EXTENDED REPORT

Tissue inhibitor of metalloproteinase-3 differentially binds to components of Bruch's membrane

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Background: Sorsby's fundus dystrophy (SFD) is caused by mutations in tissue inhibitor of metalloproteinase (TIMP)-3 and, with the exception of early onset, is similar to age-related macular degeneration. The pathological features of this condition relate to the accumulation of TIMP-3 in Bruch's membrane.

Aims: To compare the extracellular membrane-binding characteristics of wild-type and four SFD-mutant TIMP-3s.

Methods: COS-7 cells were transfected with wild-type, Ser-181, Gly-167, Ser-156 and Tyr-168 SFD-mutant TIMP-3 cDNA. The TIMP-3 proteins subsequently synthesised were harvested, analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, semiquantified by ELISA and used in binding assays on the basis of the retention of the wild-type and SFD-mutant TIMP-3 proteins by components of Bruch's membrane.

Results: SFD-mutant TIMP-3s could not be distinguished from wild-type TIMP-3 by the extents to which they aggregated or adhered to type-I collagen, type-IV collagen, fibronectin, laminin, elastin, chondroitin sulphates A, B and C, and heparin sulphate. Of these macromolecules, the wild-type and SFD-mutant TIMP-3s exhibited greatest affinity for elastin and laminin.

Conclusion: The similarity in the physical and extracellular membrane-binding characteristics of wild-type and SFD-mutant TIMP-3s indicates that these properties are not responsible for the difference in timing of onset of SFD and age-related macular degeneration.

Sorsby's fundus dystrophy (SFD)¹ is a rare autosomal dominant macular dystrophy caused by point mutations in exon 5 of the gene for tissue inhibitor of metalloproteinase-3 (TIMP-3) on chromosome 22.²⁻³ The pathological fundal features are similar to those of age-related macular degeneration (ARMD), but occur at a younger age.⁴⁻⁶ An early sign of SFD is the deposition of lipid and proteinaceous material in Bruch's membrane.⁷⁻⁸ These deposits are similar to the drusen found in Bruch's membrane of patients with ARMD,⁹ in that they are rich in TIMP-3 and associated with thickening and, eventually, break formation.⁷⁻⁸ Through these breaks, neovascular vessels, derived from the choriocapillaris, eventually invade the sub-retinal pigment epithelial (RPE) space. The ensuing subretinal haemorrhage from the vascular tissue leads to macular scarring and irreversible loss of central vision.

TIMP-3 is one of the naturally occurring inhibitors of the matrix metalloproteinases (MMPs). Atypically, it adheres to the extracellular membrane (ECM) and, possibly accounting for its apoptotic properties,¹⁰⁻¹² is a more efficient inhibitor of the aggrecanases, ADAM-10, ADAM-12 and tumour necrosis factor α -converting enzyme. Despite this, as a consequence of its MMP inhibitory function, TIMP-3 may limit ECM degradation in healthy eyes. In eyes with SFD and ARMD, excessive accumulation of mutant and wild-type TIMP-3, respectively, might also induce thickening of Bruch's membrane and the consequential pathology.

The ability of TIMP-3 to bind to ECM is a function of its C-terminal domain.¹³ Although wild-type TIMP-3 can be extracted from the rat postpartum uterus with sulphated glycosaminoglycans,¹⁴ in eyes with SFD TIMP-3 colocalises specifically with elastin.⁷ As part of a wider investigation of the mechanism of TIMP-3-induced retinal pathology, we compared the binding properties of wild-type TIMP-3

proteins and four of the known SFD-mutant TIMP-3 proteins to purified components of Bruch's membrane.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium (DMEM) and OptiMEM, fetal calf serum (FCS), glutamine and Lipofectamine were obtained from Invitrogen (Paisley, UK). All general reagents, a penicillin, streptomycin, amphotericin antibiotic or antimycotic solution, purified ECM macromolecules, horseradish peroxidase (HRP)-conjugated secondary antibodies and their substrates diaminobenzidine and 3,3',5,5'-tetramethyl benzidine (TMB) were obtained from Sigma (Surrey, UK). A polyclonal TIMP-3 antibody, raised against loop 1 of human TIMP-3, was obtained from Chemicon (Hampshire, UK).

