Sorsby's fundus dystrophy mutant tissue inhibitors of metalloproteinase-3 induce apoptosis of retinal pigment epithelial and MCF-7 cells

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Abstract C-terminal domain tissue inhibitor of metalloproteinases-3 (TIMP-3) mutations cause the rare hereditary blindness Sorsby's fundus dystrophy (SFD), which involves loss of retinal pigment epithelial (RPE) cells. Since wild-type TIMP-3 causes apoptosis, we investigated whether SFD TIMP-3 might kill RPE and other cells. Plasmid-mediated overexpresion of Ser-156, Gly-167, Tyr-168 and Ser-181 SFD mutant TIMP-3 decreased RPE viability to 22 ± 8, 20 ± 6, 32 ± 5, 30 ± 12% (SFD mutants all $P < 0.01$ versus wild-type 50 ± 8%) and similarly increased propidium iodide staining and in situ end labelling. Adenovirus-mediated overexpression of the Gly-167 mutant also caused RPE apoptosis dose-dependently. Apoptosis of RPE cells might therefore contribute to the pathology of SFD.

Key words: Tissue inhibitor of metalloproteinases; Apoptosis; Macular dystrophy

1. Introduction

The four tissue inhibitors of metalloproteinases (TIMPs) have a common structure with three N-terminal and three C-terminal disulphide bonded loops that divide the protein into two domains [1]. TIMPs share the ability to inhibit most members of the matrix degrading metalloproteinase (MMP) family (MMPs), which mediate matrix turnover during physiological and pathological tissue remodelling [1]. Pro-apoptotic effects have been identified for TIMP-3 [2,3] and, more recently, TIMP-4 [4]. The basis of apoptosis induced by TIMP-3 has yet to be fully defined, although it is a property of the N-terminal domain and requires the critical N-terminal cysteine residue necessary for inhibition of MMPs [5].

Sorsby's fundus dystrophy (SFD) is a rare autosomal dominant retinal degeneration which becomes apparent in the fourth decade of life [6]. An early sign is the deposition of lipids and protein in the Bruch's membrane, which separates the retinal pigment epithelium (RPE) from its blood supply, the choriocapillaris [7]. These abnormal deposits thicken the Bruch's membrane and may impede the transport of essential metabolites from the choriocapillaris to the RPE, thus causing RPE dysfunction and cell loss [8]. In addition or consequentially, neovessels invade through the Bruch's membrane and the ensuing sub-retinal haemorrhaging leads to macular scarring and irreversible loss of central vision [9]. In this respect, SFD is similar to age-related macular degeneration (ARMD), which ranks as the most common cause of blindness in developed countries.

Patients with SFD have mutations in exon 5 of the TIMP-3 gene [10–14]. SFD mutant TIMP-3 proteins have gained or lost a cysteine residue in their C-terminal region, thus generating a free SH group. TIMP-3 is synthesised by RPE cells and accumulates in the Bruch's membrane [15–22]. Since SFD mutant TIMP-3 proteins contain an intact N-terminal domain we hypothesised that they might possess the ability to cause apoptosis of RPE cells.

2. Materials and methods

2.1. Reagents and cells

All chemicals, unless otherwise stated, were obtained from Sigma Chemical. Culture media (MEM, DMEM and OptiMEM), foetal calf serum (FCS), glutamine, a penicillin/streptomycin antibiotic mix and Lipofectamine™ were obtained from Gibco BRL. Anti-cytokeratin 18 and anti-pancytokeratin fluorescein-conjugated monoclonal antibodies were obtained from Sigma Chem. Anti-TIMP-3 polyclonal antibody, raised against loop I of TIMP-3 in rabbit, was obtained from Chemicon. Plasmids containing the cDNAs of wild-type and SFD mutant TIMP-3 under a metallothein promoter were generously donated by Professors D. Edwards and G. Murphy (University of Norwich, UK). These were cleaved with the *Sma*I enzyme and subcloned into pCI.neo vectors under a cytomegalovirus early immediate promoter to enhance expression of the TIMP-3 proteins in our transfection system. Additional plasmids for transfections were prepared with plasmid mini kits (Quiagen). Alternatively, wild-type and mutant (Gly-167) TIMP-3 were subcloned into adenovirus shuttle vector pal200 (a generous gift from G. Wilkinson, University of Wales, College of Medicine, UK) downstream of the cytomegalovirus immediate early promoter. Replication-deficient recombinant adenoviruses were produced through homologous recombination, plaque purified, grown to high titre and infectious titre, determined as described [3,23]. All TIMP-3 plasmids and adenovirus inserts were fully sequenced on an applied biosystems automated DNA sequencer to verify the mutations and to ensure that no further mutations had been introduced.

