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Expression of TIMP3 mRNA is elevated in retinas affected by simplex retinitis pigmentosa

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Abstract To explore the molecular and cellular mechanisms associated with photoreceptor death in retinitis pigmentosa (RP), we have investigated altered transcriptional activity in RP retinas by a differential cDNA screening approach. We identified a clone (K222) showing over-expression in simplex RP retinas compared with controls. K222 encodes a partial cDNA of the human tissue inhibitor of metalloproteinases-3 (TIMP3) gene, a member of a family of genes implicated in extracellular matrix (ECM) remodelling. Increased expression of TIMP3 in degenerating RP retinas may reflect restructuring of the ECM architecture, and disruption of photoreceptor–matrix interactions could contribute to activation of apoptotic cell death processes.

Key words: Retinitis pigmentosa; Retina; Tissue inhibitor of metalloproteinase; Extracellular matrix; Gene expression; Apoptosis

1. Introduction

Retinitis pigmentosa (RP) is the term given to a heterogeneous group of retinal diseases, affecting approximately 1 in 4000 people, in which progressive degeneration of the photoreceptors occurs, resulting in night blindness and contraction of the visual fields (reviewed in [1]). The genetic basis for a substantial proportion of cases is clear: transmission may be via autosomal dominant, autosomal recessive, or X-linked modes, and in an increasing number of dominant and certain recessive forms the causal genetic defects have been identified, occurring in genes expressed primarily in the photoreceptors. However, simplex or isolated cases also frequently occur, and can account for up to 58% of all RP types according to some studies [2].

The cellular mechanisms leading from the gene defects to photoreceptor cell death in RP are poorly understood. Recent investigations of mouse models of RP have indicated that the final stages of photoreceptor demise occur via apoptosis [3–5], but the earlier triggers of this process are unknown. In seeking to gain insight into these mechanisms, we have analysed gene expression in retinas affected by RP using the technique of differential screening of cDNA libraries. We previously detected increased expression in RP retinas of the mRNA for the multifunctional glycoprotein clusterin [6], the gene for which is widely over-expressed in apoptotic and neurodegenerative tissues. Here we report identification of a second differentially expressed gene, tissue inhibitor of metalloproteinases-3 (TIMP3), which shows increased mRNA expression in retinas affected by simplex RP, and discuss the possible significance of this finding in relation to the mechanisms of photoreceptor degeneration.

2. Experimental

2.1. Tissues

Control human eyes from adult donors with no history of ocular disease were obtained from the UK Transplant Support Service (Bristol); control brain was from the MRC Brain Bank Laboratory, Cambridge; and other control tissues were from St. Thomas' Hospital. Eyes from patients (or their relatives) affected by RP were obtained through the British Retinitis Pigmentosa Society (BRPS) Eye Donor Scheme. Enucleated eyes or other tissues were dissected and stored frozen in liquid nitrogen or at −70°C until used for total RNA extraction. The postmortem interval (pmi) between death and freezing was recorded where possible. For donors of control tissues, the details were as follows: Donor NR1 (normal retina), female, age 77 years, pmi less than 8 h; Donor NR2 (normal retina), female, age 89 years, pmi = 11.5 h; Donor NR3 (normal retina), male, age 67 years, pmi = 7 h; Donor NBr (normal brain), male, age 34 years, pmi = 6 h; Donor NLv (normal liver), male, age 58 years, pmi = 36 h.

For the donors of retinal tissues obtained through the BRPS Donor Scheme, the details were as follows: Donor RP1, male, age 79 years, pmi not greater than 24 h, simplex RP; Donor RP2, male, age 81 years, pmi = 32 h, simplex RP; Donor RP3, female, age 85 years, pmi = 11.5 h, carrier of X-linked RP with pigmentary deposits in retina and histological evidence of some photoreceptor loss; Donor RP4, female, age 92 years, pmi = 26 h, unaffected (latent) carrier of RP.