Wild-type and SFD-mutant TIMP-3 plasmids

The point mutations in the C-terminal domains of four SFD-mutant TIMP-3 proteins (Ser-181, Gly-167, Ser-156 and Tyr-168) all generated cysteine residues and hence free sulphhydryl (-SH) groups. Plasmids containing the cDNAs of wild-type and SFD-mutant TIMP-3s were subcloned into pCI.neo vectors under a cytomegalovirus early immediate promoter to enhance expression of the TIMP-3 proteins. All TIMP-3 plasmids were completely sequenced to verify the mutations and to ensure that no further mutations had been introduced.

Abbreviations: ARMD, age-related macular degeneration; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular membrane; FCS, fetal calf serum; GAG, glycosaminoglycan; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; RPE, retinal pigment epithelial; SDS, sodium dodecyl sulphate; SFD, Sorsby's fundus dystrophy; TIMP-3, tissue inhibitor of metalloproteinase-3; TMB, 3,3',5,5'-tetramethyl benzidine

Experimental cell line

COS-7 cells (American Tissue Culture Collection, Manassas, Virginia, USA) do not succumb to TIMP-3-induced apoptosis and can produce relatively large quantities of this protein. Routinely, these cells were maintained in DMEM supplemented with 10% FCS (vol/vol) and antibiotic or antimycotic solution and incubated at 36°C in a moist atmosphere of 95% air and 5% CO₂. The culture medium was changed every 3–4 days.

COS-7 cell transfection

An optimised method of transfecting RPE cells using Lipofectamine (Invitrogen, Paisley, UK) was followed.¹⁵ Briefly, trypsinised COS-7 cells were plated out in 25 cm³ flasks at the same density ($\sim 2.5 \times 10^5$ cells/flask) and incubated in DMEM containing 10% (vol/vol) FCS until they achieved around 80% confluence. The cell cultures were then washed twice with OPTI-MEM (Invitrogen, Paisley, UK) and incubated in OPTI-MEM (1.5 ml) containing Lipofectamine (30 μ l) and 6 μ g of TIMP-3 plasmid DNA. Cultures from controls contained only Lipofectamine (30 μ l). An equal volume of DMEM containing 20% (vol/vol) FCS was added after 6 h. This was replaced with DMEM containing 10% (vol/vol) FCS after a further 18 h. The transfected cells were left for 48 h to synthesise TIMP-3 protein.

TIMP-3 protein collection and characterisation

The matrices and COS-7 cells of the control and transfected cultures were homogenised in 0.05 M Tris HCl, pH 7.4, 1% wt/vol sodium dodecyl sulphate (SDS; 100 μ l/well), and centrifuged. Samples of the solubilised proteins were then reduced with mercaptoethanol (2% vol/vol), boiled for 2 min, electrophoretically fractionated on SDS polyacrylamide (10% wt/vol) gels,¹⁶ western blotted on to polyvinylidene difluoride membranes (Millipore) and immunostained using diaminobenzidine as the HRP substrate.

TIMP-3 quantification by ELISA

Although TIMP-3 standards of known concentrations were not available, the relative amounts of the wild-type and SFD-mutant TIMP-3 proteins harvested were determined by ELISA.

Aliquots of the TIMP-3 samples, diluted with 0.05 M Tris HCl, pH 7.4, were pipetted into wells of a 96-well plate. After 16 h, blocking solution (0.05 M Tris HCl, pH 7.4, containing 150 mM NaCl, 5% vol/vol FCS, 2 mM mercaptoethanol and 0.02% NaN₃) was added to these and control wells that did not contain TIMP-3 for 6 h. This was followed by addition of the anti-TIMP-3 antibody and the HRP-conjugated secondary antibody. Stringent washing procedures were followed before and after incubation with each antibody. Finally, aliquots of a solution containing TMB were added. The kinetics of its oxidation were followed at 370 nm at 30°C, using a Molecular Devices Spectromax Spectrophotometer (Sunnyvale, CA, USA). Before storage at –20°C, the wild-type and SFD-mutant TIMP-3 proteins were diluted to the same, although unknown, concentration for experimentation.

