Sorsby’s fundus dystrophy mutations impair turnover of TIMP-3 by retinal pigment epithelial cells†

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Sorsby’s fundus dystrophy (SFD) is an autosomal dominant degenerative disease of the retina, caused by
mutations in exon 5 of the gene for tissue inhibitor of metalloproteinases-3 (TIMP-3). The mechanism by
which these mutations give rise to the disease phenotype is unknown. In an attempt to identify common
properties of these molecules that might underlie the disease phenotype, a range of SFD mutants were
expressed from human retinal pigment epithelial (RPE) cells. This showed that resistance to turnover,
resulting from intermolecular disulfide bond formation, was a common property of all the SFD mutants exam-
ined, providing a possible explanation for the increased deposition of the protein observed in eyes from SFD
patients. In contrast, SFD mutants varied in their ability to inhibit cell-surface activation of matrix metallopro-
tenase-2 (MMP-2), a potent mediator of angiogenesis, ranging from being fully active to totally inactive.
These data show that increased deposition of active TIMP-3, rather than dysregulation of metalloproteinase
inhibition, is likely to be the primary, initiating event in SFD.

INTRODUCTION

Sorsby’s fundus dystrophy (SFD) is a fully penetrant,
autosomal dominant degenerative disease of the macula, the
central area of the retina responsible for high resolution and
color vision. Initial manifestation of the disease can range
from the third to the sixth decades and proceeds with rapid
loss of central vision followed by progressive loss of
peripheral vision (1). The disease is characterized by
extracellular deposits (drusen) and thickening of Bruch’s
membrane, the basement membrane separating the retinal
pigment epithelium (RPE) from its blood supply, the
choriocapillaris. This is accompanied by atrophy of the chori-
capillaris, RPE and photoreceptors. Visual loss is usually
accelerated by sub-retinal neo-vascularization leading to
retinal detachment (2), although in some patients this does
not occur and loss of central vision is attributed solely to
retinal atrophy (3,4). Although itself rare, phenotypically,
SFD is very similar to age-related macular degeneration
(AMD), the most common cause of blindness in the elderly
developed countries (5).

Unlike AMD, which has a complex etiology, SFD is caused
by specific mutations in the tissue inhibitor of metalloprotei-
nases-3 (TIMP-3) gene, one of a family of four endogenous
inhibitors of matrix metalloproteinases (MMPs) that play a
role in regulating turnover of the proteinacious extracellular
matrix (ECM) (6). Structurally and functionally, TIMPs can
be separated into their N-terminal and C-terminal domains
(N-domain and C-domain), each containing six cysteine
residues that form three disulfide bonds (7–9). While

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†This article is dedicated to our colleague Kevin Langton, who died suddenly on 19th December 2004. We all miss his intellect, practical skills and
sense of humor.

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sharing this domain structure, TIMP-3 has several unique properties. Specifically, it is associated with the insoluble ECM (10), it is an efficient inhibitor of the aggreganases (11) and an inhibitor of certain ADAM (a disintegrin and metalloproteinase domain) proteinases that are involved in the processing of several type-I membrane protein ecto-domains, including tumor necrosis factor-

The N-domain of TIMP-3 alone (N-TIMP-3) is sufficient to mediate its metalloproteinase inhibitory activities and, at high level, can induce apoptosis in some cell types (13,14). RPE cells constitutively express high levels of TIMP-3 mRNA, and TIMP-3 protein is a component of the normal Bruch’s membrane (15,16).

