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Analysis of differentially expressed genes in retinitis pigmentosa retinas

Altered expression of clusterin mRNA

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The molecular and cellular processes underlying photoreceptor degeneration in retinitis pigmentosa (RP) are unknown. We have investigated gene expression in diseased retinas using differential hybridization screening of a retinal cDNA library with probes derived from normal and RP retinal RNA. Most differential clones detected corresponded to transcripts absent from the dystrophic state, including e.g. opsin. However, one clone was noticeably increased in RP in comparison with the control: partial sequencing showed it encoded clusterin. Increased expression of clusterin has been identified in several cases of tissues undergoing apoptosis (programmed cell death), and our finding suggests that the degenerative changes in advanced RP may represent another example of apoptosis, possibly with common causative mechanisms.

Retinitis pigmentosa; Clusterin; Programmed cell death; Apoptosis

1. INTRODUCTION

Retinitis pigmentosa (RP) is the term for a heterogeneous group of inheritable retinal diseases in which degeneration of the photoreceptor cells results in night blindness and progressive contraction of the visual fields. The diseases, which together affect 1–2 in 5,000 of most populations studied, may be transmitted in autosomal dominant, autosomal recessive, and X-linked modes [1]. Sporadic (simplex) cases also occur. The genetic defects responsible for a number of autosomal dominant cases have been identified: the majority are point mutations distributed through the coding region of the gene for the visual pigment protein, rhodopsin, but display no obvious relationship between altered amino acid residue and the known functional significance of rhodopsin structure [2–4]. Mutations in other genes expressed in the photoreceptors, including peripherin, have also been associated with dominant RP [5,6]. In spite of the rapidly increasing identification of RP-associated mutations, however, the links between the genetic faults and the molecular and cellular processes which result in photoreceptor degeneration in all types of RP remain poorly understood.

Insight into the pathology of RP may be gained by an analysis of the transcriptional activity in diseased retinas. We have previously reported RP-related abnormalities in the expression of specific retinal mRNAs by Northern blot and in vitro translation analysis [7]. Here we describe an extinction of these studies by the use of differential screening of a retinal cDNA library with cDNA probes derived from normal and RP RNA, and discuss the significance of abnormally expressed genes in the dystrophic retina in relation to possible mechanisms of cellular degeneration.

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2. EXPERIMENTAL

2.1. *Tissues*

Normal human eyes were obtained through the UK Transplant Service, Bristol. Enucleated eyes were dissected and the retinas removed; these were stored frozen in liquid nitrogen until RNA extraction. Dystrophic retinas were frozen in liquid nitrogen through the UK RP Eye Donor Scheme. The post-mortem interval (pmi) between death and freezing was recorded in each case. RNA was extracted from the retinas of 3 donors with ocular disease, two of whom had retinitis pigmentosa (DYS2 and DYS3), and a third who had hereditary optic atrophy (DYS1):

Donor DYS1: Female, age 55 years, pmi not > 24 h. Hereditary optic atrophy, based on medical history; normal dark adaptation and no pigmentary abnormalities of retina. Daughter also affected.

Donor DYS2: Male, age 65 years, pmi = 22 h. Father known RP sufferer. Son (only child, age 30 years) normal. Probable autosomal dominant RP with reduced penetrance.

Donor DYS3: Male, age 81 years, pmi = 32 h. No history of visual disorder in family. Probable simplex RP.

RNA was also extracted from the retinas of 4 normal donors with no known history of eye disease:

Donor CON1: Male, age 54 years, pmi = 26 h.
Donor CON2: Male, age 64 years, pmi = 25 h.
Donor CON3: Male, age 70 years, pmi = 38 h.
Donor CON4: Male, age 72 years, pmi = 33 h.

2.2. *RNA extraction and cDNA probe synthesis*

Total cellular RNA was extracted from frozen tissues using the guanidinium isothiocyanate/caesium chloride method [8]. Single-stranded cDNA (ssecDNA) probes were prepared by reverse transcrip-

tion of total retinal RNA (DYS3 and CON4) using the following conditions (D. Edwards, personal communication): in a 50 μl reaction volume containing 5 μg total RNA; 50 mM Tris–HCl, pH 8.3, at 43°C, dATP, dTTP and dGTP at 0.96 mM each, 3.2 μM dCTP, 150 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 20 μg/ml oligo $dT_{(12-18)}$, 1.85 MBq α-[^{32}P]dCTP at 110 TBq/mmol and 25 U RAV2 reverse transcriptase (Amersham Int., UK), incubated for 2 h at 43°C, and the reaction stopped with 2 μl 0.5 M EDTA and 2 μl 10 M NaOH. The RNA was hydrolysed by heating the mixture at 65°C for 30 min, the ssDNA products neutralized with 1 M HCl and separated from unincorporated nucleotides by spun column (Sephadex G-50) chromatography. The activities of the probes were determined [9].