Retinal tissue was obtained from eyes, donated with research per-

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Abbreviations: RPE, retinal pigment epithelium; SFD, Sorsby's fundus dystrophy; TIMP, tissue inhibitor of metalloproteinase

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mission to the Bristol CTS Eye Bank. The age of the donors was under 40 yr and the RPE-choroid sheets were dissected from the ocular globes within 36 h of death.

The human adenocarcinoma cell line, MCF-7, was obtained from the American Tissue Culture Collection.

2.2. Tissue culture

Human RPE-choroid sheets dissected from ocular globes were fragmented using sterile forceps and a scalpel in a small quantity of MEM supplemented with 10% v/v FCS, glutamine and the antibiotic mixture in 5-cm-diameter Petri dishes. The fragmented tissue was then transferred into 25-cm^3 culture flasks and left for a minimum period of 1 h at 36°C in a 5% CO_2 incubator to allow the tissue to adhere to the flasks. Additional MEM (2 ml) was then added to each flask. After 7 days incubation under the same conditions, this was removed and replaced with MEM containing 2.5% v/v FCS to prevent proliferation of fibroblasts. Subsequently this media was replenished every 3–4 days, until the cell cultures became established. To propagate these cultures, the cells were washed with Ca^{2+}-free PBS, trypsinised, collected by centrifugation at 12 000 rpm for 3 min at room temperature, resuspended in fresh MEM medium containing 10% v/v FCS and reseeded into multiple 75-cm^3 culture flasks. For obtaining the cells used experimentally, this passaging procedure was never repeated more than twice. The purity of the RPE cell cultures was checked by immunostaining for cytokeratins 4, 5, 6, 8, 10, 13 and 18 and pan-cytokeratin 18 using corneal keratocyte cultures as a control cell line [21].

MCF-7 cells were maintained at 37°C in DMEM supplemented with 10% FCS (v/v) and antibiotics. All established cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2.

2.3. Methods of transduction

Prior to transfection, trypsinised RPE and MCF-7 cells were plated out, in triplicate, into six-well plates at densities of $\sim 2.5 \times 10^5$ cells/well and incubated in DMEM media containing 10% v/v FCS until the cells achieved around 80% confluence (approximately 24 h). For lipofection, the cells were washed twice with OptiMEM, and then incubated with OptiMEM-containing plasmid DNA and Lipofectamine™ (1 ml total volume). After 6 h an equal volume of DMEM containing 20% v/v FCS was added. After a further 18 h, the medium was replaced with 2 ml DMEM containing 10% v/v FCS and the transfected cells were left for a further 48 h. Alternatively, cells were incubated with adenovirus overnight and then the virus was washed off and the cells incubated in fresh medium for 48 h.

2.4. Quantification of the viable cells number and cell death

Triplicate cell cultures were trypsinised and mixed with an equal volume of trypan blue dissolved in 0.9% saline (0.2% w/v) and the cells that excluded the dye were counted with a haemocytometer. Alternatively, propidium iodide (1 mg/ml stock solution, diluted 1:4000 for use) was added to each well. Permeable were counted microscopically at ×400 magnification in three visual fields. Total cells numbers were subsequently counted after rendering all cells leaky with formalin (10% v/v in PBS), and the percentage permeability was calculated.