2.2. RNA extraction, Northern and RNA dot blot analysis, and reverse transcription PCR

RNA extraction and Northern blotting were as previously described [6]. For dot blots, 1 μg samples were denatured in formamide/formaldehyde solution and spotted onto nylon membrane (Amersham Int., UK) according to the manufacturer's instructions. Isolated cDNA inserts or control β-actin cDNA were labelled with α-[^{32}P]dCTP using the Megaprime 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. Quantification of autoradiographic signals (maximum peak values) was performed using a laser densitometer (LKB, Sweden). Analysis of expression by reverse transcription PCR was performed on 1 μg samples of control retinal RNA, using random hexamers to prime cDNA synthesis [7]. Oligonucleotide primers for PCR were obtained through the Human Genome Mapping Project Resource Centre, Harrow, UK, and from Pharmacia Biotech, Sweden.

2.3. Differential hybridization screening and clone analysis

The detection of differentially expressed human retinal cDNAs in a λgt11 library (Clontech) was based on the method of Leonard et al. [8] and has been previously described [6]. Cloned cDNA inserts were amplified by PCR using vector-specific primers, and subcloned into the vector pCRII (Invitrogen) for sequencing using automated sequencers (A.L.F., Pharmacia Biotech, Sweden; 373A, Applied Biosystems, USA). Sequences were analysed using the Genetics Computer Group (Wisconsin) Version 7 (1991) program, made available through the Human Genome Mapping Project Resource Centre, Harrow, UK.

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2.4. Hybrid cell line DNAs and Southern blotting

Rodent–human hybrid cell line DNAs and control human and rodent genomic DNAs (BIOS Laboratories, Inc., USA; ~4 μg quantities) were digested with *Eco*RI and Southern blotted, prior to hybridization with the ^{32}P-radiolabelled cDNA insert and autoradiography using standard protocols [9]. The human chromosome content of the hybrids was as follows: Hybrid #683: chromosomes 1, 5 (with multiple deletions), 12, 14, 19, 21, 22; #803: 4, 5, 8, 22, X; #1099: 1, 5 (with deletions), 13, 19, 21, 22; #811: 8, 17, 18; #1079: 3, 5; #423: 3 only.

3. Results

Differential screening of the human retinal cDNA library using single-stranded cDNA probes from control and simplex RP-affected retinal RNAs gave rise to a panel of 35 clones, the analysis of some of which, including clusterin, has been reported [6]. In this study, we isolated by vector-primer PCR a further cDNA insert (K222) which showed differential signal intensity between simplex RP and control retina RNAs when used to probe an RNA dot blot (Fig. 1A) or Northern blot (Fig. 2A). When expressed as a ratio of signal intensity to the control actin probe (Fig. 1B), a 5- to 10-fold over-expression of K222 was detected in retinal RNAs from donors with simplex RP compared to those from unaffected donors or from the donor with carrier status for X-linked RP (Fig. 1C). The mini-Northern blot resolved two major hybridizing transcripts, of approximately 4.5 and 2.5 kb (Fig. 2A). High levels of expression were detected in the RP retinal RNA; faint hybridizing bands were visible in the other tissue RNAs on the original autoradiograph. The high expression in RP was not due to variable loading or integrity of the RNA as the corresponding plot normalized to actin expression shows (Fig. 2B,C).