2.3. *Differential screening of cDNA library*

The method was adapted from that of Leonard et al. [10]. A normal human retinal cDNA library in λgt11 (Clontech) was plated out on host *E. coli* Y1090 cells at low density (approx. 3,000 pfu per 14 cm petri dish). Single nylon filter disc (Amersham Int., UK) plaque lifts were taken from each of 6 plates, and processed through denaturing, neutralizing and UV cross-linking according to the manufacturer's recommendations. Following washing and pre-hybridization, the filters were hybridized at 42°C for 40 h, with either the RP or control retinal ssDNA probes, using approximately equal amounts of radioactivity for each screening (4.8×10^6 cpm in total). The filters were autoradiographed following the first hybridization, stripped off the probe, autoradiographed to confirm probe removal, re-prehybridized and hybridized with the second probe, and autoradiographed again. Plaques showing differing intensities of hybridization signal with the two probes were picked using the wide end of a Pasteur pipette, and subjected to a second round of screening. Single, well-isolated plaques corresponding to differentially-expressed retinal cDNA clones were picked and used for further analysis.

2.4. *Analysis of differential cDNA clones*

DNA preparations from liquid cultures of isolated recombinant lambda phage were digested with *Eco*RI, and subjected to agarose gel electrophoresis and Southern blotting for hybridization with probes for known retinal genes [9]. Oligonucleotide probes (19mers) for the detection of opsin and S-antigen were synthesized based on published sequences [11,12] by Dr. A. Northrop (AFRC, Babraham) and end-labelled with γ-[^{32}P]ATP using T4 polynucleotide kinase [13]. The probes were hybridized separately with the blot and autoradiographed after washing to high stringency. Inserts from clones not showing hybridization with these probes were subcloned into M13 vectors for sequencing by the dideoxy method [14] using the Sequenase version 2.0 kit (United States Biochemicals).

2.5. *Northern blot analysis*

Three microgram samples of total cellular RNA extracted from human retinas were denatured and electrophoresed in a 1.3% agarose formaldehyde mini-gel [15], blotted onto nylon membrane and fixed by UV illumination.

Isolated cDNA inserts from differentially expressed clones were labelled with α-[^{32}P]dCTP using the Multiprime kit (Amersham Int., UK) according to the manufacturer's protocols, and hybridized separately with the Northern blot in the presence of 50% formamide at 42°C overnight. The blot was washed at varying stringencies (see legend to Fig. 2) and autoradiographed. The blot was stripped between probings and probe removal confirmed by autoradiography.

3. RESULTS AND DISCUSSION

Screening of the human retinal cDNA library filters using probes reverse transcribed from control and RP-affected retinal RNA revealed 35 primary differential hybridization signals. Analysis of 6 differential clones confirmed by secondary screening is presented here. Of these, 5 gave strong signals with the control probe and weak or absent signals with the RP probe. Since it is the photoreceptor cells that are primarily affected in RP, it is probable that these clones correspond to messenger RNAs expressed mainly in this layer, although altered expression in other neural layers cannot be excluded. Support for the former possibility was obtained by probing a Southern blot of the inserts with a labelled oligonucleotide probe specific for human opsin: two clones (K111 and K554) contained complementary sequences (Fig. 1). Two other clones (K511 and K611) cross-hybridized with the oligonucleotide probe for S-antigen (not shown), which is another gene abundantly expressed in photoreceptors. A fifth clone (K411), not detected by these probes, whose insert was obtained by PCR amplification using vector specific primers, was used to probe a Northern blot of retinal RNAs (Fig. 2a). It detected an abundant transcript of approximately 2.0 kb, and a faint, possibly precursor transcript in 3.0 kb, in normal retinal RNA. These transcripts were completely absent from the retinal RNA of the two cases of advanced RP (donors DYS2 and DYS3) but were clearly detectable in the case of optic atrophy (donor DYS1). When the blot was hybridized with a cDNA probe specific for actin, bands of similar intensity corresponding to transcripts of approximately 2.3 kb were detected in each RNA sample, indicating that the dif-

