TIMP3 mutation in Sorsby's fundus dystrophy: molecular insights

Zheng Li, Michael P. Clarke, Michael D. Barker and Norman McKie

Sorsby's fundus dystrophy (SFD) is a rare autosomal dominant disorder that results in degeneration of the macular region of the retina, with onset usually in the fourth to fifth decade of life. It leads to the rapid loss of central vision, often followed by further loss of peripheral vision. SFD shares several pathological features commonly found in the 'wet' or exudative form of age-related macular degeneration (AMD), the most common cause of blindness in the elderly in developed countries. These phenotypic similarities have led to SFD being proposed as an acceptable genetic model for AMD. Whereas AMD appears to have a complex aetiology, with both genetic and environmental factors playing a role, SFD has been shown to be a single-gene disorder, linked to mutations in exon 5 of the tissue inhibitor of metalloproteinases 3 (*TIMP3*) gene on chromosome 22q12-q13. This review confines itself to a discussion of the known biochemical properties of the wild-type and SFD TIMP3 proteins and attempts to relate these to the pathology encountered in SFD patients. We also discuss briefly how, despite the lack of inherited mutations in the structural gene, the TIMP3 protein might play a role in the onset and progression of AMD.

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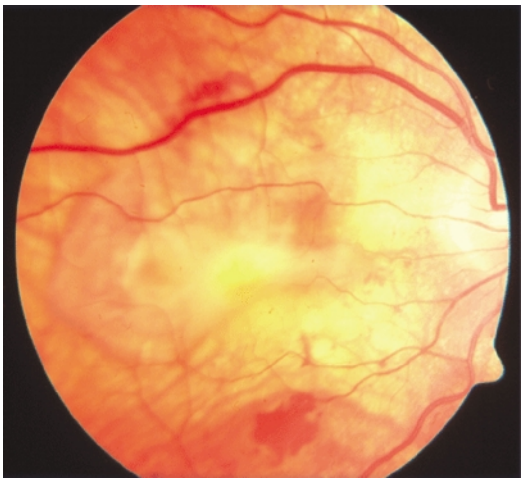
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Sorsby's fundus dystrophy (SFD) is a rare autosomal dominant condition that clinically manifests itself as a late-onset loss of central vision due to degeneration of the macula (the central region of the retina) (Refs 1, 2). The age of onset of SFD can vary considerably within families but typically ranges from the third up to the seventh decade with the vast majority of cases occurring around the beginning of the fifth decade (Ref. 3). Individuals with SFD usually present in clinic because of rapidly evolving loss of central vision in one eye, but often describe a history of increasing night blindness (nyctalopia) over several years. Loss of central vision in one eye is followed, within highly variable time intervals, by loss of central vision in the fellow eye, leading to severe visual impairment. Subsequent deterioration of remaining visual function is common.

SFD has been shown to be a single-gene disorder, linked to mutations in exon 5 of the tissue inhibitor of metalloproteinases 3 (*TIMP3*) gene on chromosome 22q12-q13 (Ref. 2). SFD appears to be fully penetrant in all families studied but can vary considerably in phenotype, both within and between family members. This article reviews the biochemical properties of the wild-type and SFD TIMP3 proteins and attempts to relate these to the pathology encountered in SFD patients.

Clinical features of SFD

The macula is responsible for high-resolution vision, and has distinct anatomical and physiological properties. The retinal pigment epithelium (RPE) is supported by a specialised multilaminar basement membrane known as Bruch's membrane, which separates the RPE from the underlying blood supply (the choroid). The main histopathological features of SFD are an increased deposition of proteinaceous and lipid material in Bruch's membrane (these extracellular deposits are known as drusen), and a disciform macular scar with associated haemorrhage is usually visible during fundus examination of affected individuals (Ref. 4) (Fig. 1). This potentially leads to impaired transport of essential nutrients and metabolites from the choroid, resulting in RPE cell atrophy and cell death. New blood-vessel growth through breaks in Bruch's membrane can also result in leakage of blood and the initiation of aberrant matrix repair processes, resulting in scarring and further macular damage.



Features of the retinal defect in Sorsby's fundus dystrophy

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Figure 1. Features of the retinal defect in Sorsby's fundus dystrophy. A typical fundus photograph of the eye from a patient with Sorsby's fundus dystrophy, showing the large central scotoma (scar tissue) in the macular (central) region of the retina.

Although the majority of cases of SFD lose central vision as a result of neovascular (wet-type) macular degeneration, different manifestations of the phenotype are seen and the disease can also be atrophic (dry) or show geographic macular atrophy as a prominent feature (Ref. 4).

The inheritance of mutations in the coding sequence of *TIMP3* has been demonstrated to segregate with SFD in all known cases (Ref. 5). In a few families, other pathological conditions have been seen to segregate with SFD, such as myotonic dystrophy and a severe form of periodontal disease. However, it is notable that no consistent associations have been found between SFD and nonocular pathology; in fact, our own clinical experience of SFD patients is that, apart from their vision, their health remains excellent into old age.

Phenotypic similarities to age-related macular degeneration

Interest in SFD research has grown considerably over the past decade because of phenotypic similarities between this rare condition and the much more common age-related macular degeneration (AMD) (Ref. 6). These similarities

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include subretinal neovascularisation, photoreceptor cell death, the formation of drusen, thickening of Bruch's membrane and structural disruption of the RPE. These age-related changes can lead to visual impairment with accompanying degeneration of the retina, at which stage it is referred to as AMD. It is defined as a degeneration of the macula after 50 years of age that cannot be attributed to an obvious systemic or ocular cause. It occurs in two forms: geographic atrophy (atrophy of the RPE and photoreceptors) and neovascular (scarring resulting from growth of new blood vessels and accompanying fibrous tissue through Bruch's membrane). Although there are several inherited diseases that cause macular dysfunction and progressive macular degeneration [e.g. Stargardt's disease, Best's disease and X-linked juvenile retinoschisis (XLRS)], these conditions are usually easily distinguishable from AMD on the basis of their morphology. However, SFD and autosomal dominant drusen (Malattia leventinese) can mimic the clinical findings in both forms of AMD and are primarily distinguished from AMD on the basis of age of presentation and family history. AMD is currently the leading cause of registering as blind in the elderly of developed countries and is emerging as a significant public health problem, heavily compromising a patient's quality of life and increasingly proving to be a significant burden on the healthcare and support services (Ref. 7).