Currently, nine independent TIMP-3 mutations have been identified in SFD patients, eight of which affect exon 5 that encodes the C-domain of the molecule. Seven of these lead to the introduction of an extra cysteine residue [S156C (17), G166C (18), G167C (19), Y168C (20), S170C (21), Y172C (22) and S181C (20)], whereas the eighth introduces a premature stop codon that deletes most of the molecule’s C-domain (E139X) (4). The ninth SFD TIMP-3 mutation is to the acceptor site of the intron 4/exon 5 junction and was identified in a family suffering from a novel late onset form of the disease (23). Previous work in our laboratory showed that both full-length (S156C, G166C and S181C) and truncated SFD mutants (E139X) shared a common protein phenotype when expressed transiently from COS-7 cells, a simian kidney fibroblast line (9,24). Specifically, all were ECM-bound MMP inhibitors but had a tendency to form disulfide bonded aggregates, presumably because all contain an odd number of cysteines (13 for the full-length and nine for the truncated mutant). Aggregation of SFD-TIMP-3 proteins, including mutations G167C and Y168C that we did not analyze, expressed from transfected baby hamster kidney (BHK) cells has also been reported elsewhere (25). Immunohistochemical analyses of eyes from SFD and AMD patients showed that Bruch’s membrane and drusen of these eyes were enriched in TIMP-3 protein when compared with non-diseased controls (26–28). In addition, there is no evidence for an increase in TIMP-3 mRNA expression in either SFD (29) or AMD (30) eyes. These data have led us to hypothesize that SFD-TIMP-3 protein aggregates accumulate in Bruch’s membrane and ultimately prove toxic (24). However, such a mechanism has remained speculative.

We have therefore characterized a representative group of SFD-TIMP-3 mutations expressed by human RPE cells with the aim of identifying common features that may underlie the SFD phenotype. By directly measuring the turnover of TIMP-3 and SFD-TIMP-3 protein in the matrix of human RPE cells, we show that in all cases mutation induces multimerization, rendering a proportion of the SFD proteins resistant to degradation and providing a mechanism for TIMP-3 accumulation in the disease. However, SFD mutants vary in their ability to inhibit cell-surface activation of proMMP-2, some being as effective as the wild-type molecule, whereas others are completely ineffective in this regard. We conclude that dysregulation of proMMP-2 activation is not essential for the development of SFD, but rather that SFD mutations initiate disease by a build-up of multimeric protein that is resistant to turnover.

RESULTS

Expression of TIMP-3His and AlaTIMP-3His by ARPE-19 cells

As ARPE-19 cells, transfected with empty vector (pCI.neo), express a background level of 24 kDa non-glycosylated TIMP-3 (Fig. 1A and B, lane 1) in their ECM, wild-type TIMP-3 was expressed with a C-terminal six-histidine tag (TIMP-3His) to differentiate it from the endogenous protein through its decreased mobility. TIMP-3His was detected as a 28 kDa band in the cells’ ECM (Fig. 1A, lane 2) and conditioned media (data not shown) by western blotting and was an active MMP inhibitor on reverse zymograms (Fig. 1B, lane 2). Thus, the presence of the C-terminal tag does not appear to affect the properties of TIMP-3 in RPE cells in agreement with a number of biochemical studies (11–13). Densitometric quantification revealed that this recombinant protein was expressed at a level ~2-fold greater than the endogenous ARPE-19 derived TIMP-3. However, the levels of the endogenous 24 kDa TIMP-3 protein in both control and TIMP-3His cells ECMs were identical (Fig. 1A), indicating that increased levels of TIMP-3 do not affect the expression of the endogenous TIMP-3 gene.

As a control for MMP-2 expression and activation (discussed subsequently), TIMP-3His was also expressed with an N-terminal alanine residue (AlaTIMP-3His), a change known to abolish the MMP inhibitory activity of TIMP-2 without affecting the structure or other properties of the protein (31). Western blotting showed that the expression and localization of AlaTIMP-3His is essentially identical to TIMP-3His (Fig. 1A, lane 3). However, this protein was not detected by reverse zymography (Fig. 1B, lane 3) and is, therefore, inactive against MMPs. The AlaTIMP-3His protein was expressed at a level ~5-fold greater than the endogenous TIMP-3, and its over-expression did not affect the level of the endogenous protein when compared with the levels seen in control ECM (Fig. 1A).

Turnover of TIMP-3 and SFD-TIMP-3 by RPE cells

In order to determine the rate of turnover of the wild-type and SFD mutant forms of TIMP-3 by RPE cells, the transfected cells were removed from the plates non-enzymatically and replaced with untransfected ARPE-19 cells and then the loss of the recombinant TIMP-3 proteins determined by western blotting and densitometry. As endogenous wild-type TIMP-3 is expressed by the untransfected cells, the level of this protein remains fairly constant with time, providing an additional loading control (Fig. 2A–G). Around 80% of the wild-type TIMP-3His protein was removed from the ECM during the first day and <1% remained after day 4 (Fig. 2A). As TIMP-3His could not be detected in cell conditioned media at any point during its removal from the ECM (data not shown), it is clear that this protein is being actively turned over, rather than simply being displaced from the ECM by endogenous ARPE-19 cell TIMP-3. Endogenous TIMP-3 was not detectable in the conditioned media at any time point.