In situ end labelling (ISEL) technique was carried out on cells, were plated out in triplicate on sterile glass coverslips. Cells were fixed in 10% v/v formalin for 10 min and washed twice in TE buffer, (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The cells were incubated for 15 min at 20°C with DNA end labelling reagents (Boehringer kit containing 50 mM Tris–HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM dithiotreitol, 0.01 mM dATP, dCTP, dGTP, 0.01 mM biotinylated-dUTP) and 8 U/ml Klenow enzyme (Promega, UK). Cells were rinsed in TE and endogenous peroxidase activity was inhibited by incubation with 2% H_2O_2 for 5 min. After further washing, biotin was labelled with Extravidin™ peroxidase in 10% FCS/PBS (1:200 dilution). Incubation in diaminobenzidine was used to distinguish positive nicked DNA from negative DNA.

2.5. Detection and quantification of wild-type TIMP-3 and SFD TIMP-3 proteins

After transfection, the RPE and MCF-7 cells and their matrices were harvested in PBS containing 2% w/v SDS (100 μl/well). After centrifugation the solubilised proteins in these samples were collected and stored at −20°C. Samples of these proteins were reduced with mercaptoethanol (2%), boiled for 2 min and aliquots (20 μl) were diluted 20-fold with PBS and, using a dot blotter (Biorad), loaded onto pre-wetted PDVF membranes in triplicate alongside serially diluted reference TIMP-3 samples. The membranes were blocked in buffer containing 5% w/v milk powder and 5% v/v mercaptoethanol incubated with the primary antibody raised against loop-1 of human TIMP-3 (Chemicon) and HRP-conjugated secondary antibodies, and the antibody/antigen complexes were visualised using Luminol (ECL Kit, Amersham Pharmacia) as the HRP substrate. The intensities of the dots that appeared on photographic film were determined using a UVP gel documentation system and their image analysis software (UVP, Cambridge, UK).

3. Results

We used plasmid constructs containing cDNA for wild-type and four SFD TIMP-3 mutants (Ser-156, Gly-167, Tyr-168 and Ser-181) to compare human RPE cells with MCF-7 adenocarcinoma cells, which are known to be susceptible to the apoptotic effects of wild-type TIMP-3 [3]. In preliminary experiments, approximately 25% and 20%, respectively, of cultured RPE and MCF-7 cells were transfected under the same conditions with green fluorescent protein. Lipofectamine™ itself reduced viable cell numbers of RPE and MCF-7 cells to $73 \pm 2\%$ and $72 \pm 2\%$ ($n = 4$, $P < 0.001$), irrespective of the concentration of plasmid DNA added (1 or 2 μg). All subsequent transfections were carried out using Lipofectamine™ and a total of 2 μg plasmid DNA, while Lipofectamine™ alone served as the experimental control.

Transient transfections of wild-type and Ser-156, Gly-167, Tyr-168 and Ser-181 SFD mutant TIMP-3s all led to secretion of discrete species of the expected sizes and no other bands by Western blotting (results not shown). We therefore used the simpler dot blotting technique for quantification. Similar amounts of wild-type and Ser-156, Gly-167 or Ser-181 mutant proteins were found in the respective cultures of RPE and MCF-7 cells (Fig. 1, in all cases $P > 0.2$). With the Tyr-168 mutant cDNA TIMP-3 levels were lower and not significantly different than those in the respective untransfected, control cultures of RPE and MCF-7 cells (Fig. 1, $P > 0.2$).

Total viable RPE and MCF-7 cell numbers were significantly decreased by either wild-type or SFD mutant TIMP-3 (Fig. 2A, $P < 0.001$ in all cases). Overexpression of wild-type TIMP-3 reduced the number of viable RPE and MCF-7 cells by $50 \pm 9\%$ and $57 \pm 6\%$, respectively ($P = 0.07$). In addition, as indicated in Fig. 2A, overexpression of the SFD mutant TIMP-3 proteins killed significantly more RPE cells than the wild-type TIMP-3. In the case of the MCF-7 cells, only the Ser-181 and Gly-167 mutants caused significantly more cell death than wild-type TIMP-3.