The structure and function of TIMPs

TIMP3 is a member of a family of four proteins (TIMP1, -2, -3 and -4) that are the natural inhibitors of matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases that exist in both secreted and membrane-bound forms. TIMPs have been fully reviewed recently and a large and comprehensive literature is available (Ref. 8 and citations therein). The balance between MMPs and TIMPs regulates remodelling of the extracellular matrix (ECM) and thus plays a key role in a wide range of physiological processes, including embryonic development, connective tissue remodelling, wound healing, glandular morphogenesis and angiogenesis. An imbalance in MMP activity has been implicated in pathological conditions such as cardiovascular disease, arthritis and cancer.

Structurally, TIMPs can be divided into two broad domains, each comprising three disulphide-bonded loops. The more closely conserved N-terminal domain has been shown to be sufficient for MMP inhibition, whereas the C-terminal domains are more divergent and appear to be important in imparting individual differences in specificity to each TIMP family member. These differences in functionality, together with their unique patterns of expression and regulation, strongly support the thesis that each TIMP family member plays a unique physiological role in addition to the common function of metalloproteinase inhibition.

Although the activities of the TIMP family members show a large overlap with respect to inhibition of MMPs, clear distinctions are emerging. TIMP1, as an example, is a poor inhibitor of the membrane-bound [membrane-type (MT)] MMPs (Ref. 9). TIMP3, in particular, is capable of inhibiting additional enzymes from different subgroups within the metalloproteinase clan, as discussed in detail in the next section. As well as binding to active metalloproteinases, all of the TIMPs show selective binding to the inactive (pro-) forms of the gelatinases through their C-terminal domains: TIMP1 binds pro-MMP9; TIMP2 and TIMP4 (Ref. 10) bind pro-MMP2; and TIMP3 binds both proenzymes. Paradoxically, by binding both pro-MMP2 (via its C-terminal domain) and MT1-MMP (via its N-terminal domain), TIMP2 plays a major role in the activation of pro-MMP2: by recruiting it to MT1-MMP on the cell surface it is then activated by a second MT1-MMP molecule (Ref. 11). TIMP3 is also able to activate pro-MMP2 (and therefore possibly pro-MMP9) in a similar manner through MT3-MMP (Ref. 12). A role for TIMP1 and TIMP4 in proenzyme activation has not been demonstrated. Whereas TIMP4 is able to bind both MT1- and MT3-MMP, binding does not result in activation of bound pro-MMP2 (Ref. 13). Although extracellular metalloproteinases might be their primary target, other activities have also been ascribed to the TIMPs and these are commonly related to roles in angiogenesis; this is addressed later in the article with respect to TIMP3, but TIMP1 and TIMP2 may also play a significant role (Refs 14, 15).

TIMP3 as a special case within the TIMP family

In evolutionary terms, mammalian *TIMP3* is believed to be the most conserved of all the *TIMP* genes, and functional evidence for this has been provided by a comparative study on

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Drosophila melanogaster TIMP protein (Ref. 16). In vertebrates, the retina and kidney appear to be the major sites of TIMP3 expression during development and therefore would be expected to be sites affected by inherited mutation (Ref. 17). Several points relating to the cell biological or biochemical properties of TIMP3 are discussed here that distinguish it from the other TIMP proteins. It is important to consider whether any of these properties might be relevant to the role TIMP3 plays in the very specific genesis of SFD pathology in humans.

TIMP3 is the only member of the TIMP family that localises to the ECM and it binds particularly well to sulphated glycosaminoglycan (GAG) molecules (Ref. 18), which are a common constituent of many ECMs including that of the retina. Although binding of TIMP3 to purified sulphated GAGs in vitro appears to be largely mediated through the N-terminus, binding of TIMP3 to natural ECM produced by cells is largely mediated by its C-terminal region (Refs 19, 20), which in turn is the site of all known SFD mutations. Some aspects of ECM binding are addressed later in this review with regard to the TIMP3 protein in SFD, as there are indications of alterations in this property for mutant TIMP3. In addition, it has been documented that *TIMP3* is controlled at the transcriptional level by epigenetic means such as promoter methylation and this has significance for its role, particularly in tumour biology (Ref. 21). To date, this gene silencing of *TIMP3* has not been addressed in the retina.

Overexpression of TIMP3 triggers apoptosis in several cell types, including RPE cells (Ref. 22). Whether such levels of TIMP3 occur under normal physiological conditions is uncertain; however, it could be particularly relevant to SFD or AMD pathology if, as believed, TIMP3 concentrations in Bruch's membrane increase over time (Refs 23, 24, 25). Induction of apoptosis appears to be an MMP-dependent property of TIMP3 (Ref. 26) and it is clear that, in the AMD retina at least, a large portion of the TIMP3 remains in an active form (Ref. 27). Aggregates of TIMP3 protein have also been detected in drusen but no data on inhibitory activity have yet been reported (Ref. 28). TIMP3 also appears to play a role in inhibiting the proteolytic shedding of several different membrane protein ectodomains (Ref. 29). Since shedding of the FAS ligand has been shown to be particularly relevant to retinal neovascularisation, the role of TIMP3 as a shedding enzyme inhibitor and the effect of SFD mutations on the protein might be pivotal in this tissue (Ref. 30).

