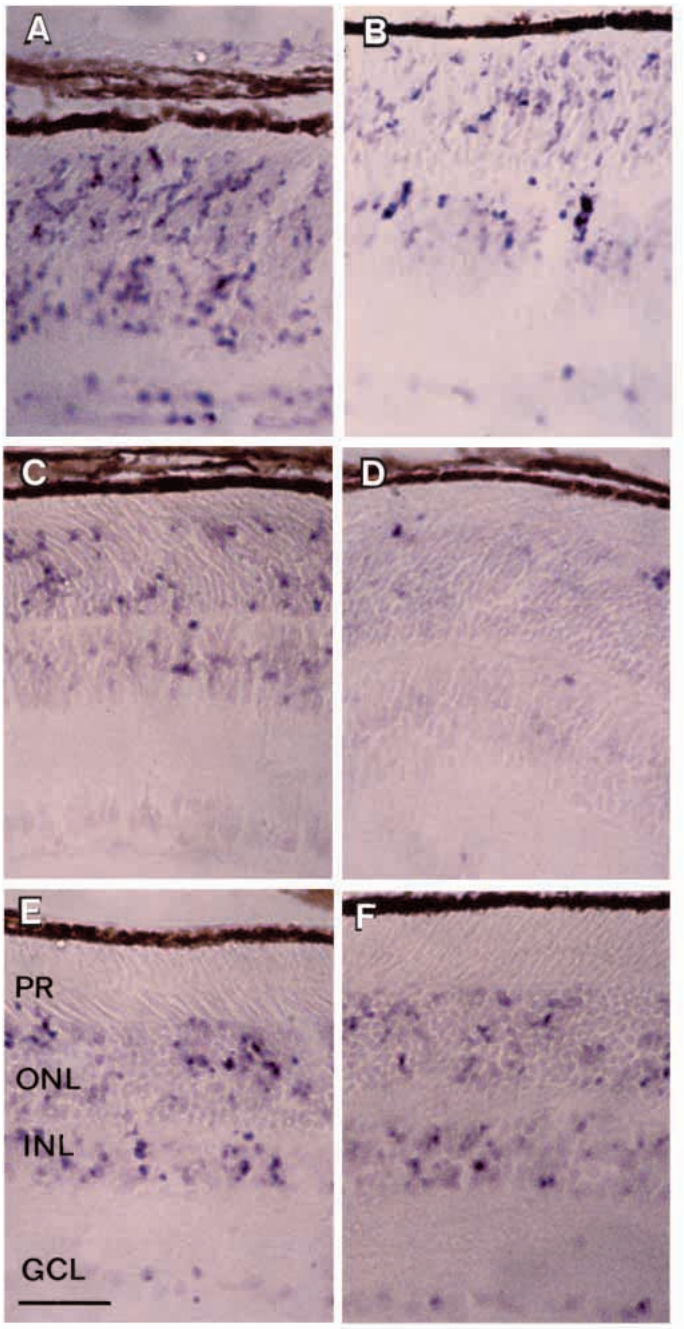


Fig. 6. Light micrographs of 1 μm retinal sections of adult control (A) and transgenic (B) mice. (A) is representative of the central part of the retina and (B) corresponds to the peripheral retina. No morphological differences were detected between control and transgenic animals from corresponding regions of the retina. Electron micrographs of adult control (C,E) and transgenic (D,F) retinas. Rod outer and inner segments connected by cilia are shown in (C,D). Rod photoreceptor nuclei in the outer nuclear layer are displayed in (E,F). ×3000. GCL, ganglion cell layer; INL, inner nuclear layer; IS, photoreceptor inner segments; ONL, outer nuclear layer; OS, photoreceptors outer segments; RPE, retinal pigment epithelium. Bar, 100 μm (A,B).

IRBP is expressed at a very low level in a subgroup of pinealocytes. Overall, it appears that the IRBP promoter was the best candidate to investigate the full effect of clusterin expression on developing and mature photoreceptors, since its expression is initiated before terminal differentiation.

Our study shows that in the transgenic mouse strain



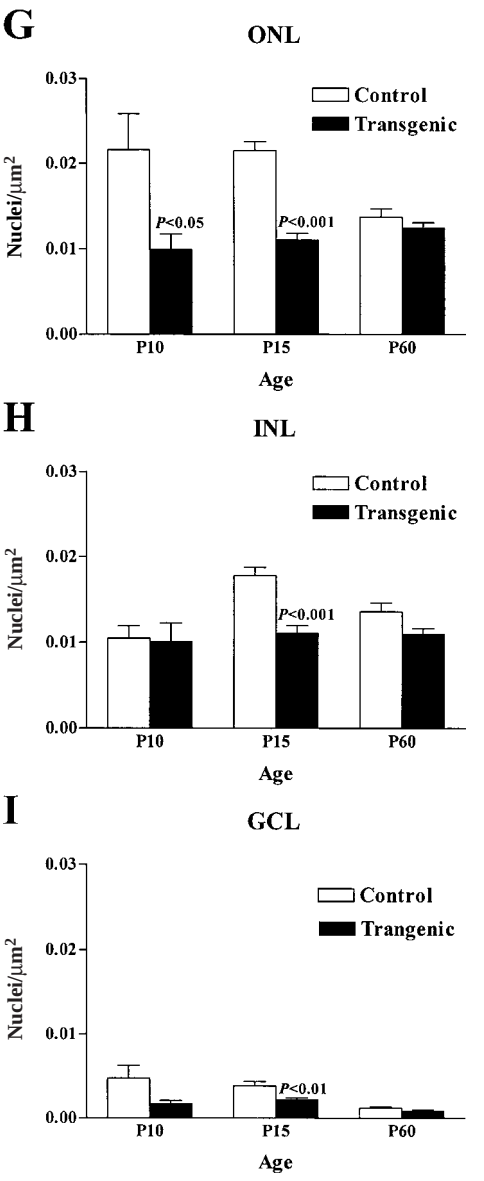


Fig. 7. TUNEL staining in control (A,C,E) and transgenic (B,D,F) retinal sections at P10 (A,B), P15 (C,D) and P60 (E,F); bar, 100 μm. Quantitative data are presented in G-I. Abbreviations as in Fig. 6.

