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Langton, K.P., McKie, N., Curtis, A. et al. (4 more authors) (2000) A novel tissue inhibitor of metalloproteinases-3 mutation reveals a common molecular phenotype in sorsby's fundus dystrophy. *Journal of Biological Chemistry*, 275 (35). pp. 27027-27031. ISSN 0021-9258

<https://doi.org/10.1074/jbc.M909677199>

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A Novel Tissue Inhibitor of Metalloproteinases-3 Mutation Reveals a Common Molecular Phenotype in Sorsby's Fundus Dystrophy*

Received for publication, December 1, 1999, and in revised form, May 12, 2000
Published, JBC Papers in Press, June 14, 2000, DOI 10.1074/jbc.M909677199

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Sorsby's fundus dystrophy (SFD) is a dominantly inherited degenerative disease of the retina that leads to loss of vision in middle age. It has been shown to be caused by mutations in the gene for tissue inhibitor of metalloproteinases-3 (TIMP-3). Five different mutations have previously been identified, all introducing an extra cysteine residue into exon 5 (which forms part of the C-terminal domain) of the TIMP-3 molecule; however, the significance of these mutations to the disease phenotype was unknown. In this report, we describe the expression of several of these mutated genes, together with a previously unreported novel TIMP-3 mutation from a family with SFD that results in truncation of most of the C-terminal domain of the molecule. Despite these differences, all of these molecules are expressed and exhibit characteristics of the normal protein, including inhibition of metalloproteinases and binding to the extracellular matrix. However, unlike wild-type TIMP-3, they all form dimers. These observations, together with the recent finding that expression of TIMP-3 is increased, rather than decreased, in eyes from patients with SFD, provides compelling evidence that dimerized TIMP-3 plays an active role in the disease process by accumulating in the eye. Increased expression of TIMP-3 is also observed in other degenerative retinal diseases, including the more severe forms of age-related macular degeneration, the most common cause of blindness in the elderly in developed countries. We hypothesize that overexpression of TIMP-3 may prove to be a critical step in the progression of a variety of degenerative retinopathies.

arating the retinal pigment epithelium from its blood supply, the choriocapillaris. This is accompanied by atrophy of the choriocapillaris, retinal pigment epithelium, and photoreceptors and subretinal neovascularization that can lead to retinal detachment. Although SFD is rare, it is of particular interest as it closely resembles the more severe (exudative or "wet") form of age-related macular degeneration (AMD), the most common cause of blindness in the elderly of developed countries.

SFD has been shown to be caused by mutations in the gene for tissue inhibitor of metalloproteinases-3 (TIMP-3) (2). The members of the TIMP family, of which there are currently four (TIMPs 1–4) (3–6), are the natural inhibitors of matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases that exist in both secreted and membrane-bound forms. The balance between MMPs and TIMPs regulates remodeling of the extracellular matrix (ECM) and thus plays a key role in a wide range of physiological processes that include embryonic development, connective tissue remodeling, wound healing, glandular morphogenesis, and angiogenesis.

Structurally, TIMPs can be divided into two major domains, each comprising three disulfide bonded loops (7, 8). The highly conserved N-terminal domain has been shown to be sufficient for MMP inhibition (9–11), whereas the C-terminal domains are more divergent and appear to be important in imparting individual differences in specificity to each TIMP family member (12), including, for example, their different affinities for pro-MMPs (the inactive precursors of MMPs) (13–16). Likewise, we have shown that a C-terminally deleted TIMP-3 is still an effective MMP inhibitor, whereas its ability to bind to the ECM, a unique feature of TIMP-3, largely resides in the C-terminal domain (11).

With one recent exception (17), all previously described mutations to TIMP-3 in SFD change residues in exon 5 (which encodes the C-terminal domain of the molecule) into cysteines; however, the role such mutations play in the disease process is unknown. It was widely assumed that these mutations would severely disrupt protein folding, leading to the conclusion that the disease was probably attributable to insufficient expression of TIMP-3 from the remaining normal allele. Nevertheless, the possibility remained that the mutant protein itself may give rise to the disease phenotype. Support for this latter hypothesis was strengthened by recent work from our laboratory that showed that the most common TIMP-3 mutation observed in SFD, S181C, gave rise to a molecule that retained its ability to both inhibit MMPs and localize to the ECM yet, unlike wild-type TIMP-3, formed dimers (11).