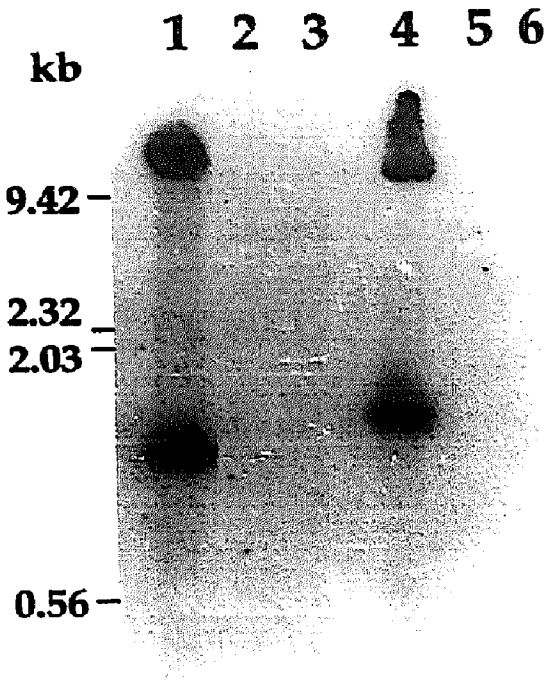

Fig. 1. Southern blot of *Eco*RI-digested DNA isolated from differentially-expressed clones, probes with γ-^{32}P-labelled oligonucleotide probe complementary to human opsin. Lanes 1–6: clones K111, 411, 511, 554, 611 and 661, respectively. Four size markers are shown. The upper hybridizing bands correspond to residual undigested recombinant DNA.

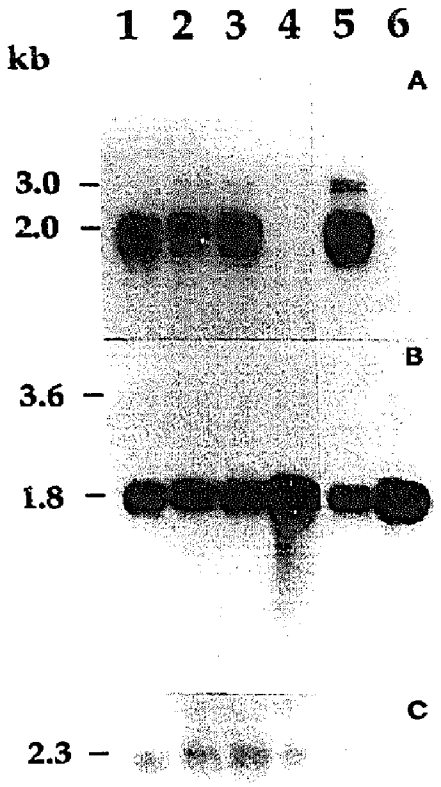

Fig. 2. Northern blot of normal and dystrophic total retinal RNA (3 μg per lane) probed with α-^{32}P-labelled differential and control cDNA probes. Lanes 1–6: RNA from donors CON1, DYS1, CON2, DYS2, CON3, DYS3, respectively. Probes: (a) K411 (opsin); (b) K661 (clusterin) and (c) actin, washed to stringencies of (a) 1 × SSPE, 0.1% SDS, 65°C; (b) 2 × SSPE, 0.1% SDS, 42°C; (c) 1 ×SSPE, 0.1% SDS, 42°C. (1 × SSPE is 0.18 M NaCl, 10 mM sodium phosphate pH 7.7, 1 mM disodium EDTA.) The actin signal (c) is relatively reduced owing to repeated reprobing and stripping of the blot.

ferences in expression of the K411 transcripts were not due to variation in sample loading or integrity (Fig 2c). Subcloning and partial sequencing of K411 showed that it also encoded human opsin. The lack of opsin mRNA expression in advanced RP supports our previous observations of reduced opsin in in vitro translation product from RP retinal RNA [7], and presumably reflects the substantial loss of photoreceptors from the diseased retinas. In contrast, the case of optic atrophy shows essentially normal expression of opsin mRNA, concordant with the normal dark-adaptation seen in this donor shortly before her death, and suggests that she had retained a largely intact population of photoreceptors.

