SCIENTIFIC REPORT

Matrix bound SFD mutant TIMP-3 is more stable than wild type TIMP-3

Mohammed A Majid, Valerie A Smith, Andrew C Newby, Andrew D Dick

Br J Ophthalmol 2007;**91**:1073–1076. doi: 10.1136/bjo.2006.113225

Background: Sorsby's fundus dystrophy (SFD) is a degenerative retinopathy characterised by accumulation of mutant TIMP-3 protein in Bruch's membrane.
Aim: To compare the stability of matrix bound SFD mutant TIMP-3s with wild type TIMP-3.
Methods: COS-7 cells were transfected with plasmids containing wild type, Ser 181, Gly-167, Ser-156, and Tyr-168 TIMP-3 cDNA. The cells and their matrices were subsequently harvested and homogenised. After measuring the bound wild type and SFD mutant TIMP-3 concentrations by ELISA, aliquots of the homogenates were heated to 100˚C. The rates of denaturation of the TIMP proteins at this temperature were monitored by reverse zymography.
Results: Over a period of 24 h at 100˚C the biological activity of both wild type and SFD mutant TIMP-3 was lost. Over a period of 6 h at this temperature the biological activity of the SFD mutant TIMP-3s was fully retained whereas that of the wild type TIMP-3 was lost.
Conclusion: Matrix bound SFD mutant TIMP-3s are thermodynamically more stable than wild type. This may explain why SFD starts earlier in life than age related macular degeneration.

Sorsby's fundus dystrophy (SFD)[1] is a rare retinal disorder that is clinically similar to age related macular degeneration (ARMD) but starts earlier in life, usually during the fourth decade.[2 3] In 1994, Weber *et al* discovered a genetic link between SFD and DNA markers on chromosome 22q13-qter.[4] Simultaneously, Apte *et al* mapped the TIMP-3 gene to chromosome 22q12.1-q13.2.[5] Because of the co-localisation of TIMP-3 and SFD on chromosome 22, Weber *et al* subsequently investigated TIMP-3 as a candidate gene for SFD and mapped TIMP-3 point mutations in patients with SFD.[6] Although both SFD and ARMD are characterised by the accumulation of TIMP-3 in Bruch's membrane,[7 8] and several more TIMP-3 point mutations have since been reported in patients with SFD,[9–13] none have been found in patients with ARMD.[14 15]

TIMP-3 consists of two structural domains, each of which is folded into three loops constrained by three disulphide bonds.[16] The N-terminal domain is required for metalloproteinase inhibition and the induction of apoptosis,[17 18] the C-terminal domain for ECM binding.[19] Most SFD TIMP-3 mutations introduce an additional cysteine residue in the C-terminal domain. In these scenarios and one where the C-terminus has been truncated,[20] the net result is the existence of an unpaired -SH group and the potential to form S-S bridges with other SFD-mutant TIMP-3 proteins or extracellular matrix (ECM) components.

The physical and ECM binding characteristics of wild type and SFD mutant TIMP-3 are similar[21] and cannot therefore account for the difference in timing of onset of SFD and ARMD. Apart from enhanced sensitivity of retinal pigment epithelial (RPE) cells to the apoptotic effects of SFD mutant TIMP-3,[22] other plausible explanations for this are that matrix bound SFD mutant TIMP-3 is more resistant to proteolytic degradation[23] or thermodynamically more stable than wild type TIMP-3. The latter of these possibilities is the subject of the work described in this paper.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and OptiMEM, fetal calf serum (FCS), glutamine, and Lipofectamine™ were obtained from Invitrogen (Paisley, UK). A polyclonal anti-TIMP-3 antibody raised against loop-1 of human TIMP-3 in rabbit was obtained from Chemicon (Chandlers Ford, UK). All other reagents were obtained from Sigma-Aldrich (Poole, UK).

Experimental cell line

COS-7 cells were obtained from the American Tissue Culture Collection. Routinely, they were maintained in DMEM supplemented with 10% FCS (vol/vol) and an antibiotic/antimycotic solution and incubated at 36℃ in a moist atmosphere of 95% air/5% CO_2. The culture medium was changed every three to four days.