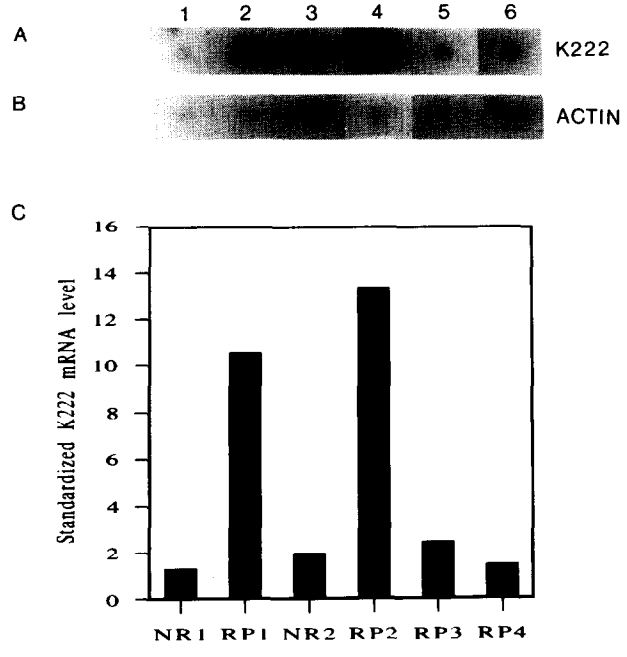

Fig. 1. RNA dot blot analysis of K222 expression in human tissues. One microgram samples of RNA from: 1, control retina, NR1; 2, dystrophic retina, RP1; 3, control retina, NR2; 4, dystrophic retina, RP2; 5, retina from affected carrier, RP3; 6, retina from unaffected donor, RP4. Probed with (A) K222 cDNA; (B) actin cDNA; the weak actin signal is a consequence of repeated reprobing of the blot. (C) Expression of K222 standardized to that of actin for each sample by laser densitometric analysis.

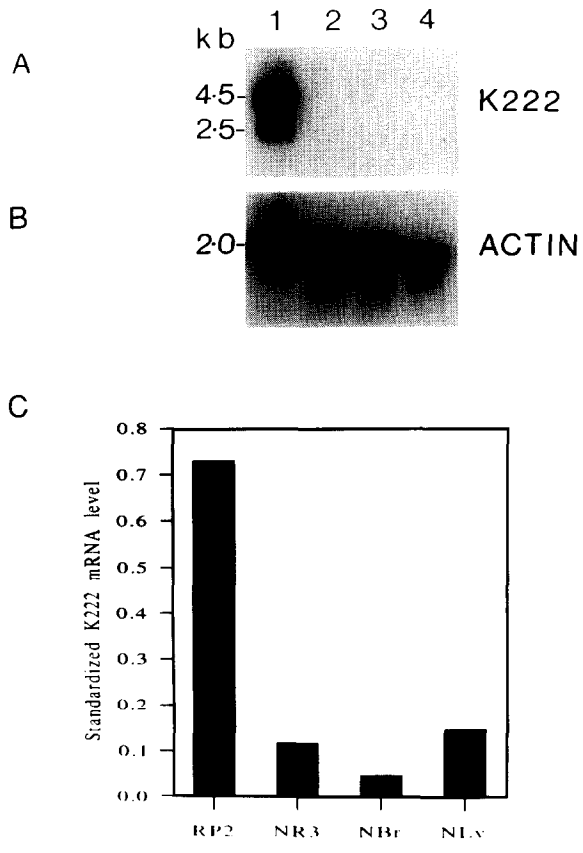

Fig. 2. Northern blot analysis of K222 expression. Samples, ~3 μg each, of RNA from: 1, dystrophic retina RP2; 2, control retina NR3; 3, control brain (cortex) NBr; 4, control liver NLv. Probed with (A) K222 cDNA; (B) actin cDNA. (C) Expression of K222 (4.5 kb transcript) standardized to that of actin using laser densitometric analysis.