This report describes the biochemical characterization of several other TIMP-3 mutations, including the identification of

Sorsby's fundus dystrophy (SFD)¹ is an autosomal, dominant degenerative disease of the macula, typically leading to loss of central vision in middle age (the fourth or fifth decade) (1). The disease is characterized by extracellular deposits (drusen) and thickening in Bruch's membrane, the basement membrane sep-

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[†] Supported by the Arthritis Research Campaign.

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¹ The abbreviations used are: SFD, Sorsby's fundus dystrophy; AMD, age-related macular degeneration; CAPS, 3-(cyclohexylamino)propane-sulfonic acid; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

an SFD family² with a completely novel TIMP-3 mutation in which a residue in exon 5 is changed to a stop codon, resulting in the deletion of most of the C-terminal domain of the molecule. These data provide strong evidence of an active role for mutant TIMP-3 in SFD and a possible link to other degenerative retinal diseases.

EXPERIMENTAL PROCEDURES

DNA Samples—Blood was obtained from three affected members and one unaffected member of a recently identified SFD family, and genomic DNA was extracted by standard methods. DNA samples from normal individuals and a patient shown to have the S181C mutation were also isolated as controls.

Polymerase Chain Reaction—Polymerase chain reaction primers for exon 5 of the TIMP-3 gene were synthesized as described previously (18). Amplifications were performed in 50- μ l reactions using 15 pmol of each primer, 200 μ M dNTPs, 4% formamide, 500 ng of genomic DNA, and 1–2 units of *Taq* DNA polymerase (Promega) in the recommended buffer (50 μ M KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl₂). Cycling was carried out on a Perkin-Elmer DNA Thermal Cycler 480 as follows: initial denaturation at 94 °C for 4 min followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min 72 °C, with a final extension at 72 °C for 10 min.

Restriction Digest and Single Strand Conformation Polymorphism Analysis—After checking for successful amplification, 25 μ l of each reaction mix was digested with 6 units of *Pst*I (New England Biolabs) in the supplied buffer for 3–18 h at 37 °C. 10 μ l of single strand conformation polymorphism loading buffer (98% formamide, 10 mM EDTA, 20 mM NaOH, 0.05% bromphenol blue, 0.05% xylene cyanol) were added to 10 μ l of each digest, and the samples were denatured at 94 °C for 5 min, placed immediately on ice to reduce duplex formation, and then loaded onto each of two gels: (a) 1 \times MDETM (FMC Corp.) in 0.6 \times TBE buffer with 2% glycerol, and (b) 8% acrylamide (49:1 acrylamide:bisacrylamide) in 0.6 \times TBE. Gel a was run in 0.6 \times TBE at ambient temperature for 18 h at 200 V. Gel b was run in 0.6 \times TBE at 4 °C for 16 h at 170 V. Gels were silver-stained by standard methods.

DNA Sequencing—Polymerase chain reaction products amplified as above, from an unaffected control and an affected patient, were sequenced using SequenaseTM polymerase chain reaction product sequencing kit (Amersham Pharmacia Biotech) and the forward primer.

Expression Plasmid Construction—Construction of pCI.neo vectors (Promega) containing wild-type TIMP-3 and the S181C SFD mutant has been described previously (11). S156C, G166C, and E139X were constructed using the QuickChange (Stratagene, Cambridge, United Kingdom) site-directed mutagenesis kit using the wild-type TIMP-3-pCI.neo as a template and overlapping oligonucleotide primers containing the appropriate mutation. Plasmids for transfection were prepared with Plasmid Mini or Maxi kits (Qiagen). All TIMP-3 constructs were fully sequenced on an Applied Biosystems automated DNA sequencer to confirm the changes and ensure that no further mutations had been introduced.

COS-7 Cell Transfection—COS-7 cells were routinely cultured in Dulbecco's modified Eagle's medium + 10% fetal calf serum in a 37 °C, 5% CO₂ humidified incubator. Transfections were performed by DEAE-dextran/dimethyl sulfoxide shock (19) with the inclusion of 0.5% FCS in the transfection solution. 48 h after transfection, the cells were transferred to serum-free Dulbecco's modified Eagle's medium and allowed to grow for a further 48 h. Cells and ECM were then harvested according to Ref. 20. However, for the Western blots, immediately after removing the cells and prior to harvesting the ECM, duplicate plates were also incubated for 60 min at room temperature with 10 mM freshly prepared iodoacetamide to alkylate any free sulfhydryl groups. The protein concentrations of the fractions were determined using the BCA protein assay reagent (Pierce), and equal protein amounts were analyzed by protease/substrate SDS-polyacrylamide gel electrophoresis.