Under non-reducing conditions, all SFD mutants were present as a mixture of monomers, novel dimers and high
molecular weight aggregate forms, which ran at the top of the gels (Fig. 2B, D and F, day 0). Reduction largely abolished the multimeric species and augmented the relevant monomer species (Fig. 2C, E and G, day 0). Densitometric quantification of the relative expression levels revealed that expression of the mutants was between 1.5- and 3-fold greater than that of the endogenous TIMP-3. Comparison of the levels of endogenous TIMP-3 present in the matrices from control and E139X cells revealed that over-expression of this mutant protein did not affect the expression of the endogenous wild-type TIMP-3 (Fig. 2C). Determination of the effect of the other mutants on the level of endogenous TIMP-3 was not possible because of co-migration of the monomeric forms of the mutant protein. Dimeric forms of the full-length mutants were active on reverse zymograms (Fig. 2H), but determination of the activity of E139X was not possible due to the co-localization of the dimeric form with endogenous TIMP-3 expressed by ARPE-19 cells (24). However, as both monomer and dimer forms of E139X protein from insect cells are active on gelatin reverse zymograms (32), it is likely that this protein is also active when expressed from ARPE-19 cells. These data are in good agreement with that for expression by COS-7 cells (9,24), BHK-TK cells (25) and transgenic 'knock-in' mice (33), as well as fibroblasts from S156C (33), E139X and S181C patients (32).

Analysis of the mutants removal under non-reducing conditions appeared more complex, with various high molecular weight bands remaining after 4 days, although their levels declined during the assay, especially over the first day (Fig. 2B, D and F). As reduction of E139X gives rise to a novel monomer band, these conditions were used for accurate assessment of the total amount of mutant remaining (Fig. 2C). Reduction of S156C and S181C did not give rise to any novel bands (Fig. 2E and G), therefore the high molecular weight species present on the non-reduced gels, which all co-migrate with the endogenous TIMP-3 protein upon reduction, were used to quantify turnover of these proteins (Fig. 2D and F).

A plot of the removal of the wild-type and SFD mutant TIMP-3 species, averaged from three separate experiments, over the 4-day assay period is shown in Figure 2I. This shows that initially the mutants were turned over rapidly, with ~65% of E139X, 50% of S156C and 60% of S181C being removed during the first day. However, after day 2, the rate of removal slowed dramatically leaving ~20% of E139X and S181C and 25% of S156C, following 4 days incubation (Fig. 2I). Accurate quantification of mutant protein levels at later time points was not possible due to the differentiation of the ARPE-19 cells in response to prolonged confluence (34,35), leading to increased deposition of insoluble cytoskeletal proteins in the matrix fraction and preventing accurate comparison of protein levels with earlier time points. However, even after 7 days, high molecular weight TIMP-3 complexes were still visible by western blotting as exemplified by the E139X mutant (Fig. 2B and C).

The data for the wild-type TIMP-3His fit a one-phase exponential decay model with a predicted half-life of ~9.5 h. The calculated half-life of the mutant protein removed during the first 2 days was similar to that of the wild-type molecule (between 9 and 10.5 h). However, in contrast, any further reduction from days 2 to 4 was too small to be statistically significant. Results for longer incubation periods (up to 7 days) indicated there may have been a very gradual loss of the remaining fraction with time; however, the possibility remains that a fraction of SFD-TIMP-3 protein is totally resistant to turnover.