Comparison of the partial sequence of clone K222 (Accession No. X77690) against the EMBL and GenBank Databases (releases 39.0 and 83.0, respectively) showed ~79% similarity with a cDNA corresponding to the mouse homologue of a recently described gene termed tissue inhibitor of metalloproteinases-3 (TIMP3; Accession No. Z30970 [10]). Three sequences of the human TIMP3 cDNA have been reported [11–13], as well as the additional characterization of TIMP3 cDNAs from mouse [14] and chicken [15], but K222 did not show significant similarity to any of these. This was due to the region of similarity with the first murine clone extending 3′ beyond the end of these published sequences (Fig. 3). In order to confirm that K222 corresponded to a 3′ extension of the human TIMP3 sequence, we used primers derived from our own and from the published human sequence (relative positions shown in Fig. 3) in a reverse transcription PCR applied to human retinal RNA. A single product of the predicted size, ~1.4 kb, was amplified and subcloned into pCRII (data not shown). Partial sequence analysis of this clone, K222TA2 (Accession No. X80791), indicated that the product corresponded to a 3′ extension of the reported human TIMP3 cDNAs (Fig. 4), divergence beginning at nucleotide 1229 based on the sequence of Silbiger et al. [12]. The novel human sequence reported here shows ~60% identity with the murine TIMP3 cDNA of Sun et al. [10]. We also used K222 as a probe on Southern blots of rodent–human hybrid cell lines to test whether the cDNA mapped to chromosome 22, as estab-

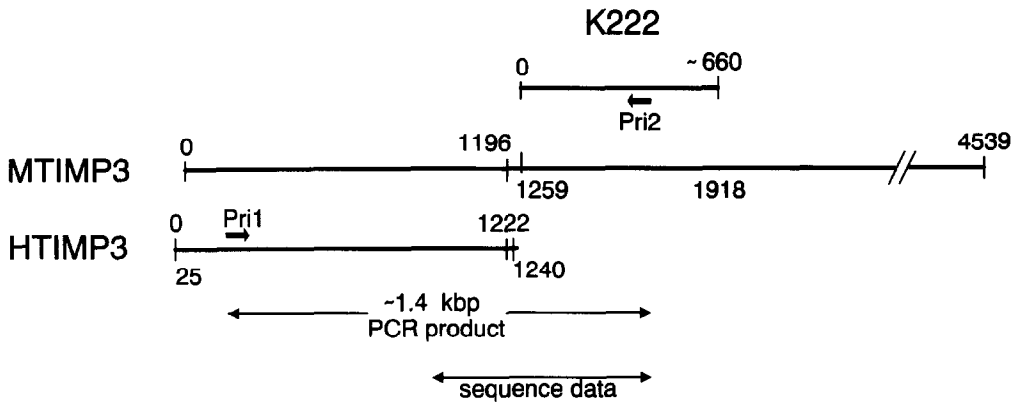

Fig. 3. Schematic representation of sequence alignment between clones of mouse TIMP3 (MTIMP3, [10]), human TIMP3 (HTIMP3, [12]) and clone K222. Numbers indicate base positions (0 at the 5′ end). Primers Pri1 and Pri2 used for reverse transcription PCR are indicated by arrows (not to scale), and the sequenced region of the resulting PCR product (see Fig. 4) is also shown.

lished for human TIMP3 [11]. A single hybridizing band of 2.4 kb was detected in human genomic DNA, with no detectable bands in rodent DNA (Fig. 5). The pattern of positive and negative hybrids confirmed mapping of K222 to human chromosome 22.

4. Discussion

In extending our analysis of altered gene expression associated with the dystrophic state in human retinas affected by retinitis pigmentosa, we identified over-expression of a clone, K222, occurring in retinal RNA from donors with simplex RP in contrast to controls and to affected carrier and unaffected donors. Partial sequence data from clone K222 showed 79% similarity with a cloned cDNA of the mouse homologue of tissue inhibitor of metalloproteinases-3 (TIMP3) [10], although the sequence did not overlap that of published human TIMP3 cDNAs. Our data indicate that K222 most likely corresponds to part of the 3′ extension of an alternative transcriptional species of the human gene, since (i) reverse transcription PCR of human retinal RNA using primers from the K222 and human TIMP3 sequences amplifies a single product of the predicted size, incorporating a sequence identical up to the poly(A) terminus of the human cDNA; and (ii) mapping of K222 to chromosome 22 accords with the results of Apte et al. for the localization of the human TIMP3 gene [11]. The unusually high similarity of the putative untranslated 3′ sequence between the human and mouse clones (~60–80% over extended regions of 200 nucleotides or more) also parallels similarities observed by Leco et al. [14] between mouse and chicken TIMP3 cDNAs in comparing the 3′-untranslated sequences of (presumably) the shorter transcriptional forms. As suggested by these investigators, the similarities may reflect regulatory functions of the 3′ region, such as stabilization of TIMP3 transcripts or modulation of their translation.