Protease/substrate SDS-Polyacrylamide Gel Electrophoresis (Reverse Zymography)—Samples were analyzed on 12 or 15% acrylamide protease/substrate gels according to Ref. 21, except that phorbol myristate acetate-stimulated U937 cell-conditioned media, at a final concentration of 10% (v/v), was used as a source of gelatinase activity (22), and gelatin was incorporated into the gel at a final concentration of 2.5 mg/ml. Following staining with Coomassie Brilliant Blue (Sigma), inhibitory species were visualized as dark blue bands against a clear

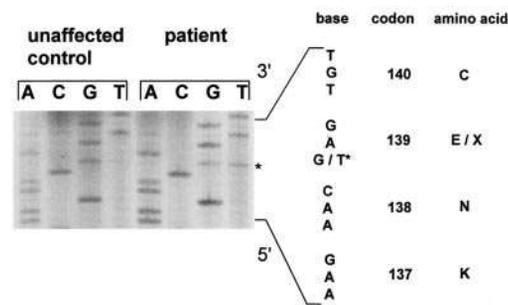


FIG. 1. Section from a sequencing gel of DNA from a normal and SFD patient. All of the affected members of the new SFD family have a thymidine instead of a guanosine in one allele at the first base of codon 139, resulting in the transformation of a glutamate residue into a stop codon.

background.

Immunoblotting—Reduced or nonreduced (heated for 10 min at 95 °C with or without 2% 2-mercaptoethanol respectively) samples were separated on 12% SDS-polyacrylamide gel electrophoresis gels with biotinylated molecular weight markers (Amersham Pharmacia Biotech) prior to blotting onto polyvinylidene difluoride membrane (Pierce & Warriner UK Ltd.) in 10 mM CAPS + 10% methanol (pH 11.0) at 60mA overnight. Membranes were blocked by incubation with Tris-buffered saline, pH 7.5 (TBS) + 6% skim milk powder + 0.2% Tween-20 for 6 h. Rabbit anti-TIMP-3 (first loop) antibody (Sigma) was diluted (1:1000) in TBS + 6% skim milk powder + 0.05% Tween-20 (antibody dilution buffer) and incubated with the blots overnight at 4 °C. Membranes were washed extensively in TBS and TBS + 0.05% Tween-20, prior to incubation with horseradish peroxidase-labeled secondary antibody (DAKO) (1:6,000) and streptavidin-horseradish peroxidase conjugate (1:1, 500) (Amersham Pharmacia Biotech) in antibody dilution buffer for 1 h. Membranes were then washed as before and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

Genetic Mapping

Three single strand conformation polymorphism band patterns were found: one in the normal control and unaffected family member, one in the three affected individuals, and a third in an individual known to have the common S181C mutation. Direct DNA sequencing showed a G to T transversion in one allele in the first base of codon 139 in the three affected individuals (Fig. 1), resulting in a TAG termination codon replacing the normal GAG glutamic acid codon (E139X).

The presence of the same E139X mutation in patient III5 (Fig. 2), as well as the mother and daughter (patients II2 and III1 respectively), indicates that it is not a *de novo* mutation in this latter family but must have come from either I1 or I2.

Biochemical Characterization

S156C, G166C, and S181C SFD Mutations—Mock-transfected COS-7 cells (pCI.neo alone) express 2 or 3 functional MMP inhibitors in the ECM with approximate molecular masses in the range of 23–29 kDa (Fig. 3, lane A). These masses correspond to those previously reported for unglycosylated and glycosylated TIMP-3, respectively (23), and the inhibitors are presumed to be endogenously expressed TIMP-3. This conclusion is confirmed by Western blotting (Fig. 4a, lane A) and by the fact that these bands are greatly intensified in COS-7 cells transfected with wild-type TIMP-3 (Figs. 3 and 4a, lane B).

ECM from cells transfected with the various SFD mutants all showed up to four additional higher molecular mass MMP-inhibitory species in the range of approximately 45–60 kDa that also cross-reacted with the anti-TIMP-3 antibody (Figs. 3 and 4a, lanes C–E). There were, however, differences in the intensities of the higher mass bands between the three mutants tested, which was particularly apparent on the (more

² M. P. Clarke, K. W. Mitchell, N. McKie, M. D. Barker, and J. A. Goodship, manuscript in preparation.