COS-7 cell transfection with wild type and SFD mutant TIMP-3 cDNA

The method of transfecting COS-7 cells with plasmids mixed with lipofectamine and containing wild type and the Ser-181, Gly-167, Ser-156, and Tyr-168 SFD mutant TIMP-3 cDNA inserts has been reported.[21] Post-transfection, together with controls that had been incubated with lipofectamine only, the cells were incubated at 36℃ for 48 hours to maximise TIMP-3 protein production, harvested with their matrices using a cell scraper, then washed and homogenised in 0.05 M Tris HCl, pH 7.4. After centrifugation and resuspension, their relative TIMP-3 contents were determined by enzyme linked immunosorbent assay (ELISA).[21] Before storing at −20℃ the transfected cell/matrix samples were adjusted to the same TIMP-3 concentration.

Thermal denaturation of samples

Aliquots of the cell and matrix samples (25 µl) were pipetted into polymerase chain reaction (PCR) cuvettes and covered with silicon oil (15 µl) to prevent evaporation. They were then heated at 100℃ for specified periods in a thermal cycler (GRI, Braintree, UK), snap frozen, and stored at −20℃.

TIMP-3 analysis by reverse zymography

Reverse zymography is a method of visualising bioactive TIMP.[24] Briefly, 12% SDS-polyacrylamide gels[25] were prepared that contained gelatin (1 $mg.ml^{-1}$) and dispase (optimised to

Abbreviations: ARMD, age related macular degeneration; ELISA, enzyme linked immunosorbent assay; RPE, retinal pigment epithelial; SFD, Sorsby's fundus dystrophy

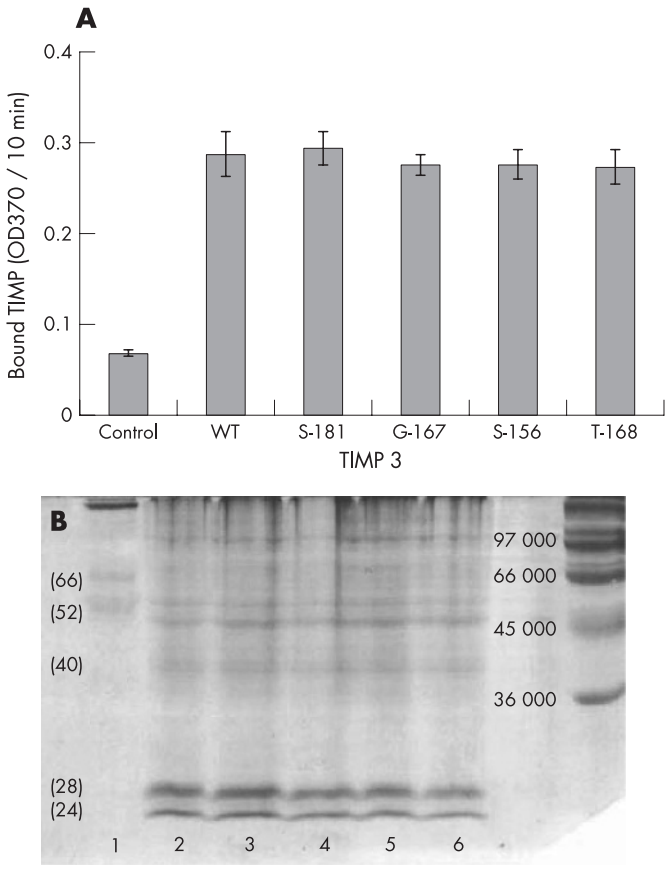

Figure 1 (A) Relative estimates of the amounts of matrix bound TIMP-3 recovered from control and transfected COS-7 cell cultures. (B) Reverse zymogram profiles of the TIMP-3 solubilised from cell and matrix samples of control COS-7 cultures (lane 1) and COS-7 cultures transfected with wild type TIMP-3 cDNA (lane 2) and the Ser-181 (lane 3), Gly-167 (lane 4), Ser-156 (lane 5), and Tyr-168 (lane 6) mutant TIMP-3 cDNAs. Numbers in parentheses are the calculated molecular weights ($\times 10^{-3}$) of visualised TIMP-3 bands.