The tissue inhibitors of metalloproteinases (TIMPs) are a family of polypeptides which bind and inhibit matrix metalloproteinases (MMPs) such as collagenases and gelatinases (for review see e.g. [16]). The controlled expression of the proteases and their inhibitors is critical for the regulation of the structure

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Clone K222TA2   TTCTTGCAAATTTAGCACTTGGAACATTTAAAGAAAGGTCTATGCTGTCATATGGGGTTT   60
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human TIMP3     TTCTTGCAAATTTAGCACTTGGAACATTTAAAGAAAGGTCTATGCTGTCATATGGGGTTT  1075

K222TA2         ATTGGGAACTATCCTCCTGGCCCCACCCTGCCCCTTCTTTTTGGTTTTGACATCATTCAT   120
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TIMP3           ATTGGGAACTATCCTCCTGGCCCCACCCTGCCCCTTCTTTTTGGTTTTGACATCATTCAT  1135

K222TA2         TTCCACCTGGGAATTTCTGGTGCCATGCCAGAAAGAATGAGGAACCTGTATTCCTCTTCT   180
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TIMP3           TTCCACCTGGGAATTTCTGGTGCCATGCCAGAAAGAATGAGGAACCTGTATTCCTCTTCT  1195

K222TA2         TCGTGATAATATAATCTCTATTTTTTTAGGAAAACAAAAATGAAAAACTACTCCATTTGA   240
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TIMP3           TCGTGATAATATAATCTCTATTTTTTTAGGAAAAAAAAAAAAAAA                 1240

K222TA2         GGTTGTAATTCCCACCCCTCTTGCTTCTTCCCCACCTCACCATCTCCCAGACCCTCTTCC   300

                CTTTGCCCTTCTCCTCCAATACATAAAGGACACAGACAAGGAACTTGCTGAAAGGCCAAC   360

                CATTTCAGGATCAGTCAAAGGCAGCAAGCAGATAGACTCAAGGTGTGTGAAAGATGTTAT   420

                ACGCCAGGAGCTGCCACTGCATGTCCCAACCAGACTGTGTCTGTCTGTGTCTGCATGTAA   480

                GAGTGAGGGAGGGAAGGAAGGAACTACAAGAGAGTCGGAGATGATGCAGCACACACACAA   540

                TTCCCCAGCCCAGTGATGCTTGTGTTGACCAGATGTTCCTGAGTCTGGAGCAAGCACCCA   600

                GGCCAGAATAACAGAGCTTTCTTAGTTGGTGAAGACTTAAACATCTGCCTGAGGTCAGGA   660

                GGCAATTTGCCTGCCTTGTACAAAAGCTCAGGTGAAAGACTGAG                   704
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Fig. 4. Partial sequence of the cloned PCR product (K222TA2) amplified using primers 1 and 2 indicated in Fig. 3. The sequence is deposited in the GenBank and EMBL Databases with the Accession Number X80791. The sequence shows 100% identity with the 3′ end of the human TIMP3 cDNA [12] to base 1229, numbered as in the original publication.