Effect of SFD proteins on MMP-2 secretion and activity

Activation of proMMP-2 by membrane-type-MMPs (MT-MMPs) plays a major role in angiogenesis (36), a phenomenon often encountered in SFD. Although TIMP-3 can inhibit this process in vitro (37), it has been reported that the S156C protein has a reduced MMP-inhibitory activity (38), possibly explaining authors’ observation that cloned S156C-transfected ARPE-19 cells demonstrated a marked increase in the secretion and MT-MMP-mediated activation of proMMP-2. As might be expected, these cells also had an increased ability to migrate and stimulate angiogenesis. However, as this property has only been ascribed to the S156C mutant, which is associated with a disease in which angiogenesis is particularly pronounced (17), we sought to determine whether this was a unique property of the S156C mutant or a general property of all SFD proteins.

The ability of TIMP-3His and the different SFD mutant forms of the molecule to inhibit MT-MMP-mediated activation of proMMP-2 was tested by gelatin zymography of cell supernatants from transfected ARPE-19 cells before and after treatment with Concanavalin A (Con A). Con A stimulates the activation of proMMP-2, probably by up-regulating the cell-surface expression of MT-MMP (39). Normally inactive forms of proMMP-2 are also visible by zymography due to self-activation during renaturation on the SDS–PAGE gel. In our hands, in the absence of Con A, essentially identical amounts of 72 kDa proMMP-2 were secreted by each cell line (Fig. 3A). Con A treatment of control cells (pClneo) resulted in the activation of proMMP-2, as determined by a decrease in the intensity of the 72 kDa band and the appearance of two lower bands of 68 and 64 kDa, corresponding to MMP-2 with partial and full prodomain cleavage, respectively (Fig. 3B and C) (37). None of the TIMP-3 transfected cells displayed increased proMMP-2 activation when compared
with controls; in fact, expression of TIMP-3His, N-TIMP-3 and S181C significantly inhibited Con A stimulated activation (Fig. 3B and C). Although expression of AlaTIMP-3His, E139X and S156C resulted in an apparently consistent, small inhibition of proMMP-2 activation relative to control cells (Fig. 3B and C), however, this was not sufficient to reach statistical significance with the method of quantification used here. The similar effect of these three proteins on proMMP-2 activation indicates that the E139X and S156C mutants are essentially inactive against MT-MMP; however, this does not give rise to an increase in proMMP-2 expression or activation relative to controls.

DISCUSSION

In order to more clearly understand the role of TIMP-3 mutations in SFD, we expressed a representative group of SFD proteins from human RPE cells. Our aim was to identify common properties of three distinct SFD mutants whose structure and associated disease phenotypes encompass the full range of SFD; first, the unique, truncated E139X protein, which is associated with disease onset in the third or fourth decades and where geographic macular atrophy is the major cause of vision loss (4); secondly, the full-length S156C mutant, which is also associated with disease onset in the third or fourth decades but in which choroidal neo-vascularization is the predominant feature (17) and thirdly, the full-length S181C protein, the prototypic SFD mutant which is associated with disease onset around the fifth decade (20).

SFD proteins expressed from human RPE cells are ECM-bound MMP inhibitors that are prone to cross-linking via the formation of aberrant intermolecular disulfide bonds. These characteristics are essentially identical to those seen when they are expressed by other cell types including patient fibroblasts from E139X, S156C and S181C pedigrees, as well as 'knock-in' transgenic mice (24,25,32,33), arguing strongly that this is the nature of the mutant protein expressed from patient RPE cells in vivo. However, this contrasts with a recent report in which S156C protein was expressed by ARPE-19 cells solely in a monomeric form that was devoid of MMP inhibitory activity (38). As that study employed the same cell line and involved a similar level of recombinant protein expression as our study, this discrepancy is surprising. It is possible that the use of a C-terminal peptide tag (FLAG) in this latter study might have interfered with folding of the mutant S156C protein, explaining this difference.

Figure 2. Analysis of the expression and turnover of TIMP-3His, E139X, S156C and S181C by ARPE-19 cells. (A, B, D and F) Non-reduced western blot analyses of TIMP-3His, E139X, S156C and S181C turnover, respectively. (C, E and G) Reduced western blot analyses of E139X, S156C and S181C turnover, respectively. These blots are representative of three independent experiments. (H) Reverse zymogram analysis of the ECM from SFD TIMP-3 transfected ARPE-19 cells. The numbers to the right of the blots/gels indicate the relative positions of molecular weight markers in kDa. (I) Densitometric quantification of the turnover of TIMP-3His (open circles), E139X (closed squares), S156C (closed circles) and S181C (closed triangles) as described. Points are the mean ± SD, n = 3. *At 4 days, the levels of all SFD mutants were significantly greater than the TIMP-3His control (P < 0.05).
However, here we find that wild-type TIMP-3 appears unaffected by a C-terminal His-tag (Fig. 1).