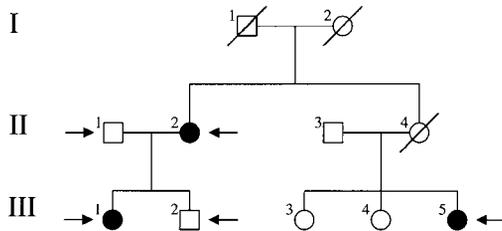


FIG. 2. **Pedigree of the SFD family.** ■ and ○, unaffected male and female, respectively; ●, affected female; *slashed square* and *slashed circle*, deceased male and deceased female, respectively, affected status unknown; →, mutation testing completed and consistent with affected status.

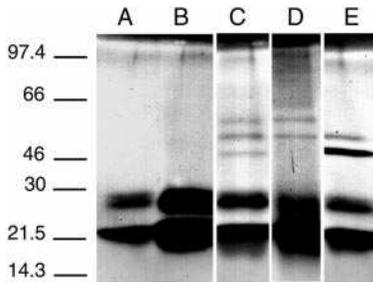


FIG. 3. **Reverse zymogram of ECM from COS-7 cells transfected with normal and previously reported SFD mutations of TIMP-3.** A, mock-transfected; B, wild-type TIMP-3; C, S156C-TIMP-3; D, G166C-TIMP-3; E, S181C-TIMP-3. Positions of molecular mass markers (in kDa) are indicated on the left.

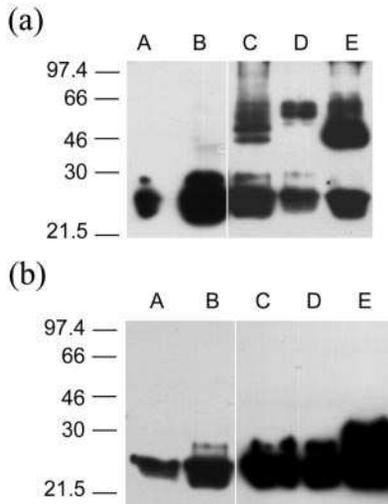


FIG. 4. **Western blots of ECM from COS-7 cells transfected with normal and previously reported SFD mutations of TIMP-3.** a, nonreduced gel; b, reduced gel. Lane A, mock-transfected; lane B, wild-type TIMP-3; lane C, S156C-TIMP-3; lane D, G166C-TIMP-3; lane E, S181C-TIMP-3. Positions of molecular mass markers (in kDa) are indicated on the left.

sensitive) Western blots (Fig. 4a, lanes C–E). S156C formed four bands, with the 50-kDa band predominating; G166C ran mainly at approximately 55 and 60 kDa; and S181C had a particularly predominant band at about 45 kDa, with lower intensity bands at approximately 50, 55, and 60 kDa. Treatment of the ECM with iodoacetamide prior to harvesting had no effect on the formation of these higher molecular mass species.

None of these higher molecular mass species were apparent on Western blots of the same samples run under reducing conditions (Fig. 4b, lanes C–E). In this case, however, there was an increase in the intensity of staining of the 23–29-kDa bands relative to mock-transfected cells.

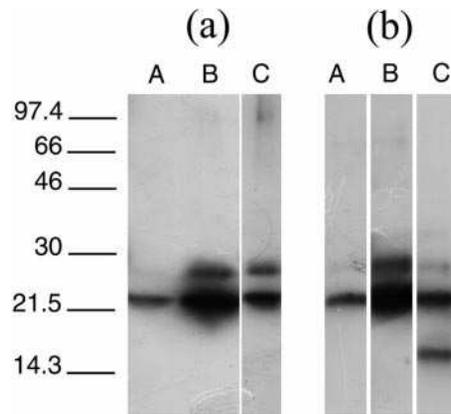


FIG. 5. **Western blots of ECM from COS-7 cells transfected with normal and E139X-TIMP-3.** a, nonreduced gels; b, reduced gels. Lane A, mock-transfected; lane B, wild-type TIMP-3; lane C, E139X-TIMP-3. Positions of molecular mass markers (in kDa) are indicated on the left.