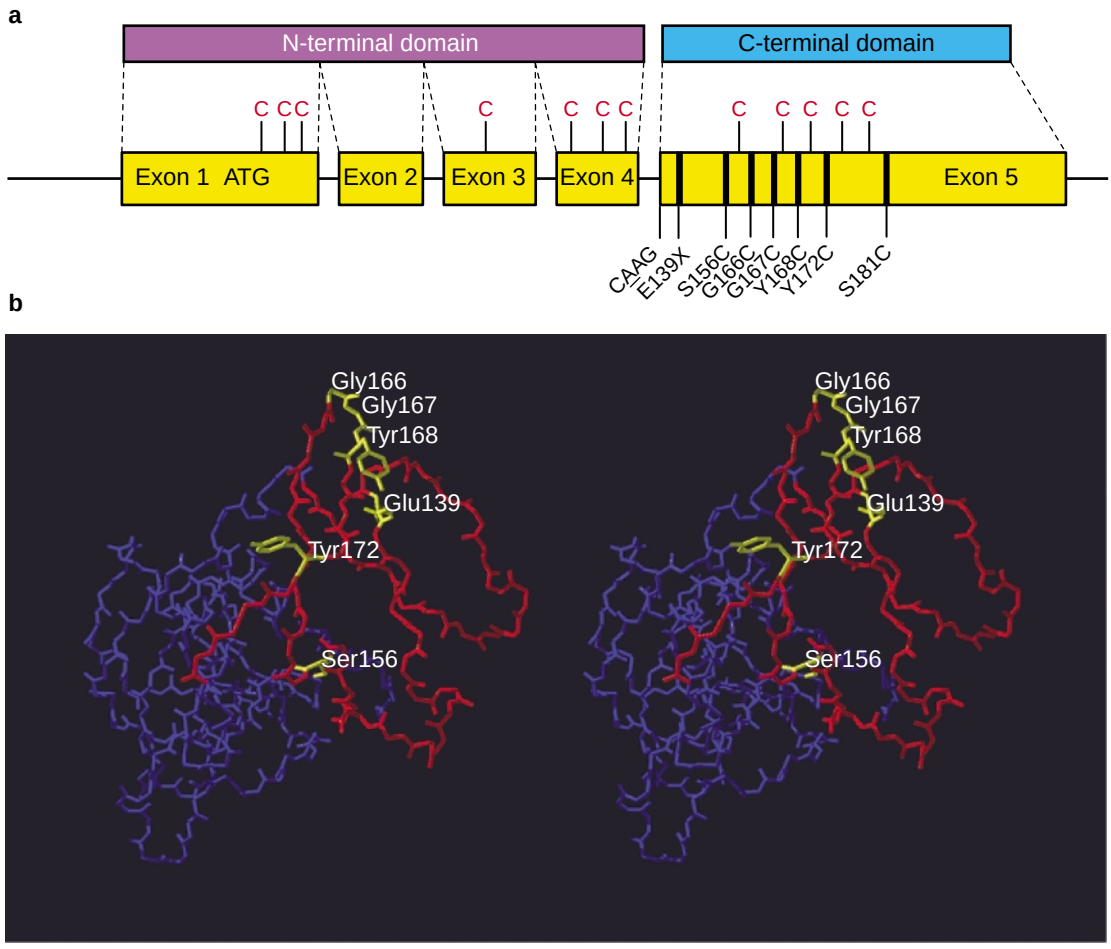
As mentioned earlier, TIMP3 has a broader inhibitory activity than the other TIMP family members, and its inhibition of ectodomain shedding appears to be a result of the ability of the molecule to inhibit certain ADAM (for 'a disintegrin and a metalloproteinase') proteinases. For example, TIMP3 inhibits the tumour necrosis factor α (TNF-α)-converting enzyme (TACE or ADAM17) and the related enzyme ADAM10 in vitro (Ref. 31). These enzymes are membrane-bound metalloproteinases that have active-site residues characteristic of the MMPs but little else in common in terms of primary structure (Ref. 32). The ability of TIMP3 to inhibit TACE also appears to play an important anti-inflammatory role in vivo (Ref. 33), which might have functional significance for disease states such as SFD or AMD. A closely related metalloproteinase family to the ADAM type, termed ADAMTS (for 'a disintegrin and a metalloproteinase with thrombospondin motifs'), also has several members that are sensitive to inhibition by TIMP3. Notably, these are the proteoglycan-degrading members ADAMTS1, ADAMTS4 and ADAMTS5, which are being investigated in considerable detail as to their role in the turnover of cartilage ECM (Ref. 34). TIMP3, probably above all other TIMPs, thus has a considerable potential to control ECM turnover wherever it is expressed with respect to several different substrates such as proteoglycans, structural collagens and several basement membrane components. It has a discrete expression profile in the retina and choroidal tissues and might act to protect different ECM proteins in this crucial structural and functional region in the eye (Ref. 35).

TIMP3 mutations causing SFD

Progress in understanding the disease mechanism of SFD was initiated by the discovery that the mutations segregating with SFD were located in the gene encoding TIMP3 (Ref. 36). The authors suggested that the observed pathology could be due to the mutations in *TIMP3* leading to dysregulation of metalloproteinase activity. This mechanistic role as far as MMPs are concerned seems unlikely (see below), but control of tissue turnover by the mutant TIMP3 has yet to be appropriately analysed because of the large number of potential candidate proteinases for which TIMP3 is a known inhibitor.