0.1 caseinolytic units/ml). Aliquots of the cell/matrix samples were mixed with SDS (1% wt/vol), glycerol (10% vol/vol), and bromophenol blue (0.001%) and together with molecular weight marker proteins were loaded into wells formed in 3% acrylamide stacking gels. Mercaptoethanol and dithiothreitol were omitted because they inhibit MMP and hence dispase activity. After electrophoresis the gels were washed in distilled water, incubated for 30 minutes in Triton X-100 (2.5% vol/vol), washed again and incubated overnight in 0.05 M Tris HCl, pH 7.4 containing 5 mM $CaCl_2$. During this time the gelatin included in the gel is digested except in the vicinities of the electrophoretically separated TIMP protein. These were visualised as blue bands on a cleared background after staining with Coomassie Blue and destaining. The molecular weights of the bands corresponding to TIMP-3 were determined from plots of Log_{10} molecular weight *v* protein standard mobility.

RESULTS

Quantification of the wild type and SFD mutant TIMP-3 produced by COS-7 cells

The wild type and SFD mutant TIMP-3s recovered in the control and transfected cell and matrix samples were quantified relative to each other by ELISA (fig 1A) and visualised by reverse zymography. As shown in fig 1B, the banding patterns of the transfected cell/matrix samples on these gels were similar and comparable to those of reduced wild type and SFD mutant TIMP-3 protein visualised on immunostained western blots.[21] In addition to the glycosylated and non-glycosylated species of M_r 28 000 and 24 000 respectively, a band of M_r 40 000 and dimers of M_r 48 000 and M_r 52 000, several other high molecular weight bands, either homo- or heteropolymer aggregates, were resolved.

TIMP-3 thermostability

Aliquots of the wild type and SFD mutant TIMP-3 protein samples were initially heated at 100°C for 24 h. When subsequently analysed by reverse zymography it was shown that although both wild type and SFD mutant TIMP-3s had denatured, in the lanes corresponding to the Ser-181, Gly-167, Ser-156, and Tyr-168 SFD mutant TIMP-3 samples only, diffuse areas of enhanced staining of M_r 36 000–24 000 remained (Fig 2A).

In subsequent experiments, the kinetics of denaturation at 100°C were followed over a period of 7 h. For wild type TIMP-3 (fig 2B), the M_r 48 000 and M_r 52 000 bands corresponding to dimerised TIMP-3 were lost within the first hour and the M_r 28 000 band, corresponding to glycosylated TIMP-3, after 4 h. The non-glycosylated M_r 24 000 band appeared to be more resilient but over the 7 h incubation period faded in intensity. In sharp contrast, the SFD mutant TIMP-3s appeared to retain their biological activity for at least 6 h at 100°C. In all cases, as shown in fig 3, A–D respectively, the M_r 52 000, 48 000, 28 000, and 24 000 bands of the Ser-181, Gly-167, Ser-156, and Tyr-168 SFD mutant TIMP-3 proteins were visualised with little or no reduction in intensity.

DISCUSSION

TIMP-3 is an inducible protein which can be produced in response to metabolic stress.[26] Although it is unknown whether the RPE cells of individuals who have or develop ARMD or SFD