E139X SFD Mutation—In contrast to the reverse zymograms and Western blots of the previously described SFD mutations, those of nonreduced ECM from COS-7 cells transfected with the E139X SFD construct did not reveal any novel high molecular mass species, although the 27-kDa species appeared to show some increase in intensity relative to the 24-kDa species on the Western blot (Fig. 5a, lane C). However, when the same sample was run under reducing conditions (Fig. 5b, lane C), there was a marked decrease in intensity of the 27-kDa species, with a corresponding appearance of a novel anti-TIMP-3 immunoreactive species at approximately 16 kDa. This corresponds almost exactly to the predicted molecular mass of monomeric E139X protein (15.9 kDa). The reduced molecule ran as a single species in keeping with the fact that the *N*-glycosylation site, which lies close to the C terminus of the wild-type molecule (see Fig. 6), is absent in this mutant. There was no change in size of the wild-type control under the same conditions (Fig. 5b, lane B).

DISCUSSION

We have identified an entirely novel TIMP-3 mutation in a family with Sorsby's fundus dystrophy. Instead of a transition to a cysteine, a glutamic acid residue is changed to a stop codon, resulting in a TIMP-3 molecule lacking a large part of the C-terminal domain (see Fig. 6). Despite this difference, the phenotype of the disease in these patients, including age of onset, is not particularly distinguishable from previously described SFD families (clinical details to be reported elsewhere), and this appears to be reflected in the biochemical characteristics of the mutant protein.

We have previously shown that the S181C SFD mutation gives rise to an MMP-inhibitory, ECM-localized TIMP-3 dimer (11). Here, we confirm that this also appears to be the case for two other SFD mutations, S156C and G166C, and it is more than likely that the other two observed SFD mutations, G167C and Y168C, being adjacent to G166, behave likewise. There were, however, some differences in the pattern of expression of S156C and G166C, relative to S181C, in that expression levels of these mutants were somewhat less than those observed for S181C, and the pattern of higher molecular mass species varied between the different mutants. In theory, the different combinations of the three monomer forms of TIMP-3 (which are due to differential glycosylation (11)) could give rise to up to six different dimers. The fact that for each mutant different dimers appeared to predominate may be a reflection of the relative positions of the free sulfhydryl residue and carbohydrate moieties. In S181C, for example, the potentially unpaired cysteine is in close proximity to the glycosylation site (Asn¹⁸⁴),

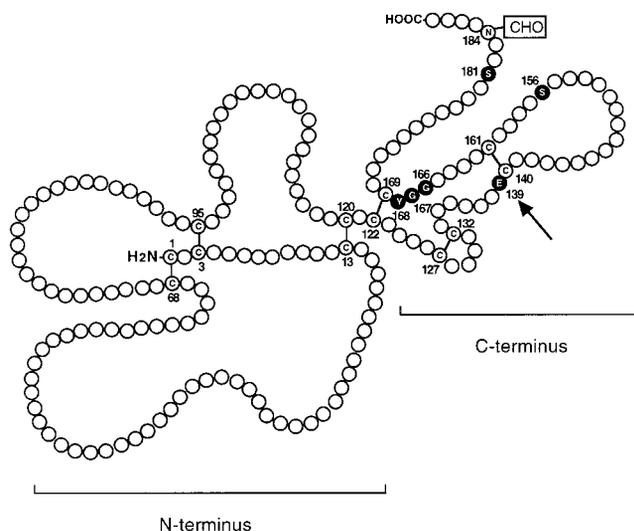


FIG. 6. **Diagrammatic representation of TIMP-3 showing key residues.** The positions of disulfide bonds are based on those deduced for TIMP-1 (7, 8). CHO denotes the *N*-glycosylation site. Residues mutated in SFD are shown in *solid black*, and the E139X mutation is indicated with an *arrow*.

possibly explaining why the smaller, less glycosylated dimer predominates.

Despite the fact that the new SFD mutation described herein results in truncation of most of the C-terminal domain of the molecule (which we have previously shown contributes significantly to the ability of TIMP-3 to localize to the ECM (11)), E139X still forms an ECM localized dimer. Although this new mutation does not give rise to an extra cysteine residue, it does result in one that is potentially unpaired (Cys¹²²; see Fig. 6), explaining the ability of the molecule to dimerize. Several features of the mutant may contribute to ECM localization. First, it lacks an *N*-glycosylation site. We have shown previously that glycosylation of TIMP-3 decreases its apparent affinity for the ECM, presumably by masking basic residues (11). Second, truncation still leaves a small section (18 residues) of the C-terminal domain that may contribute disproportionately to ECM binding. Third, dimerization will increase avidity. The fact that the E139X dimer ran somewhat anomalously at approximately 27 kDa (rather than the expected 32 kDa), in a position identical to that of the glycosylated TIMP-3 monomer, precluded confirmation that this molecule is also a functional MMP-inhibitor; however, this is very likely to be the case, as TIMP-3 truncated at Asn¹²¹ still retains its ability to inhibit MMPs (11).