These data show that TIMP-3 is a regulator of MT-MMP-mediated activation of proMMP-2 by RPE cells (Fig. 3), in agreement with its ability to inhibit recombinant MT-MMP (37). However, although the E139X and S156C mutants have apparently lost this ability, the S181C protein is comparable with the wild-type protein in this regard (Fig. 3). The inability of the S156C mutant to inhibit MT-MMP activation of proMMP-2 is in agreement with a recent study in which this protein was expressed in ARPE-19 cells (38); however, we do not observe the increase in MMP-2 secretion and activation by cells expressing the S156C protein noted in that report. Similarly, Soboleva et al. (40) failed to find any difference in MMP-2 secretion and activation between wild-type and transgenic fibroblasts derived from TIMP-3 S156C/S156C mice. This discrepancy may result from the differing characteristics of the recombinant mutant proteins displayed in these two studies that are discussed earlier. Alternatively, the use of cloned cells in the latter study (38), as opposed to the uncloned cell populations used here, may allow the isolation of more invasive variants of the ARPE-19 cell line. Indeed, previous attempts to clone ARPE-19 cells have led to marked changes in cell phenotype (35). Nevertheless, these data clearly demonstrate that impaired ability to inhibit activation of proMMP-2 by MT-MMP is not a conserved property of SFD mutants and that dysregulation of this process is not essential to the development of the disease. This conclusion is supportive of the fact that angiogenesis is not always a feature of SFD (3,4). Furthermore, the loss of this ability does not correlate with the extent of sub-retinal neo-vascularization manifested by individuals bearing these specific mutations, because the E139X mutation is associated with a disease in which neo-vascularization is rarely observed (4).

This article presents the first direct evidence that SFD-TIMP-3 is resistant to removal by RPE cells. Strikingly, a portion (∼20%) of three different SFD-TIMP-3 molecules, including the novel truncated E139X mutant, was resistant to turnover. This resistant material decayed many times more slowly than the rest of the mutant protein or the wild-type molecule and some may be totally resistant to turnover (Fig. 2). Thus, assuming its expression remains constant, a gradual accumulation of mutant TIMP-3 will occur. With regards to expression over time, it has been demonstrated that expression of TIMP-3 by RPE cells is constitutive (15,16) and is not significantly affected during aging (30). Whereas in this study, several fold increases in the TIMP-3 content of transfected cells ECMs did not decrease the secretion of endogenous TIMP-3 from these cells (Fig. 1A), indicating that increasing extracellular concentrations of TIMP-3 do not effect the expression of this protein. Taken together, these findings indicate that the increased TIMP-3 content of SFD retinas results from a gradual accumulation of the portion of the mutant protein that is resistant to turnover. This gain of function fits well with the dominant inheritance of SFD (1).

These conclusions that SFD mutations play an active role in the disease process in this way differ from those made recently by Soboleva et al. (40), using the TIMP-3 S156C/S156C fibroblasts mentioned earlier. In that report, the authors observe differences in morphology and metabolism between wild-type and TIMP-3 S156C/S156C fibroblasts, the latter bearing a closer similarity to those derived from TIMP-3 knock-out (TIMP-3−/−) mice. They also state that there is no difference in wild-type and S156C TIMP-3 turnover, when examined over 48 h, and therefore conclude that SF pathogenesis is due to loss of function mutation in TIMP-3. Although it is possible that the SFD mutations have an as yet undetermined role in disease, it is clear these differences in morphology and metabolism are not due to differences in TIMP-3 content.
effect on TIMP-3 function, it seems unlikely that the disease could be attributed simply to a loss of function as, unlike the TIMP-3<sup>S156C</sup>S156C fibroblasts, patients’ cells will also be expressing fully functional TIMP-3 from the normal allele. These observations are also solely based on the S156C mutation and so cannot necessarily be assumed to apply to all SFD mutations. In addition, while the authors state that there is no difference in turnover between the wild-type and the mutant protein, this conclusion is based on the turnover of these proteins over a shorter time period than that used here (48 versus 96 h). In fact, their published data do actually appear to show more TIMP-3 immunoreactive material in ECM from S156C/S156C cells at both 24 and 48 h than that in the wild-type cells. A crude densitometric analysis of these bands reveals ~5% of the wild-type protein remaining at 48 h, whereas >20% of the S156C protein remains at the same time point, closely paralleling our observations. Continuation of their study over a longer time period may well have revealed a more significant difference between the turnover of these molecules.