The interaction of TIMP-3 and the SFD-mutant TIMP-3s with ECM macromolecules

Preparation of samples

Bruch's membrane contains elastin, type-I collagen, type-IV collagen, fibronectin and laminin. Elastin is a highly insoluble protein. It was either suspended in 1 M acetic acid (solubilises the α -chains) or dissolved in 1 M KOH (generates the β -chains), at a concentration of 0.2 mg/ml, unless otherwise stated. Samples of the other components listed were dissolved in 0.05 M acetic acid (type-I collagen), 10 mM

acetic acid (type-IV collagen) or phosphate-buffered saline (laminin and fibronectin) at a final concentration of 0.2 mg/ml.

The chondroitin sulphates A, B and C, and heparin sulphate were also assayed as potential TIMP-3 ligands. Stock solutions of these glycosaminoglycans (GAGs) were prepared in phosphate-buffered saline at a concentration of 0.2 mg/ml.

TIMP-3 binding assays

These assays were based on the retention of TIMP-3 by the selected components of Bruch's membrane after extensive washing in high ionic strength buffer containing the detergent polyethylene sorbitan monolaurate (Tween-20, Sigma-Aldrich, Poole, Dorset, UK). Aliquots of each component (150 μ l) were pipetted into several wells of a 96-well plate. After 16 h at 4°C, each well was filled with blocking solution (0.05 M Tris HCl, pH 7.4, 150 mM NaCl, 5% vol/vol FCS). This was removed after 6 h at room temperature, and aliquots of wild-type and SFD-mutant TIMP-3 proteins were added to the wells and removed after 16 h at 4°C. The plates were then washed thoroughly in 0.05 M Tris HCl, pH 7.4, 150 mM NaCl, 1% Tween-20. The anti-TIMP-3 antibody and the HRP-conjugated secondary antibody were added sequentially, and the oxidation of TMB followed at 370 nm at 30°C. Readings were routinely taken after 10 min, while the kinetics were still linear.

Statistical treatments

All data are expressed as mean (standard deviation (SD)). Unless otherwise stated, the two-tailed Student's *t* test for unpaired data was used to determine correlative significance.

RESULTS

Analysis of the TIMP-3 proteins produced by the transfected COS-7 cells

The representation of a TIMP-3 immunostained western blot (fig 1) shows that the characteristic TIMP-3 bands at *M_r* 50 000, 28 000, and more weakly at 24 000, were present in both wild-type and SFD-mutant TIMP-3 protein extracts of transfected COS-7 cells and their matrices. These represent the dimerised, glycosylated and unglycosylated forms, respectively. Other bands visualised were ascribed to either degraded TIMP-3 ($\sim M_r \sim 15$ 000) or aggregated/undissociated TIMP-3 (*M_r* >50 000).

Figure 1 Western blot of immunostained wild-type and Sorsby's fundus dystrophy-mutant tissue inhibitor of metalloproteinase-3 proteins extracted from transfected COS-7 cell cultures.

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

The relative amounts of wild-type and SFD-mutant TIMP-3 recovered in the transfected COS-7 cell cultures were determined by ELISA. The results obtained (fig 2) indicated that the yield of wild-type TIMP-3 in the transfected cultures was approximately three times that of control cultures, and that the SFD-mutant TIMP-3 protein yields were similar and significantly slightly more than wild-type TIMP-3 ($p < 0.05$).

Interaction of wild-type TIMP-3 with elastin

Elastin is known to survive longer than other matrix proteins in eyes with ARMD and in eyes with SFD; its immunoreactivity coincides with that of TIMP-3.⁷ Preliminary work was carried out to establish that elastin binds TIMP-3 *ex vivo* and, because of its insolubility, included an investigation of the effects of using increasing concentrations of homogeneous suspensions of elastin, prepared in acid or KOH, to coat the ELISA plate wells. From these experiments, it was noted that an elastin concentration of 5.0 mg/ml achieved maximal TIMP-3 binding; at 0.5 mg/ml the percentage of maximal binding was 69.6 and 67.7, respectively, for acid-prepared and KOH-prepared elastin. Thus, to compare the extent to which wild-type and SFD-mutant TIMP-3 adhered to individual components of Bruch's membrane, they were all assayed at a fixed concentration of 0.2 mg/ml.

Interaction of wild-type and SFD TIMP-3s with selected components of Bruch's membrane

The results of this study indicated that the wild-type and SFD-mutant TIMP-3s could not be distinguished by their ability to differentially bind acid-prepared and KOH-prepared elastin, type-I collagen (containing no cysteine), type-IV collagen (containing cysteine), fibronectin and laminin (fig 3). However, in addition to the acid-prepared or KOH-prepared elastin, the substrate that retained the greatest quantity of the applied TIMP-3 proteins relative to the plastic of uncoated wells was laminin (table 1).