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Figure 2. *TIMP3* mutations in Sorsby's fundus dystrophy. (a) The exon–intron structure of the tissue inhibitor of metalloproteinases 3 (*TIMP3*) gene showing the Sorsby's fundus dystrophy (SFD) mutations in the C-terminus as black lines, and cysteine residues (C). Exons 1–4 encode the N-terminal domain and exon 5 alone encodes the entire C-terminal domain of TIMP-3. The splice acceptor site mutation is indicated at the end of the intron between exons 4 and 5. (b) Stereo structural representation of TIMP3, produced by modelling TIMP3 on the crystal structure of TIMP2 using SwissModel and then rendering the image using POV-Ray ray tracer. The residues affected by mutation are shown in yellow and are labelled.

To date, geneticists have discovered eight independent *TIMP3* mutations resulting in SFD. Seven of these affect the *TIMP3* coding sequence in exon 5 and one lies in the intron between exon 4 and exon 5 (Fig. 2a). Six of the eight mutations convert a serine, tyrosine or a glycine residue in the C-terminus to cysteine. These are located at the following sites: S156C (Ref. 37), G166C (Ref. 38), G167C (Ref. 39), Y168C (Ref. 38), Y172C (Ref. 40), and S181C (Ref. 36). In each of these cases, the result of the mutation on the protein is the addition of an unpaired cysteine residue in the C-terminus. The seventh mutation introduces a stop codon at position 139 (E139X) in exon 5 (Ref. 41). The position of this stop codon at residue 139 in the *TIMP3* gene also results in an odd number of cysteine residues in the C-terminus of the corresponding protein. It has been shown that this C-terminally truncated allele is expressed in patient cells (Ref. 42). The eighth mutation is also likely to affect exon 5 but does so by the introduction of a single base (A) in the splice

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acceptor site at the junction of exon 4 and exon 5 (CAG to C**A**AG; splice acceptor site underlined) in a Japanese pedigree (Ref. 43).

As already mentioned, variations in the clinical presentation of SFD are frequently seen and range quite widely across different families. The age of onset of pathology varies according to the type of mutation in the pedigree, with some S156C and G166C mutations presenting earliest (second decade) and the splice acceptor mutation presenting latest (sixth decade). Most of the others cluster around the end of the fourth and beginning of the fifth decade. The S156C and G166C mutations show prominent neovascularisation in some family members and the splice acceptor mutation causes a phenotypically distinct SFD with normal peripheral retina and absence of nyctalopia (Ref. 44).

It is not yet known what the consequences are for the TIMP3 protein in the Japanese pedigree. Splice-site mutations can in some cases lead to total loss of functional mRNA. In SFD, this seems an unlikely mechanism, because of the dominant mode of inheritance of pathology (i.e. the mutant protein has pathology-associated functionality) and because of the observation that TIMP3-knockout mice do not exhibit an obvious retinal phenotype (Ref. 45). These observations support an active role for the mutant SFD TIMP3 protein. It is possible, however, that this splice-site mutation results in a decrease in the amount of TIMP3 mutant protein produced, giving rise to the delayed onset of the disease in these patients. Whereas splice-site mutations can lead to exon skipping, this normally occurs when the mutation affects an internal exon, and so is unlikely in this case where the last exon in the *TIMP3* gene is affected. In an alternative scenario, the aberrant retention of intron 4 owing to read through of the 5' splice donor site would result in an unpaired cysteine residue in the encoded protein. Such a mutant protein would also lose the normal ECM-binding domain in the C-terminus, providing an alternative explanation for the delayed onset of the disease in this family. Another possibility is the activation of a cryptic splice site in exon 5; however, this cannot be properly identified without a full and detailed analysis of the splicing pattern in the patients concerned. Since the N-terminus of TIMP3 is unlikely to be affected in these patients, any protein produced probably still functions as an active proteinase inhibitor.

In the C-terminus of TIMP3, three of the SFD mutations are in consecutive residues (numbers 166, 167 and 168) and two others are close by (172 and 181), suggesting a region of the *TIMP3* gene that might be predisposed to mutation. Mutations in this region may affect TIMP3–MMP or other TIMP3 protein interactions and thus contribute to the SFD disease phenotype (Fig. 2). The E139X mutation, which is predicted to occupy a space close to the other affected residues, would result in the total loss of these C-terminal residues and so might be predicted to give rise to a similar phenotype to the main cluster of affected residues by deletion rather than by alteration of the C-terminal domain tertiary structure. It is also noteworthy that the chemically more-reactive thiol group in cysteine residues substitutes in the mutations for the less-reactive hydroxyl group in both serine and tyrosine residues and the unreactive group of glycine in all known cases to date. The substitution of these residues for cysteine would increase the susceptibility of the TIMP3 protein to oxidative crosslinking and perhaps further oxidative damage involving amino acid residues juxtaposed by this oligomerisation.

Although loss of function for the TIMP3 protein in SFD cannot be ruled out entirely, studies to date indicate that the two most likely scenarios for the effect of *TIMP3* mutations on pathology result from either a gain of function for the mutant protein or from a dominant-negative effect where the mutant TIMP3 interferes with the functioning of TIMP3 encoded by the normal allele. These are considered later in this review.

The biochemical properties of SFD TIMP3 proteins

The biochemical properties of SFD TIMP3 proteins S156C, G166C, G167C, Y168C and S181C have been studied using the simian fibroblast cell line COS-7 (Refs 19, 41) and baby hamster kidney (BHK) cells (Ref. 46). In addition, S156C TIMP3 has been characterised in the human RPE cell line ARPE-19 (Ref. 47). In the COS-7 and BHK cells, the SFD TIMP3 proteins produced were found, like the wild-type protein, to be active metalloproteinase inhibitors that localised to the ECM. However, in all cases, a significant proportion of the mutant protein formed disulphide-bonded dimers and higher molecular weight aggregates. Expression of the E139X TIMP3 protein in COS-7 cells also resulted in the formation of ECM-localised dimers (Ref. 41) that, when expressed in insect cells,

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retained inhibitory activity against MMP2 (Ref. 42). This led to the proposal of a common molecular phenotype for SFD TIMP3 proteins in which dimerisation and retention of inhibitory activity were unifying features (Ref. 19). However, in contrast to these data, S156C TIMP3 expressed by the RPE cell line ARPE-19 did not dimerise and showed no inhibitory activity (Ref. 47). Furthermore, expression of the S156C SFD TIMP3 protein in this cell type was demonstrated to increase secretion and activation of pro-MMP2 and pro-MMP9 and thus to increase the potential for matrix turnover, which in vivo might influence angiogenesis. This was an interesting finding since it was the first study on an SFD TIMP3 protein in a retinal cell type and opened up the possibility that the SFD TIMP3 protein behaved differently in these cells (arguably, these cells are closer in phenotype, in a developmental sense, to normal RPE cells). The discrepancy between the RPE-derived data and the other published findings requires further evaluation.