examined, clusterin transgene mRNA expression was localized only to the photoreceptor cells, confirming that the 1.3 kb fragment of the IRBP promoter can be used to produce photoreceptor-specific gene expression in transgenic mice as previously described (Liou et al., 1990; Yokoyama et al., 1992). The increased clusterin immunoreactivity signal in the PR outer segments of the transgenic animals at P15 suggests that, in spite of the fact that the antibody used did not differentiate between the transgene and the endogenous protein, the transgene protein is most probably expressed in the target PRs.

In control retinas, the distribution of clusterin mRNA and protein was consistent with previous reports (Jomary et al., 1993a,b, 1995). In contrast, since both mRNA and protein detection signals were decreased in the inner part of the transgenic retinas from postnatal day 15, endogenous clusterin expression seems to be repressed in these areas. Furthermore, preliminary studies using quantitative RT-PCR have indicated that at P10, the majority of clusterin mRNA is derived from transcription of the transgene. These findings suggest that the reduction of the endogenous gene expression could be due to a feedback regulatory process induced by the ectopically expressed clusterin in the PRs. Given that no difference is observed before P15, the decrease of endogenous clusterin expression in the inner part of the retina most likely results from a complex regulatory mechanism induced by the transgene expression. Reduction of clusterin mRNA levels by cAMP in Leydig tumour cell lines (Pignataro et al., 1992) has

been shown to be due to an increase in the degradation of its mRNA through synthesis of a destabilizing protein(s) and not to a decrease in its transcriptional activity (Rosemblit et al., 1996). Therefore, it could be postulated that clusterin transgenic expression in the photoreceptor cells may have disturbed phosphodiesterase activities, resulting in an increase of retinal cAMP, which in turn has an inhibitory effect on the endogenous gene. An assessment of cAMP levels in the transgenic retinas will help to test this hypothesis.

Moreover, the presence of detectable clusterin protein in transgenic neural retinas at P15 and P60 in spite of low total clusterin mRNA levels could suggest that the protein persists for a long period of time. Since evidence of an intracellular form of clusterin has been reported in smooth muscle cells (Thomas-Salgar and Millis, 1994) and in apoptotic MCF-7 cells (Wilson et al., 1995), it could be postulated that the transgene expression we observed in this study corresponds to a non-secreted form of the protein (Reddy et al., 1996). This may arise from an altered spatio-temporal pattern of expression, governed by the IRBP promoter, resulting in modified post-translational processing. Alternatively, several putative initiation sites are present in the clusterin sequence (Wilson et al., 1995), and the initiation of translation could originate from a site located after the hydrophobic signal sequence, in the transgenic animals. The clusterin would then lack the N-terminal hydrophobic signal peptide typical of secretory proteins and would remain intracellular. Western blot and immunohistochemistry analysis using specific antibodies (Lakins et al., 1998) will be needed to determine which isoforms of clusterin are expressed in the transgenic animal.

Our present study demonstrates that clusterin transgene expression in PRs does not alter the differentiation and maturation of these cells, as shown by their normal ultrastructural morphology. Further analysis, such as electroretinography, will be required to establish whether there has been any effect on retinal function. However, in spite of the similarities in morphology of control and transgenic retinas, differences were observed in the patterns of staining of apoptotic nuclei. The staining in transgenic retinas at P10 and P15 was markedly lower than in controls, suggesting that clusterin expression in the PRs reduced the frequency of apoptotic activation. This observation strengthens the growing evidence that clusterin is not causally implicated in apoptosis. The apparent absence of effect of apoptotic suppression on retinal morphology suggests that compensatory mechanisms are operating to prevent retinal dysplasia. Previously, Michel and collaborators (1997) clearly showed that clusterin-expressing cells do not die by apoptosis in an experimental model of neurodegeneration. Similarly, in human inherited retinal degeneration and models of such diseases, increased expression of clusterin has been reported in surviving cells, suggesting a role in neuronal protection (Jomary et al., 1993a,b, 1995). Increased levels of clusterin in Alzheimer's disease have been proposed to decrease the neurotoxicity of amyloid-β by attenuating its aggregation (Boggs et al., 1996). Clusterin in these situations has been proposed to remove cellular debris resulting from apoptosis, protecting bystander cells from cytolysis by debris-activated complement (French et al., 1992). Given that clusterin is a potent complement inhibitor (Jenne and Tschopp, 1989; Kirszbaum et al., 1989), and that no inflammation is detected in areas that undergo apoptosis, clusterin's primary function has been postulated to restrict inflammation (Kounnas et al., 1995). Our findings that cells overexpressing clusterin do not undergo apoptosis in an animal model of retinal light-induced photoreceptor degeneration also support this hypothesis (Jomary et al., 1999).

In summary, the present study clearly demonstrates that clusterin overexpression does not induce apoptosis in the transgenic retinas, and may even act transiently to inhibit apoptosis. Studies of the effects of light-induced photoreceptor degeneration in these transgenic animals will assist in establishing whether clusterin can function in an unequivocally cytoprotective role.

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