A further clone (K661) gave moderately strong signals with the control ssDNA probe, but stronger signals with the RP probe. Digestion with *Eco*RI released two fragments, of 850 and 170 bp. Use of the larger fragment as a probe on the Northern blot of retinal RNA detected a major abundant transcript of approximately 1.8 kb present in both normal and RP RNA; a minor, possible precursor mRNA at 3.6 kb was also visible in some lanes on the original autoradiograph (Fig. 2b). Stronger signals were seen with both cases of advanced RP (donors DYS2 and DYS3) than with the controls, confirming the differential screening result. Again, with the case of optic atrophy (DYS1), expression of this gene approximated normal levels. Both *Eco*RI fragments of K661 were subcloned into M13 and sequenced wholly or partially. Comparison of the sequences with the EMBL and GenBank databases showed that both contained parts of the coding sequence of the human clusterin gene (>99.5% identity; differences assumed to be cloning artifacts), known variously, also, as SP-40,40, complement lysis inhibitor (CLI), sulphated glycoprotein 2 (SGP-2), testosterone-repressed message-2 (TRPM-2), and apolipoprotein J [16–20]. The size of the major retinal transcript is in agreement with reports of mRNAs of human clusterin in other tissues (1.9–2.0 kb [20,21]). Internal cleavage of the K661 insert had occurred at the *Eco*RI site at base 856 of the published sequence of clusterin (as SP-40,40) [16].

Clusterin is a 70–80 kDa sulphated glycoprotein comprised of two nonidentical polypeptides of M_r 40,000 linked by disulphide bonds. The polypeptides are proteolytically derived from a precursor protein translated from a single mRNA transcript [16]. Clusterin is present at concentrations of 50–100 μg/ml in normal human plasma and at about 10-fold higher concentrations in seminal plasma, and is expressed in a variety of tissues [16–18]. Our report describes the first observation of clusterin mRNA expression in retina, and the strength of hybridization signal on the Northern blot indicates it is a highly abundant transcript even in the normal tissue. Clusterin is assumed to be multifunctional, and in blood associates with components of the complement system [17]; it also appears to interact with apolipoprotein A1, suggesting possible involvement in lipid transport [20,22]. Most significantly for our own observations, an increase in expression of clusterin mRNA or protein has been observed in several instances of tissues undergoing programmed cell death (apoptosis) [19,23–25]. The increase in clusterin expression in the retinas of patients with advanced RP suggests that the associated photoreceptor degeneration may be another instance of programmed cell death, possibly with molecular and cellular mechanisms in common with these reported examples.

Apoptotic events characteristically include endonuclease-induced fragmentation of genomic DNA into nucleosomal unit oligomers, followed by irreversible morphological changes, including nuclear disintegration, cell surface blebbing, and cellular fragmentation into a cluster of membrane-bound apoptotic bodies [26]. While nuclear DNA changes have, to our knowledge, never been examined in RP, ultrastructural studies of RP retinas show, in addition to photoreceptor loss, severe membranous distortion and vesiculation, reactive

gliosis of Muller cells, and variably, autophagocytic vacuoles, epiretinal membrane formation, and extensive and heterogeneous extracellular deposits in the region of Bruch's membrane [27,28]. We have previously shown that the gliotic process in RP is linked to an increase in expression of the mRNA for the intermediate filament component and glial cell marker, glial fibrillary acidic protein (GFAP) [7]. It is possible that elevated clusterin expression is also associated with glial proliferation or activity, either as a result of the increased contribution of this cell type to the total tissue mass of the degenerate retina, or as a specific intracellular increase in response to the adjacent dystrophic photoreceptor cells. In this regard, observations of the concomitant elevation of clusterin and GFAP mRNA levels in Alzheimer-diseased hippocampus [21], and of similar transcriptional changes in the hippocampus of castrated rats [29], are of particular relevance. Further, McNeill et al. [30] detected the clusterin homologue, SGP-2, in the degenerating dentate gyrus of adrenalectomized rats, as punctate deposits in the molecular layer, and in the glial cells juxtaposed to surviving neurons, but not in the granule neurons exhibiting signs of degeneration. SGP-2 was not detected in corresponding brain regions of intact rats or adrenalectomized rats not showing granule cell loss.

At least 3 possible roles for elevated clusterin expression in tissue degeneration may be hypothesized (see also e.g. [22,30]): (i) a direct causal involvement in the cascade of events leading to apoptosis in the cells where clusterin transcription increases; (ii) an 'anti-suicide' action, as a response of the cell to retard the committed progression of the cell death program; or (iii) a response not of the dying cells but of their immediate neighbours, possibly as part of a repair or tissue remodelling mechanism. We suggest that the parallel molecular and cellular changes in glial cells in neurodegenerative states, including perhaps advanced RP, may correlate most closely with hypothesis (iii). In these cases, the glia, which are generally regarded as important supportive elements of neurons, may upregulate clusterin expression to supplement its activity possibly as a lipid scavenger to redistribute or recycle lipid membrane components [22], or as a transporter of lipids. Analysis of normal and degenerating retinas, including in situ and immunocytochemical approaches, is in progress to help resolve these questions.

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