It might be that S156C TIMP3 has a lower level of expression in the ARPE-19 cell line and thus the S156C dimer was not visualised using the zymography techniques employed. However, the monomeric form retains inhibitory activity in two other cell types as discussed above, and it is difficult to explain why this protein should appear to lose activity solely in an RPE cell type. It could be argued that expression of inhibitory, multimerised SFD TIMP3 protein is an artefact of heterologous overexpression in COS-7 and BHK cells; however, E139X, S156C and S181C proteins expressed from their normal promoter context by fibroblasts from SFD patients retain these properties (Refs 42, 48). Likewise, the possibility that these contrasting results are due to retinal expression is not borne out by 'knock-in' studies in transgenic mice where S156C TIMP3 from the RPE in vivo also forms inhibitory multimers (Ref. 49). An alternative explanation for these contrasting results may be the fact that the S156C TIMP3 expressed by Anand-Apte and colleagues (Ref. 47) had a C-terminal epitope tag that could conceivably affect multimerisation or activity. These RPE cell data are further contradicted by Edwards and colleagues, who assayed the S156C TIMP3 protein and showed retention of inhibitory activity (Ref. 46).

The possibility that overexpression of S156C TIMP3 induces metalloproteinase activity, which, as a cellular phenotype would certainly be pro-angiogenic, is counterintuitive with respect to microscopic findings relating to Bruch's membrane. Bruch's membrane is known to thicken with ECM deposits in both SFD and AMD (Refs 23, 27), which is difficult to reconcile with increased proteolytic activity induced as a consequence of the expression of a mutant SFD *TIMP3* allele. Furthermore, this increased proteolytic activity was not observed in fibroblasts from SFD patients heterozygous for the S181C and the E139X mutations in SFD *TIMP3* genes (Refs 42, 48). Neovascularisation is a prominent and early-onset feature in the S156C SFD pedigree (Ref. 38) that may warrant some explanation. It is possible that the differences seen reflect the different genetic background of the families concerned, rather than functional differences in the mutant protein itself. Consanguineous marriages in this pedigree might lead to an increased possibility of a recessive allele exerting a modulatory effect on the mutant *TIMP3* allele in relation to angiogenesis. The SFD family G166C also has some members with prominent retinal neovascularisation and evidence for consanguinity in the pedigree. Repetition of the RPE cell expression experiments is currently under way in our laboratory in an attempt to clear up this apparent discrepancy.

What certainly appears to remain a common feature from the vast majority of the published data is that SFD TIMP3 proteins form matrix-associated dimeric and higher oligomeric forms that retain inhibitory activity against a variety of MMP enzymes.

Implications of SFD TIMP3 biochemistry for retinal pathology?

From a pathological perspective, the accumulation of extracellular debris in the SFD retina intuitively tells us that the SFD TIMP3 protein is likely to be inhibiting extracellular proteinases responsible for protein turnover in this compartment. This has to be qualified, since the inhibitory activity of SFD TIMP3 was largely determined by reverse zymography on gelatin gels where the predominant proteinases in such assays were MMP2 or MMP9. Other studies have shown that the S156C TIMP3 protein retains activity against MMP3 and MMP13 (Ref. 46) in in vitro assay systems. A major direction for future studies will be to determine whether or not inhibitory activity of SFD TIMP3 proteins is retained for ADAM or ADAMTS family members

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also controlled by wild-type TIMP3. For example, several of the ADAMTS family members are also expressed in the RPE cell type ARPE-19 (Ref. 50), and some of the enzymes detected in this work are known to be involved in angiogenesis. ADAMTS1, in particular, can be inhibited by TIMP3 (Ref. 51) and has been demonstrated to have anti-angiogenic properties. If the mutant TIMP3 has altered inhibitory properties towards ADAMTS1 or other metalloproteinase(s) involved in new vessel formation, then downstream effects on subretinal neovascularisation might be a consequence. The matrix accumulation seen in SFD might thus be the result of increased inhibition towards some particular group or groups of enzymes intimately involved in retinal ECM turnover, which might include members of the ADAM, ADAMTS and MMP families of proteinases. Whereas binding to most soluble MMPs is in any case essentially irreversible, multimerised TIMP3 might have unforeseen effects on membrane-bound enzymes such as TACE or MT-MMPs, which could result in crosslinking. Clearly, in a dynamic proteinase activation cascade, minor alterations of the kinetic properties of individual proteinases by an inhibitor might have profound effects on ECM homeostasis.