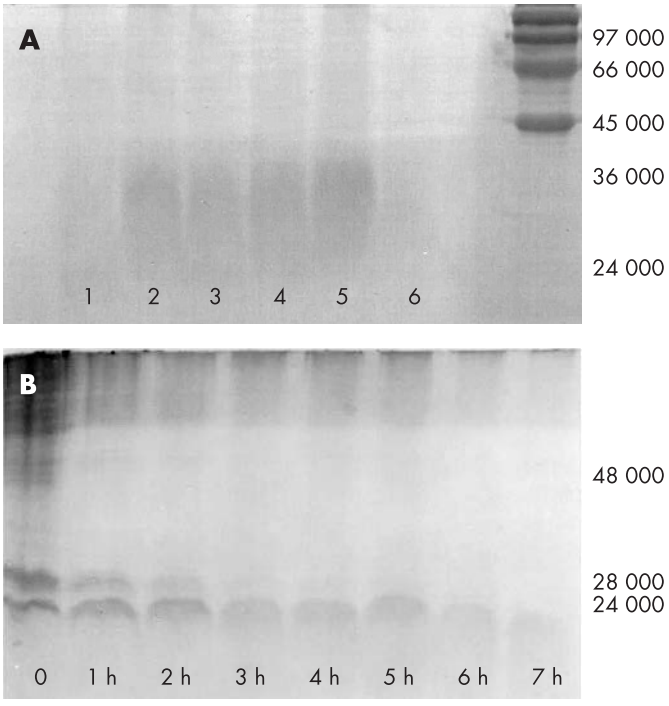

Figure 2 (A) Reverse zymograms of wild type TIMP-3 from transfected COS-7 cell cultures (lane 1), the Ser-181 (lane 2), Gly-167 (lane 3), Ser-156 (lane 4), and Tyr-168 (lane 5) mutant TIMP-3s, and TIMP-3 from control COS-7 cell cultures (lane 6). These proteins were solubilised from their matrices after heating for 24 hours at 100°C. No discrete bands of bioactive TIMP-3 remained after this treatment. (B) Time course of matrix bound wild type TIMP-3 heated at 100°C and sampled hourly over a period of 7 h.

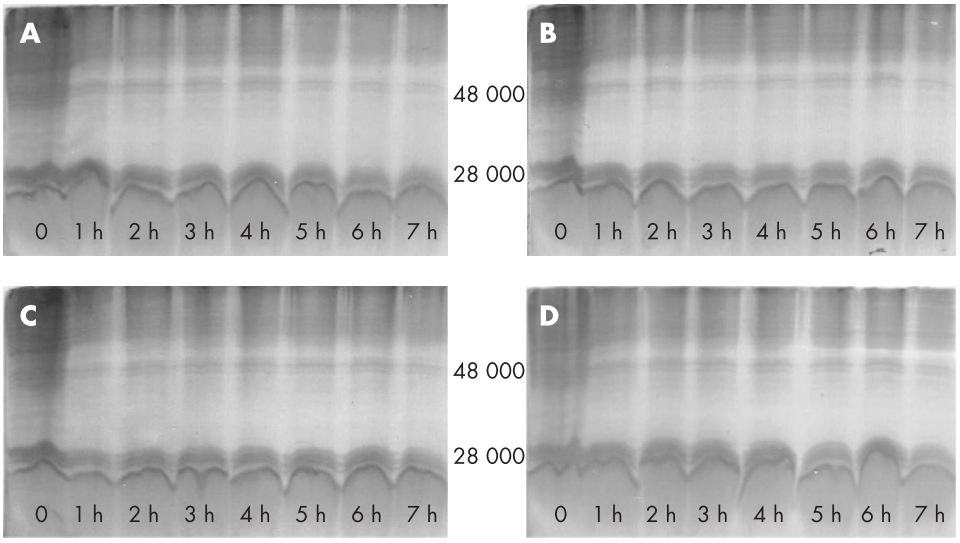

Figure 3 Denaturation time courses of matrix bound SFD mutant TIMP-3 at 100°C over a period of 7 h. (A) Ser-181 mutant TIMP-3. (B) Gly-167 mutant TIMP-3. (C) Ser-156 mutant TIMP-3. (D) Tyr-168 mutant TIMP-3.

constitutively produce excess wild type and SFD mutant TIMP-3, respectively, or whether these proteins are induced by metabolic stress relating to the trafficking of insoluble, undegradable photoreceptor catabolites, their accumulation is central to the progression of these degenerative retinopathies.

Mechanistically TIMP-3 may play a dual role. First, it can induce apoptosis and RPE cells are more sensitive to apoptotic effects of SFD mutant TIMP-3 than wild type.[22 27] Second, by inhibiting activated MMP and protecting matrix components susceptible to proteolytic degradation, excessively accumulated TIMP-3 will limit matrix turnover and ultimately induce thickening, crack formation, RPE cell loss, ischaemia, and neovascularisation.

Wild type and SFD mutant TIMP-3s do not interact with collagen, the principal component of the inner and outer collagenous zones of Bruch's membrane. They have, however, similar affinities for elastin, the principal component of the central elastic layer where maximum disruption occurs, and laminin, a cell matrix linker located in the RPE basement membrane.[21 28] In the absence of noted differences in the ECM binding characteristics of wild type and SFD mutant TIMP-3, the difference in the timing of onset of SFD and ARMD could be caused by a reduction in the rate of turnover of the matrix bound SFD mutant TIMP-3, either because the conformation adopted is thermodynamically more stable or because it is less susceptible to proteolytic degradation.