There are several possible mechanisms by which TIMP-3 accumulation could then give rise to the symptoms of SFD. Increased TIMP-3 concentrations may directly trigger apoptosis of RPE cells (14) or lead to their loss by impairing cell attachment to the ECM (25,41). These events could in turn permit neo-vascularization through loss of the RPE-mediated attachment to the ECM (25,41). An alternative hypothesis is that TIMP-3 accumulation inhibits transport across Bruch’s membrane, either by inhibiting normal turnover of the ECM as a whole or by providing a direct physical barrier in itself. Such a barrier would impair retinal nutrition, leading to atrophy of the RPE and photoreceptor death. Neo-vascularization would then be a secondary response to retinal malnutrition and hypoxia.

Aberrant accumulation of another ECM protein, fibulin 3, also occurs in a different autosomal dominant macular degenerative disease (Malattia Leventinese) and AMD (43). Recent evidence suggests that fibulin 3 and TIMP-3 are binding partners (44), leading these authors to conclude that accumulation of fibulin 3/TIMP-3 complexes acts as a physical barrier to retinal nutrition. If this is the case, then the apparent loss of functionality of S156C TIMP-3 observed by Soboleva et al. (40) may not be of consequence in disease progression.

The question remains as to whether TIMP-3 could also play an active role in AMD, a disease that bears a striking resemblance to SFD and which also displays increased deposition of TIMP-3 protein in Bruch’s membrane and drusen (27). Recent work has demonstrated a strong association between complement factor H polymorphisms and AMD, implicating complement-mediated inflammation in disease progression (reviewed in 45). This association was, at least in part, highlighted by the observation that patients with membranoproliferative glomerulonephritis type-II, a rare kidney disease caused by uncontrolled activation of the alternative pathway of complement, often develop ocular drusen in the macula. In addition to complement components, these drusen also contain significant quantities of TIMP-3 (46). Increased deposition of TIMP-3 is also observed in a dominant form of retinitis pigmentosa having sub-RPE deposits resembling those in SFD (26) and which is caused by mutations in genes other than TIMP-3. It could well be argued, therefore, that TIMP-3 accumulates wherever Bruch’s membrane thickening or drusen occurs, presumably as a result of its tendency to bind ECM components.

However, SFD clearly demonstrates that abnormalities in TIMP-3 biology itself can cause macular degeneration. A further possibility, then, is that deposits of TIMP-3 caused by the Sorsby’s mutations trigger an inflammatory response and disease progression. Nevertheless, the possibility that increased levels of TIMP-3 in AMD also play an active role in disease progression cannot be ruled out. Given that the expression of TIMP-3 mRNA does not increase with age or Bruch’s membrane thickening (30), the increased TIMP-3 content of AMD retinas is also very likely to be the result of an accumulation of TIMP-3 protein (27). Cross-linked TIMP-3, apparently resulting from oxidation reactions, has been detected in drusen from both normal and AMD retinas (47). Thus, cross-linked TIMP-3 aggregates are a feature of both SFD and AMD and could possibly underlie the accumulation of TIMP-3 in the latter disease. Given the obvious potency of this phenomenon in SFD, it might also be an important stage in the development of AMD. Indeed, cumulative oxidative stress has been proposed to contribute to AMD (48). Disulfide-bonded TIMP-3 dimers have also been isolated from Bruch’s membrane of a normal retina (15), suggesting that wild-type TIMP-3 may spontaneously mis-fold in a similar way to SFD-TIMP-3 proteins. This phenomenon may be increased in aging where dysregulation of protein folding pathways in the endoplasmic reticulum environment is thought to occur (49,50). It is also worth noting that in AMD patients with choroidal neo-vascularization, linkage analysis has revealed strong evidence for a susceptibility locus on chromosome 22q (51), a region containing the TIMP-3 gene.