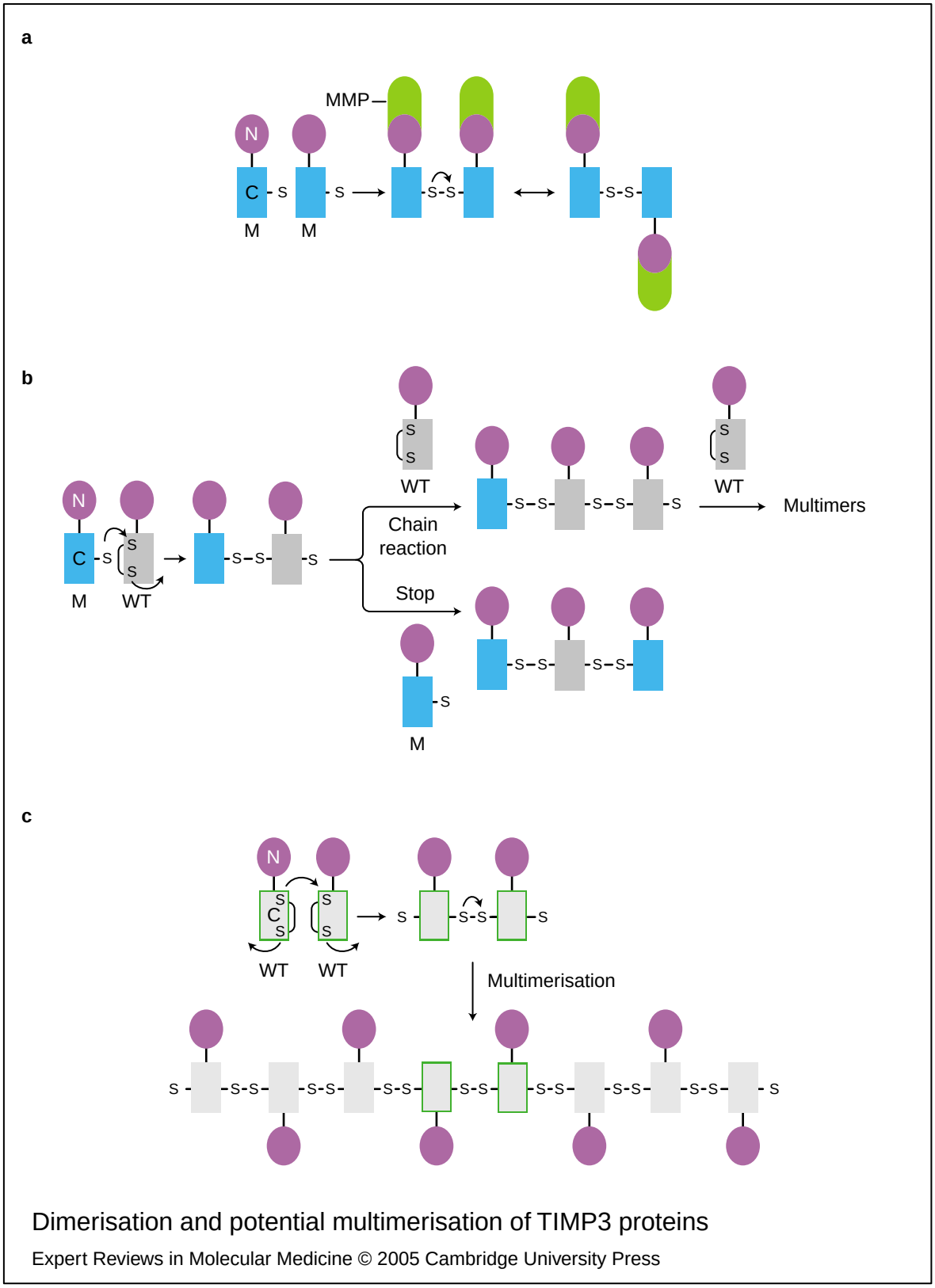
A further biochemical possibility relating to pathology in the retina is that the mutations in SFD TIMP3 might affect the binding of the mutant protein to a non-MMP target. One case that might be relevant is the reported binding of wild-type TIMP3 to vascular endothelial growth factor receptor 2 (VEGFR2; Ref. 52). If the VEGFR2-binding site of TIMP3 resides in or close to the C-terminus, then different SFD mutations in this domain might show variable loss of inhibitory activity towards the receptor. Although, for reasons already outlined, loss of functionality is unlikely to be the cause of the disease, it is conceivable that different SFD mutations might show different degrees of inhibitory activity towards this receptor, perhaps accounting for the differences in neovascularisation observed between the different SFD genotypes. Alternatively, if the mutant protein exhibited an increased affinity or avidity (as a result of multimerisation) for the ECM, this could explain the increased deposition of the protein in Bruch's membrane. Edwards and co-workers have already demonstrated an increased adhesiveness for the ECM of an SFD TIMP3 protein expressed in BHK cells (Ref. 46).

The disease phenotype of SFD could also arise because the mutant protein interferes with the functioning of the wild-type protein, the situation encountered with dominant-negative mutations (Refs 53, 54). It is clearly the case that SFD TIMP3 forms disulphide-linked dimers (Ref. 18) (Fig. 3a). It is also feasible that the unpaired cysteine residue in SFD TIMP3 could initiate nucleophilic attack on a wild-type TIMP3 molecule, leading to the formation of a heterodimer that may in turn be the pathological form of the TIMP3 molecule in SFD (Fig. 3b). Such an event has been observed for mutant forms of the epidermal growth factor receptor, although in this case there was no covalent crosslinking through cysteine (Ref. 53). Supporting this hypothesis for TIMP3 is the fact that TIMP3 aggregates in SFD are also commonly seen at higher molecular weight than would be expected for the dimeric species (Ref. 41), indicating that multimerisation is taking place. This multimerisation is unlikely to be due to two independent mutant proteins binding through