Although differences in the susceptibility of ECM bound wild type and SFD mutant TIMP-3 to proteases produced by RPE cells in culture have been detected,[23] it is uncertain which proteases normally exist in Bruch's membrane that are capable of removing the deposited TIMP-3, given that MMP-2 and MMP-9 do not degrade their inhibitory ligands. However, to test the proposition that the SFD mutant TIMP-3s are thermodynamically more stable than wild type TIMP-3, the relative rates of thermal denaturation of matrix bound wild type and the SFD mutant TIMP-3s were determined. The results of these experiments indicated that over a period of six hours at 100°C the SFD mutant TIMP-3s retained full activity, whereas that of the wild-type was lost. As the SFD mutations do not influence the ability of TIMP-3 to dimerise and form higher molecular weight aggregates when released from cell matrices with SDS and mercaptoethanol,[21] it would appear that through oxidation and homo- or heteropolymer crosslinking, the free -SH groups may stabilise matrix bound SFD mutant TIMP-3 aggregates and, in vivo, facilitate its accumulation in Bruch's membrane.

Conclusions

Evidence has been provided that matrix bound SFD mutant TIMP-3 is thermodynamically more stable than wild type TIMP-3 and this may account for the fact that the onset time of SFD is earlier than that of ARMD. Because the SFD point mutations lie within the encoded proteins, it is highly unlikely that the SFD mutant TIMP-3s are synthesised at different rates from wild type TIMP-3. It remains to be determined, however, whether the RPE cells of patients with SFD and ARMD constitutively overproduce TIMP-3.

ACKNOWLEDGEMENTS

We thank Professors D Edwards and G Murphy (Universities of East Anglia, Norwich and Cambridge, respectively) for the plasmids containing the cDNAs of wild type and SFD mutant TIMP-3s. We also thank the Wellcome Trust and the National Eye Research Centre for financial support

Authors' affiliations

Mohammed A Majid, Valerie A Smith, Andrew D Dick, Academic unit of Ophthalmology, University of Bristol, Bristol Eye Hospital, Bristol, UK
Andrew C Newby, Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, UK

Competing interests: None declared.

Correspondence to: Valerie A Smith, Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK; Val.Smith@bristol.ac.uk

Accepted 1 March 2007
Published Online First 23 March 2007

REFERENCES

1 **Sorsby A**, Mason MEJ. A fundus dystrophy with unusual features. *Br J Ophthalmol*, 1949;**33**:67–97.
2 **Capon MR**, Polkinghorne PJ, Fitzke FW, *et al*. Sorsby's pseudo-inflammatory macula dystrophy – Sorsby's fundus dystrophies. *Eye* 1988;**2**:114–22.
3 **Polkinghorne PJ**, Capon MRC, Berninger T, *et al*. Sorsby's fundus dystrophy. A clinical study. *Ophthalmology* 1989;**96**:1763–8.
4 **Weber BHF**, Vogt G, Wolz W, *et al*. Sorsby's fundus dystrophy is genetically linked to chromosome 22q13-qter. *Nat Genet* 1994;**7**:158–61.
5 **Apte SS**, Mattei MG, Olsen BR. Cloning of the cDNA encoding human tissue inhibitor of the metalloproteinase-3 (TIMP-3) and mapping of the TIMP-3 gene to chromosome 22. *Genomics* 1994;**19**:86–90.
6 **Weber BHF**, Vogt G, Pruett RC, *et al*. Mutations in the tissue inhibitor of metalloproteinase-3 (TIMP-3) in patients with Sorsby's fundus dystrophy. *Nat Genet* 1994;**8**:352–6.
7 **Fariss, RN, Apte, SS, Luthert, PJ, et al**. Accumulation of tissue inhibitor of metalloproteinases-3 in human eyes with Sorsby's fundus dystrophy or retinitis pigmentosa. *Br J Ophthalmol* 1998;**82**:1329–34.