In assessing wild-type and SFD-mutant TIMP-3 binding to chondroitin sulphates A, B and C and heparin sulphate, it was observed that slightly more of the Gly-167, Ser-156 and Tyr-168 TIMP-3s were retained by these GAGs than wild-type or Ser-181-mutant TIMP-3s (fig 4). Despite there being a significant difference ($p < 0.05$), the possibility of biological significance is doubted. Furthermore, although the amounts of wild-type and SFD-mutant TIMP-3 retained by the chondroitin sulphates and heparin sulphate were greater

Figure 2 ELISA estimates of the amount of tissue inhibitor of metalloproteinase (TIMP)-3 protein recovered from COS-7 cell cultures transfected with wild-type and Sorsby's fundus dystrophy (SFD)-mutant TIMP-3 plasmid DNA. Control and native TIMP-3 samples were obtained from COS-7 cell cultures exposed to Lipofectamine alone. The amount of protein expressed in all the SFD-mutant cultures was similar and significantly higher than the expression of wild-type TIMP-3 ($p < 0.05$ in all cases). OD₃₇₀, optical density at 370 nm.

than the amounts retained by type-I and type-IV collagen, they were all significantly less compared with elastin and laminin (table 1).

DISCUSSION

The clinical pathology of SFD, the only known condition directly attributable to a point mutation in TIMP-3, is, with the exception of time of onset, remarkably similar to that of ARMD. Both are characterised by excessive accumulation of TIMP-3 in Bruch's membrane.^{9 17-23}

TIMP-3 can induce apoptosis in several cell types,²⁴⁻²⁷ including RPE cells.²⁸ Because the RPE cells are slightly but significantly more susceptible to the SFD-mutant TIMP-3s than wild type,¹⁵ the RPE cell death or loss observed in the eyes of patients with SFD may be a primary event in the pathological progression of the disease. However, through its presence and MMP inhibitory activity, TIMP-3 also induces thickening of Bruch's membrane. The consequential metabolic starvation and ischaemia, followed by break formation and neovascular invasion, would also exacerbate RPE cell loss. Apparently, in eyes with SFD, localised RPE cell loss correlates with localised TIMP-3 loss, and RPE cell retention correlates with the retention of TIMP-3 between the RPE and elastin sublayer of Bruch's membrane.^{8 19}

The pathological properties of TIMP-3 and the SFD-mutant proteins are not well understood. However, assuming that these proteins have important roles in ARMD and SFD, respectively, the difference in age of onset could either relate to the enhanced sensitivity of RPE cells to the apoptotic effects of the SFD-mutant TIMP-3s or occur because the mutant proteins accumulate more readily in Bruch's membrane.

With respect to the second possibility, in view of a report that SFD-mutant TIMP-3 proteins more readily dimerise or aggregate than wild-type TIMP-3, reflecting differences in ECM affinity,²⁹ it was hypothesised that the free -SH groups in the carboxy-terminal regions of the SFD-mutant TIMP-3 proteins might abnormally bind ligand matrix components with free -SH groups. Despite this, however, SDS-polyacrylamide gel electrophoresis analysis of wild-type and the four SFD-mutant TIMP-3 proteins synthesised by transfected RPE cells¹⁵ or the COS-7 cells used in this study showed no quantitative differences in their banding patterns. In all cases, even after reduction, the immunostained western blots were similar and yielded many bands corresponding to high-molecular-weight aggregated protein, dimerised and monomeric TIMP-3, and degraded TIMP-3. Although differences in the affinities of wild-type and the SFD-mutant TIMP-3s for each of the Bruch's membrane component assayed were not apparent, relative to fibronectin, collagen I, collagen IV, heparin sulphate and chondroitin sulphates A, B and C, both the wild-type and the SFD-mutant TIMP-3 proteins exhibited high binding to elastin and laminin. These findings are consistent with histopathological reports that in eyes with SFD TIMP-3 immunoreactivity predominates in the central elastic layer of Bruch's membrane, the site of maximal disruption,⁸ and between the RPE and elastin sublayer of Bruch's membrane (the RPE basement membrane). Elastin, with which TIMP-3 colocalises, is the major component of the elastic layer of Bruch's membrane, and laminin is a multidomain glycoprotein that functions as a cell-ECM linker. This could be the sub-RPE component that binds the RPE-secreted TIMP-3, and through its attachment to the RPE cells the localised removal of dead RPE cells brings about the observed loss of the underlying TIMP-3 in eyes with SFD.⁸