Figure 3. Dimerisation and potential multimerisation of TIMP3 proteins. *(Legend; see next page for figure.)* (a) The interaction of two Sorsby's fundus dystrophy (SFD) tissue inhibitor of metalloproteinases 3 (TIMP3) proteins (labelled M for mutated; N and C indicate the N- and C-terminal domains of the protein, respectively). The proteins would be expected to dimerise through their unpaired cysteine residue, creating a disulphide-linked dimer. The S–S bond formed will have a certain amount of conformational flexibility, as indicated by the arrow. The size of the matrix metalloproteinase (MMP) bound is likely to influence the positioning of the TIMP3 N-termini with respect to each other in the dimer as a result of steric hinderance effects. (b) The interaction between an SFD TIMP3 protein and a wild-type (WT) TIMP3 protein. The initial interaction will produce a dimeric species but, in contrast to (a), the dimeric species would have a further unpaired cysteine residue due to nucleophilic attack across a disulphide bond in the C-terminus of the wild-type protein. The bifurcation of the pathway indicates the possible events when the M–WT dimer encounters either another SFD TIMP3, resulting in formation of a stable heterotrimer, or another WT molecule, which can result in iterative condensation. (c) The interaction between two WT TIMP3 molecules. These can interact to form a homodimer, although this would be energetically much less favourable than is the case with the SFD mutant. Multimerisation of WT TIMP3 might respond to redox balance in the retina as a patient ages, and might require catalysis by an oxidoreductase enzyme.

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Dimerisation and potential multimerisation of TIMP3 proteins

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Figure 3. Dimerisationand potential multimerisation of TIMP3 proteins. *(See previous page for legend.)*

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their unpaired cysteine residues since that reaction would be likely to be self-limiting (Fig. 3a). By contrast, if a mutant–wild-type TIMP3 dimer were formed, this would leave a free cysteine residue that could either bind a second mutant protein, leading to a stable heterotrimer, or a second wild-type protein, resulting in another free cysteine and the formation of a tetramer. This latter condensation process could be repeated indefinitely, giving rise to increasingly large TIMP3 complexes (Fig. 3b). The SFD TIMP3 protein thus initiates this condensation process and 'catalyses' the formation of larger aggregates. If such complexes have a reduced propensity for degradation then this would lead to a build up of a large inhibitory protein complex with multiple active sites that would act as a 'sink' for active metalloproteinases. This, in turn, could lead to loss of proteolytic activity in Bruch's membrane and thus to reduced ECM turnover and thickening of this membrane, as seen in both SFD and AMD. This thickening would in turn have 'knock-on' effects on metabolic exchange, potentially leading to retinal cell malnutrition and cell death. Increased concentrations of inhibitory TIMP3 in Bruch's membrane might also contribute to photoreceptor death by induction of apoptosis. The resultant hypoxia and necrosis in the RPE would be potent activators of angiogenesis, which would then be a secondary event in the disease process.

It should be noted that if the above hypothesis is true, then it could be argued that the heterozygous $TIMP3^{wild\text{-}type/S156C}$ 'knock-in mouse' described by Weber and colleagues (Ref. 49) would be more likely to exhibit symptoms of the disease than the homozygous mutant ($TIMP3^{S156C/S156C}$), which is not apparently the case in the mice studied to date. However, since neither of these transgenic animals demonstrates changes in the retina comparable with the human disease, this does not negate this hypothesis for higher mammals. Lack of distinct macular degeneration in mice is an obvious correlate of the fact that mice do not have a macula, and other, less well-defined, retinal pathological features in such transgenic mice might be compounded by the timescale necessary for pathology to develop. This in turn might be further influenced by the assumption that TIMP3 has exactly the same function in mice and humans in vivo, which might be incorrect. The obvious lack of major matrix-degrading proteinases such as MMP1 in mice is likely to reflect considerable differences in ECM turnover between these two species. Furthermore, TIMP3 immunohistochemistry of an SFD retina reveals strong staining in Bruch's membrane. The observed staining has a similar distribution to the connective tissue protein elastin, leading some researchers to propose that TIMP3 protein expressed by the RPE cell protects this central layer within Bruch's membrane (Ref. 23). This might turn out to be a very important observation since the TIMP3-homozygous knockout mouse contracts a prominent emphysema-like disorder where lung connective tissue architecture is compromised (Ref. 45).

TIMP3 and AMD: is there a connection?

The close phenotypic similarities between SFD and AMD, together with the fact that increased retinal deposition of TIMP3 are features of both diseases (Ref. 27), is suggestive that *TIMP3* may also be a candidate gene in the age-related disease. However, genetic studies have failed to find any evidence for *TIMP3* mutations in AMD patients (Refs 55, 56), although mutations in intron sequences cannot be ruled out. It is also unlikely that the build-up of TIMP3 observed in AMD results from increased transcription, as the expression of TIMP3 by RPE cells is not significantly affected during ageing or thickening of Bruch's membrane (Ref. 57).

It is our hypothesis that post-translational modification of the wild-type TIMP3 protein plays a role in AMD pathology. Recently, crosslinked TIMP3, apparently resulting from oxidation reactions, was detected in drusen from both normal and AMD retinas (Ref. 28). Thus, crosslinked TIMP3 aggregates are a feature of both SFD and AMD and may well underlie the accumulation of TIMP3 in the latter disease. Crosslinking of TIMP3 could be the result of cumulative oxidative stress, which has been proposed to contribute to AMD (Ref. 58). It is well known that early intermediates in a protein aggregation pathway can be cytotoxic (Ref. 59) and it might be that SFD TIMP3 aggregates reach a threshold of cytotoxicity as a direct consequence of mutation before wild-type TIMP3 can achieve this drastic result in the AMD patient.

The role of TIMP3 dimers and oligomers in the early stages of retinal pathology is a new avenue of research that has benefited from heterologous expression systems in which dimers and oligomers can be isolated and studied in relation to RPE cell death and toxicity (Ref. 41).

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As described above, mutation increases the oligomerisation rate of TIMP3 in SFD; in AMD, the same process might be modulated by the redox state of Bruch's membrane and might respond to redox changes that occur as the individual ages. Since disulphide-bonded TIMP3 dimers have been seen in the Bruch's membrane of a normal retina (Ref. 25), this suggests that wild-type TIMP3 might either be susceptible to enzymatic crosslinking by oxidoreductases or alternatively dimerise in a similar way to SFD TIMP3 proteins (Fig. 3c). Such wild-type–wild-type dimers would thus initiate multimerisation in a similar way to that postulated for the SFD protein in a chain-reaction condensation process.