8 **Kamei M**, Hollyfield JG. TIMP-3 in Bruch's membrane: changes during ageing and in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 1999;**40**:2367–75.
9 **Felbor U**, Stöhr H, Amman T, *et al.* A novel Ser-156 Cys mutation in the tissue inhibitor of metalloproteinase-3 (TIMP-3) in Sorsby's fundus dystrophy with unusual clinical features. *Hum Mol Genet* 1995;**4**:2415–16.
10 **Felbor U**, Stohr H, Amann T, *et al.* A second independent Tyr 168 Cys mutation in the tissue inhibitor of metalloproteinases-3 (TIMP3) in Sorsby's fundus dystrophy. *J Med Genet* 1996;**33**:233–6.
11 **Jacobson SG**, Cideciyan AV, Bennett J, *et al.* Novel mutation in the TIMP3 gene causes Sorsby fundus dystrophy. *Arch Ophthalmol* 2002;**120**:376–9.
12 **Carrero- Valunzuela RD**, Klein ML, Weleber RG, *et al.* Sorsby fundus dystrophy: a family with the Ser-181-Cys mutation of the tissue inhibitor of metalloproteinase-3. *Arch Ophthalmol* 1996;**114**:737–8.
13 **Peters AL**, Greenberg J. Sorsby's fundus dystrophy. A South African family with a point mutation on the tissue inhibitor of metalloproteinase-3 gene on chromosome 22. *Retina* 1995;**15**:480–5.
14 **Felbor U**, Doepner D, Schneider U, *et al.* Evaluation of the gene encoding the tissue inhibitor of metalloproteinases-3 in various maculopathies. *Invest Ophthalmol Vis Sci* 1997;**38**:1054–9.
15 **De La Paz MA**, Pericak-Vance MA, Lennon F, *et al.* Exclusion of TIMP-3 as a candidate locus in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 1997;**38**:1060–5.
16 **Douglas DA**, Shi YE, Sang QA. Computational sequence analysis of the tissue inhibitor of metalloproteinase family. *J Protein Chem* 1997;**16**:237–55.
17 **Lee MH**, Knauper V, Becherer JD, *et al.* Full length and N-TIMP-3 display equal inhibitory activities towards TNF-α convertase. *Biochem Biophys Res Comm* 2001;**280**:945–50.
18 **Bond M**, Murphy G, Bennett MR, Armour A, *et al.* Localisation of the death domain of tissue inhibitor of metalloproteinase-3 to the N terminus. Metalloproteinase inhibition is associated with proapoptotic activity. *J Biol Chem* 2000;**275**:41358–63.
19 **Langton KP**, Barker MD, McKie N. Localization of the functional domains of human tissue inhibitor of metalloproteinases-3 and the effects of a Sorsby's fundus dystrophy mutation. *J Biol Chem* 1998;**273**:16778–81.
20 **Clarke M**, Mitchell KW, Goodship J, *et al.* Clinical features of a novel TIMP-3 mutation causing Sorsby's fundus dystrophy; implications for disease mechanism. *Br J Ophthalmol* 2001;**85**:1429–31.
21 **Majid MA**, Smith VA, Matthews FJ, *et al.* Tissue inhibitor of metalloproteinase-3 differentially binds to components of Bruch's membrane. *Br J Ophthalmol* 2006;**90**:1310–15.
22 **Majid MA**, Smith VA, Easty DL, Baker AH, *et al.* Sorsby's fundus dystrophy mutant tissue inhibitors of metalloproteinase-3 induce apoptosis of retinal pigment epithelial and MCF-7 cells. *FEBS Lett* 2002;**529**:281–5.
23 **Langton KP**, McKie N, Smith BM, *et al.* Sorsby's fundus dystrophy mutations impair turnover of TIMP-3 by retinal pigment epithelial cells. *Hum Mol Genet* 2005;**14**:3579–86.
24 **Staskus PW**, Masiarz FR, Pallanck LJ, *et al.* The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. *J Biol Chem* 1991;**266**:449–54.
25 **Laemmli UK**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–5.
26 **Smith VA**, Matthews FJ, Majid MA, *et al.* Keratoconus: matrix metalloproteinase-2 activation and TIMP modulation. *Biochim Biophys Acta* 2006;**1762**:431–9.
27 **Majid MA**, Smith VA, Easty DL, *et al.* Adenovirus mediated gene delivery of tissue inhibitor of metalloproteinases-3 induces death in retinal pigment epithelial cells. *Br J Ophthalmol* 2002;**86**:97–101.
28 **Chong NHV**, Alexander RA, Gin T, *et al.* TIMP-3, collagen and elastin immunohistochemistry and histopathology of Sorsby's fundus dystrophy. *Invest Ophthalmol Vis Sci* 2000;**41**:898–902.