In conclusion, SFD is caused by mutations in the TIMP-3 gene that result in the accumulation of a turnover-resistant fraction of the TIMP-3 protein. Therapies directed at preventing or reversing this accumulation may be of benefit in treating these patients and could potentially be more widely applicable in AMD.

**MATERIALS AND METHODS**

**Cell culture**

The well differentiated, spontaneously arising RPE cell line ARPE-19 was obtained from the American Type Culture Collection (35). Cells were maintained as described previously (35), except that 50 μg/ml gentamicin (Invitrogen) was used as an anti-microbial agent.

**Plasmids and transfection**

The construction of plasmids pCITIMP-3, AlaTIMP-3, TIMP-3E139X, S156C, S181C and N-TIMP-3 has been described previously (9,24,52). Plasmids pCDNA6TIMP-3His and pCDNA6AlaTIMP-3His were generated by PCR amplification of either the full TIMP-3 or AlaTIMP-3 ORFs whose stop codons were replaced by an AgeI restriction site. These molecules were then ligated into vector pCDNA6V5His(a)
(Invitrogen) between its BamHI and AgeI sites. This results in the removal of the V5 peptide tag present in this vector and fuses TIMP-3 and AlaTIMP-3 to a C-terminal tag of TGHHHHHH. Plasmids were prepared using Qiagen midiprep columns. Transfections were performed using FuGENE-6 (Roche) in six-well plates, according to manufacturer’s instructions. After 48 h, cells were transferred to 100 mm culture dishes and subjected to selection with either 500 µg/ml G418 (for pCI-based plasmids) or 4 µg/ml blasticidin (Invitrogen) (for pCDNA6 plasmids). Resistant cells were expanded for experimentation. Control cells were generated as above by transfection with empty pCIneo vector.

Analysis of expression of wild-type and mutant TIMP-3

Detection of recombinant proteins was performed as described before (9), except that heparin–agarose resin (Sigma, UK) was used to concentrate wild-type and mutant TIMP-3 from conditioned media (52,53).

Metabolism of ECM-bound TIMP-3 and mutants

Cells were plated onto 100 mm dishes at ~20% confluence (five or six dishes of each transfectant) and grown to confluence. Heparin–agarose concentrated conditioned media and ECM samples were then prepared from one dish (day 0 samples) and the cells removed from the remaining dishes according to Fischer et al. (54). Freshly trypsinized, untransfected ARPE-19 cells were then plated onto these dishes at or near to confluence. Heparin–agarose concentrated conditioned media and ECM samples were then prepared at daily intervals for 4 and, in some experiments, 7 days (day 1, 2, 3, 4 and 7 samples). Protein content of the samples was determined by BCA analysis (Pierce). Equal amounts of protein were then analyzed by western blotting under reducing and non-reducing conditions. Relative band intensities were quantified using NIH-Image.

Analysis of MMP-2 expression and activation

Cells were grown to confluence in six-well plates and then transferred to serum free medium, either with or without 25 µg/ml Con A (Sigma), for 48 h. Conditioned media and cell lysates were prepared and analyzed by gelatin zymography (38). Zymograms were then dried and scanned, and relative band intensities were determined as mentioned earlier.

Reverse zymography

Protease/substrate gel electrophoresis was performed as described before (9), except that 0.25 ng/ml proMMP-9 purified from insect cells was used as a source of MMP activity.

Western blotting

Immunological detection was performed as described before (24), except that Supersignal Femto maximum sensitivity substrate (Pierce) was used to detect bound antibodies.

Data analysis

Data were plotted and analyzed using Prism (GraphPad) software. MMP levels were compared using one-way ANOVA with Tuks post-test. Half-lives were calculated, using non-linear regression, by fitting the data to one-phase exponential decay equations.

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