Protein multimerisation or aggregation is a common feature of a diverse range of human disorders, particularly neurodegenerative diseases such as Huntington's disease (reviewed by Ref. 60). Some of these diseases, including Alzheimer's disease, have familial and sporadic forms in which the familial form of the disease has a much earlier age of onset. Interestingly, TIMP1 has been implicated in a canine model of X-linked progressive retinal atrophy where degeneration in the retina precedes the clinical onset of disease (Ref. 61). It would be very interesting to see if the TIMP1 protein present in the retina in these animals had aberrantly formed dimers.

Clinical implications

Nutritional intervention with high-dosage vitamin A has been shown to reverse nyctalopia and improve visual field defects associated with SFD in patients who have already lost central vision (Ref. 39). This study indicated the presence of a serious defect in transport processes across Bruch's membrane that might be due to thickening by proteinaceous and lipid deposits. Such metabolic transport defects would directly impinge on the very metabolically active RPE cell. The use of vitamin A to prevent or treat SFD has not become widespread as the dosage of vitamin A required to have clinical efficacy is very high and could lead to toxic effects in other tissues as well as in the retina itself.

With the availability of routine genetic testing for SFD mutations, the possibility of early intervention in presymptomatic patients with vitamin A or other drugs now exists. There are, however, significant ethical issues surrounding testing for SFD mutations in asymptomatic family members in SFD pedigrees. It is not clear, for example, that all individuals with SFD mutations will go on to develop severe visual loss. Furthermore, there is no proven treatment for the disorder that could be offered as a result of positive screening. Similarly, the ethics are uncertain of testing prior to, or during, pregnancy for a nonlethal disease that has onset in middle age.

The treatment of families already suffering from SFD would certainly require a full understanding of the role that TIMP3 mutant proteins have in the development of the pathology. If SFD is caused by a build-up of mutant TIMP3 proteins that are crosslinked into dimers and oligomers, two treatment strategies suggest themselves. The first would be to attempt to reduce the propensity of the TIMP3 molecule to multimerise; high concentrations of antioxidants in the retina might be a first step towards this. Second, peptides designed to abrogate the binding of TIMP3 molecules to each other or the matrix by competing for binding sites might also be helpful. Drug delivery would have to be 'retina specific' as TIMP3 is both widely expressed and believed to be important in many other physiological processes. It is impossible at this stage to speculate what this might mean for AMD treatment because of the ill-defined role that TIMP3 has in this disease. However, if AMD pathology is connected to a build-up of TIMP3, then similar techniques designed to abrogate TIMP3 accumulation might also be helpful in slowing disease progression in this much more widespread disorder.

Research in progress and outstanding research questions

The role of TIMP3 in SFD must be considered in the light of the overlap of phenotypic features between other macular diseases such as Best's disease, Stargardt's disease and XLRS. In these latter processes, disease function is linked to genes other than *TIMP3* but the diseases still result in maculopathy. Thus, mechanistic studies on these processes, as well as on SFD, might lead to discovery of common pathways leading to retinal dysfunction. This is particularly important when considering future therapy, as information that leads to elucidation of a common molecular pathway could illuminate intervention strategies with broader clinical applicability.

Intriguingly, aberrant accumulation of a different ECM protein, EGF-containing fibulin-like ECM protein 1 (EFEMP1) or fibulin 3, also

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occurs both in the autosomal dominant macular degenerative disease Malattia leventinese and in AMD (Ref. 62). Recent evidence suggests that EFEMP1 and TIMP3 are binding partners (Ref. 63), leading the authors of this work to conclude that accumulation of EFEMP1–TIMP3 complexes acts as a physical barrier to retinal nutrition. Since Malattia leventinese and SFD can both resemble the two broad phenotypes represented on the AMD spectrum (neovascular and atrophic), this interaction between TIMP3 and EFEMP1, from a biochemical perspective, might be relevant to macular degeneration phenotypes.

The future of research on the TIMP3 protein and its role in macular disease will for the present centre on purifying and characterising the oligomeric forms of the TIMP3 protein in SFD patients and elucidating which form (or forms) of the inhibitor is responsible for the cellular pathology seen in this disease. With this knowledge, we are likely to get closer to understanding the biochemistry of the TIMP3 protein and the mechanism of onset of SFD pathology, and thus would be in a better position to investigate novel therapeutic strategies. The developing mechanistic investigations on SFD will hopefully direct further studies on AMD. Indeed, we hope research efforts will eventually underscore the opinion of the macular degeneration expert Professor Alan Bird (Institute of Ophthalmology, University of London, UK) that 'The evidence that TIMP3 plays an important role in the generation of AMD is as strong as that for Aβ-42 in Alzheimer's disease' (Ref. 64).

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Dedication

This paper is dedicated to our colleague Dr Kevin Pearce Langton who, as a PhD student of M.B. and N.M., first demonstrated dimerisation of the SFD TIMP3 protein. Kevin died suddenly in December 2004. We all greatly miss his intellect, practical abilities and excellent sense of humour: '*Ars longa, vita brevis*'.

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Further reading, resources and contacts

For more information on inhibitor proteins such as TIMP-3, see the peptidase database MEROPS and the Online Mendelian Inheritance in Man (OMIM) website:

http://merops.sanger.ac.uk/
http://www.ncbi.nlm.nih.gov (id=188826)

For clinical disease information, see the websites of the Royal National Institute of the Blind (RNIB) and the Macular Disease Society (MDS):

http://www.rnib.org.uk
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Figures

Figure 1. Features of the retinal defect in Sorsby's fundus dystrophy.
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