The fact that this entirely novel SFD mutation is also a localized dimer provides compelling evidence that dimer formation is critical to the disease process because point mutations to residues other than cysteines could equally well disrupt protein folding if the haploinsufficiency model was correct. It has recently been reported that an intron 4/exon 5 splice site mutation in TIMP-3 also gives rise to the SFD phenotype, but with a delayed age of onset (the seventh rather than fourth or fifth decade) (17). Although the consequences of such splice site mutations cannot be predicted, one possibility is the skipping of the fifth exon. This would give rise to a TIMP-3 molecule lacking the whole of the C-terminal domain but retaining an unpaired cysteine residue (Cys¹²²) and therefore the potential to dimerize. Our data predict that such a molecule would be a functional MMP inhibitor but would show poor localization to the ECM (11). Reduced ECM localization could provide a possible explanation for the delayed age of onset of the disease in these patients.

Assuming, therefore, that the mutant TIMP-3 contributes actively to the disease process, how might dimer formation affect the function of the molecule? Several possibilities exist. For example, a TIMP-3 dimer would potentially be able to cross-link membrane-bound proteins, such as the metalloproteinases membrane type 1-MMP or tumor necrosis factor- α converting enzyme (TACE) (24, 25). Whether dimerization of such molecules is functionally significant is currently unknown; however, binding in this way to membrane type 1-MMP may inhibit its ability to activate progelatinase A via TIMP-2 (24). Interestingly, the recently identified membrane type 5-MMP also appears to exist in a dimeric form (26). Alternatively, dimerization at the C terminus may alter the binding of this domain to target molecules, such as pro-MMPs; this would certainly be the case for the E139X mutant, in which this domain is largely deleted. Another alternative is that dimerization may simply inhibit normal turnover of the molecule. Support for this latter possibility has recently come from immunohistochemical analyses of eyes from SFD patients. Such eyes show an *increased* deposition of TIMP-3, particularly in Bruch's membrane (27). This does not definitively prove that the mutated protein itself is accumulating; it is possible that the mutant TIMP-3 induces overexpression from the normal allele. However, it is strong evidence against the haploinsufficiency model. In either case, as both normal and mutant molecules show MMP-inhibitory activity, the mutation may act by decreasing cell matrix turnover leading to the observed thickening of Bruch's membrane that characterizes the disease. Such thickening could in itself lead to nutrient deprivation and thus retinopathy and also stimulate neovascularization of the choroid.

An alternative possibility is that accumulated TIMP-3 has a more direct effect on the retina by triggering apoptosis. TIMP-3 has been shown to trigger cell cycle arrest and apoptosis in a variety of cell types by both MMP-dependent and MMP-independent mechanisms (28–30).

Abnormal expression of TIMP-3 in Bruch's membrane also occurs in AMD and another degenerative retinal disease, simplex retinitis pigmentosa (27, 31, 32). Although neither AMD nor retinitis pigmentosa is associated with heritable mutations in TIMP-3 (33, 34), the similarities in disease phenotype, particularly between SFD and the more severe forms of AMD, make it tempting to speculate that TIMP-3 is a causal agent linking these diseases. It is likely that localization of TIMP-3 to the ECM occurs through its interaction with glycosaminoglycans (35). One possible mechanism for TIMP-3 accumulation in AMD may, therefore, be binding to products of advanced protein glycosylation (advanced glycation end products) associated with old age. Accumulation of advanced glycation end products is greatly accelerated in diabetes, and pharmacological inhibition of advanced glycation end product formation in diabetic animals prevents many of the complications of long term diabetes, including retinopathy (reviewed in Ref. 36). It will be interesting to determine whether TIMP-3 accumulation is also associated with this disease.

In summary, we have shown that dimerization of TIMP-3 is a common factor linking the various mutations that are associated with Sorsby's fundus dystrophy. The fact that such dimers are functional, together with evidence for TIMP-3 accumulation in SFD, suggests that dimerization inhibits turnover of the molecule, which then plays an active role in the disease process. There is also a possibility that TIMP-3 accumulation, albeit by different mechanisms, is a causative agent of other degenerative retinal diseases. If this proves to be the case, then therapies directed at preventing or reversing

TIMP-3 localization may offer hope of treatment for a variety of currently incurable and debilitating eye diseases.

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