The chondroitin sulphates A, B and C, and heparin sulphate were assayed as TIMP-3 ligands because of a report that these GAGs extracted TIMP-3 from postpartum rat uterine tissue and that their sulphate groups formed ionic

Figure 3 Relative binding capacities of selected proteinaceous components of Bruch's membrane for wild-type and Sorsby's fundus dystrophy-mutant tissue inhibitor of metalloproteinase (TIMP)-3s. (A) Acid-prepared elastin, (B) KOH-prepared elastin, (C) type-I collagen, (D) type-IV collagen, (E) fibronectin, (F) laminin. Controls were prepared from COS-7 cell cultures exposed to Lipofectamine alone. OD₃₇₀, optical density at 370 nm.

Table 1 Summated percentage adherence of wild-type and Sorsby's fundus dystrophy-mutant tissue inhibitor of metalloproteinase-3s to selected components of Bruch's membrane

Matrix component	Percentage control binding	p Values relative to control
Control (well-base plastic)	100 (3.2)	—
Elastin (acid prepared)	115 (13.1)	0.049
Elastin (KOH prepared)	98 (4.5)	0.510
Type-I collagen	48 (3.2)	<0.001
Type-IV collagen	48 (4.2)	<0.001
Fibronectin	72 (4.7)	<0.001
Laminin	108 (7.2)	0.070
Chondroitin sulphate A	61.5 (9.4)	0.001
Chondroitin sulphate B	67.5 (8.2)	0.001
Chondroitin sulphate C	68.0 (9.9)	0.002
Heparin sulphate	80.4 (10.4)	0.015

bonds with arginine and lysine residues present in the N-terminal domain of TIMP-3.¹⁴ Although there were small differences in the GAG-binding capacity of wild-type and Ser-181 TIMP-3 compared with Gly-167, Ser-156 and Tyr-168 TIMP-3s, these were low in comparison with laminin and elastin. Possibly, the TIMP-3 antibody-binding epitopes could be masked in TIMP-3–GAG complexes because the polyclonal antibody used in this study binds epitopes in the N-terminal loop 1, away from the C terminal that contains the SFD mutations. However, it is the C-terminal domain that is considered to be predominantly associated with matrix–substrate binding¹³; the requirement to use guanidinium hydrochloride or SDS to extract membrane-bound TIMP-3 suggests that matrix ligation involves hydrogen bonding and non-polar interactions. Furthermore, because the N-terminal domain of TIMP-3 is responsible for metalloprotease (MMP and aggrecanase) inhibition,³⁰ GAG binding would probably prevent TIMP-3 from fulfilling this function. For these reasons, and because the TIMP proteins cannot protect GAGs against the action of hyaluronidases and other

Figure 4 Relative binding capacities of selected glycosaminoglycans for wild-type and Sorsby's fundus dystrophy-mutant tissue inhibitor of metalloproteinase (TIMP)-3. (A) Chondroitin sulphate A, (B) chondroitin sulphate B, (C) chondroitin sulphate C, (D) heparin sulphate. Controls were prepared from COS-7 cell cultures exposed to Lipofectamine alone. OD₃₇₀, optical density at 370 nm.

glycolytic enzymes, it seems unlikely that TIMP-3–GAG interactions are of particular biological significance.

In conclusion, elastin and laminin were the components of Bruch's membrane that had greatest affinity for TIMP-3. Moreover, as little difference was observed in the relative affinities of wild-type and SFD-mutant TIMP-3 for each of the ECM components assayed, or in the extent of their dimerisation or aggregation, these properties cannot account for the difference in timing of onset of SFD and ARMD. Thus, if this differential clinical feature is not related to an enhanced sensitivity of the RPE cells to the apoptotic effects of SFD-mutant TIMP-3, it is possible that the SFD-mutant TIMP-3s, once bound to the matrix, are more thermodynamically stable or resistant to proteolytic degradation than the wild type. The resultant accumulation of these TIMPs would reduce the rate of matrix catabolism and favour early thickening. These possibilities are currently under investigation.

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