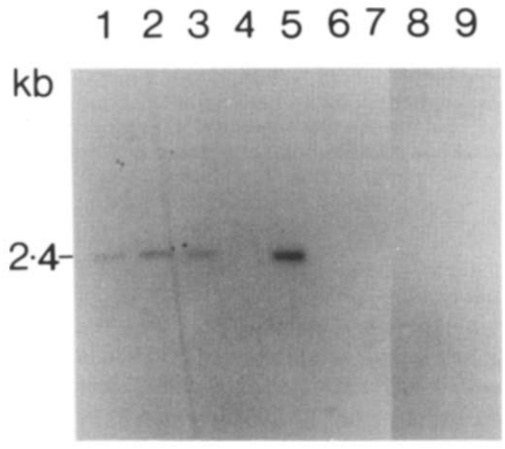

Fig. 5. Southern blot analysis of *Eco*RI-digested rodent–human hybrid cell line and control DNAs (~4 μg samples) probed with K222 cDNA. 1, hybrid #683; 2, #803; 3, #1099; 4, #811; 5, human genomic; 6, hamster genomic; 7, mouse genomic; 8, hybrid #1079; 9, #423 DNA.

and composition of the extracellular matrix (ECM), a dynamic compartment involved in a range of normal and pathological processes. Imbalances between the metalloproteinases and their inhibitors and activators, leading to increased ECM degradation, have been implicated in, for example, osteodegenerative arthritis and tumour invasion. The expression of TIMPs and of the proteases is regulated by numerous factors, including transforming growth factor-beta (TGF-β), which has a stimulatory effect on TIMP expression but inhibits metalloproteinase transcription (reviewed in [16]).

Human TIMP3 is the homologue of a gene first characterized in chicken (ChIMP-3) as the third member of the TIMP family, encoding a 21 kDa ECM protein from embryonic fibroblasts, and showing increased levels during oncogenic transformation [15,17]. The expression of human TIMP3 mRNA has been reported in a range of normal human tissues and tumour cell lines [11–13], notably at high levels in placenta. Three transcripts are detected by Northern blot, the largest of 4.4–5.5 kb, and the two smaller transcripts at 2.2–2.4 and 2.5–2.8 kb; it is likely in this study that the mini-Northern blot failed to resolve the smaller transcripts. This is the first observation of TIMP3 expression in ocular tissues, although matrix metalloproteinases and TIMP1 have been identified in cultured human retinal pigment epithelium (RPE) [18], and, in the developing neural retina, metalloproteinases have been postulated to play a role in facilitating neurite extension [19]. Recent preliminary studies have also identified MMPs and TIMP2 in the interphotoreceptor matrix and vitreous humour [20]. Three observations may be of relevance in interpreting the significance of increased TIMP3 expression in retinas affected by simplex RP. Firstly, Alexander et al. [18] reported that two cultured RPE cell lines from patients with RP showed over-expression of metalloproteinase activities, although those from other RP cases showed normal levels (the genetic types of RP were not stated). Secondly, up-regulation of genes associated with ECM turnover has been closely linked to the phenomenon of apoptosis (e.g. [21]), the mechanism responsible for the final stages of photoreceptor cell death in several rodent models of RP [3–5]. Thirdly, the recent report that disruption of cell-matrix interactions can induce apoptosis in epithelial cells ('anoikis', [22]) indicates the critical significance of maintenance of anchorage to the integrity of the cell. Our own observations, that a gene, TIMP3, implicated in ECM remodelling, is over-expressed in retinas affected by simplex retinitis pigmentosa, suggest at the very least that substantial restructuring of the retinal ECM (including presumably the interphotoreceptor matrix) is occurring in this type of disease. Potentially, the primary gene defects may lead to disruption of photoreceptor–matrix interactions, possibly via dysregulatory effects on RPE metalloproteinases. The concomitant loss of critical maintenance signals from the matrix via membrane receptors could contribute to the activation of apoptotic processes in the photoreceptors themselves. Further insight into the relationship between TIMP3 over-expression and photoreceptor degeneration may be gained by analysis of other agents modulating the integrity of the retinal extracellular matrix.

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