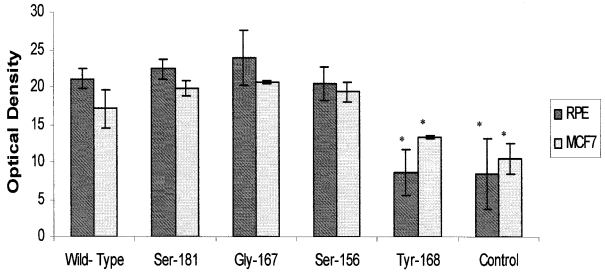

Fig. 1. Quantitative analysis TIMP-3 production. Data show the relative amounts of TIMP-3 produced by RPE and MCF-7cells transfected with plasmid containing wild-type and the SFD mutant TIMP-3 DNA inserts. *$P < 0.001$ compared with wild-type TIMP-3 production ($n = 3$ separate experiments).

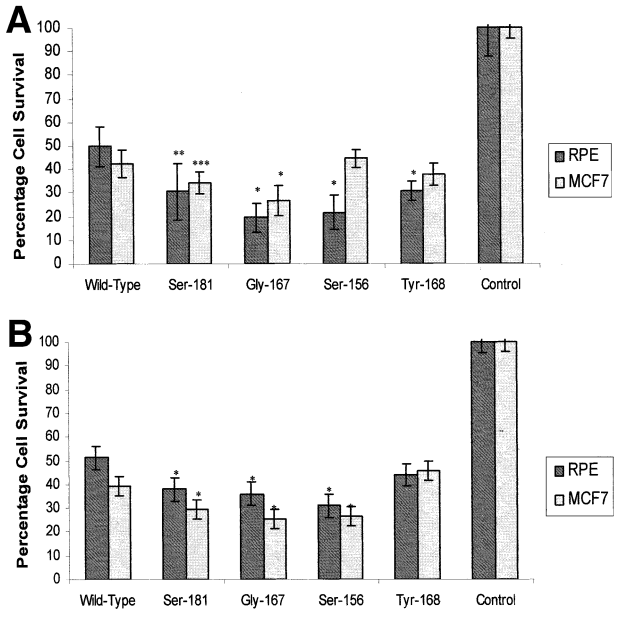

Fig. 2. Cell viability in transfected cultures. A: Relative numbers of RPE and MCF-7 cells that excluded trypan blue remaining 48 h after transfection with wild-type or SFD mutant TIMP-3 compared to cells treated with Lipofectamine alone. B: The proportion of cells with nuclei stained with propidium iodide in the same cell cultures. For comparison with panel A the values are expressed as viability, i.e. 100-percentage stained. $*P<0.001$, $**P<0.005$, $***P<0.006$ compared with wild-type TIMP-3 ($n=9$).

Staining with propidium iodide exhibited the characteristic features of apoptosis, including shrinkage and nuclear chromatin clumping (not shown). Wild-type TIMP-3 overexpression reduced RPE and MCF-7 viability measured with propidium iodide by $49\pm5\%$ and $61\pm4\%$ ($n=9$), respectively. These values are statistically significant ($P<0.001$) and support the possibility that MCF-7 cells are more susceptible to the effects of wild-type TIMP-3 than RPE cells. Each of the Ser-156, Gly-167 and Ser-181 mutants was more effective than wild-type TIMP-3, statistical significance being reached in both RPE and MCF-7 cells. Furthermore, given that Tyr-168 mutant TIMP-3 expression was low compared to the wild-type and other SFD TIMP-3 proteins, the pronounced apoptotic effect seen with this SFD mutant TIMP-3 is remarkable.

In a subset of the cell cultures, ISEL was used as additional or confirmatory evidence that the wild-type and SFD mutant TIMP-3 proteins induce apoptosis of RPE and MCF-7 cells. Fig. 3 shows the dark, condensed, ISEL-positive apoptotic RPE and MCF-7 cell nuclei. From ISEL it was calculated that wild-type TIMP-3 overexpression reduced RPE and MCF-7 viability by $48\pm3\%$ and $62\pm3\%$ (Fig. 4, $n=3$), respectively. These differences are again statistically significant ($P=<0.001$) and confirm that the MCF-7 cells are more susceptible to the apoptotic effects of wild-type TIMP-3 than RPE cells. The percentages of RPE and MCF-7 cells undergoing apoptosis in response to excess wild-type and SFD mutant TIMP-3 protein production (Fig. 4) were consistent with values of cell viability presented in Fig. 2.

We previously showed that adenovirus-mediated overex-

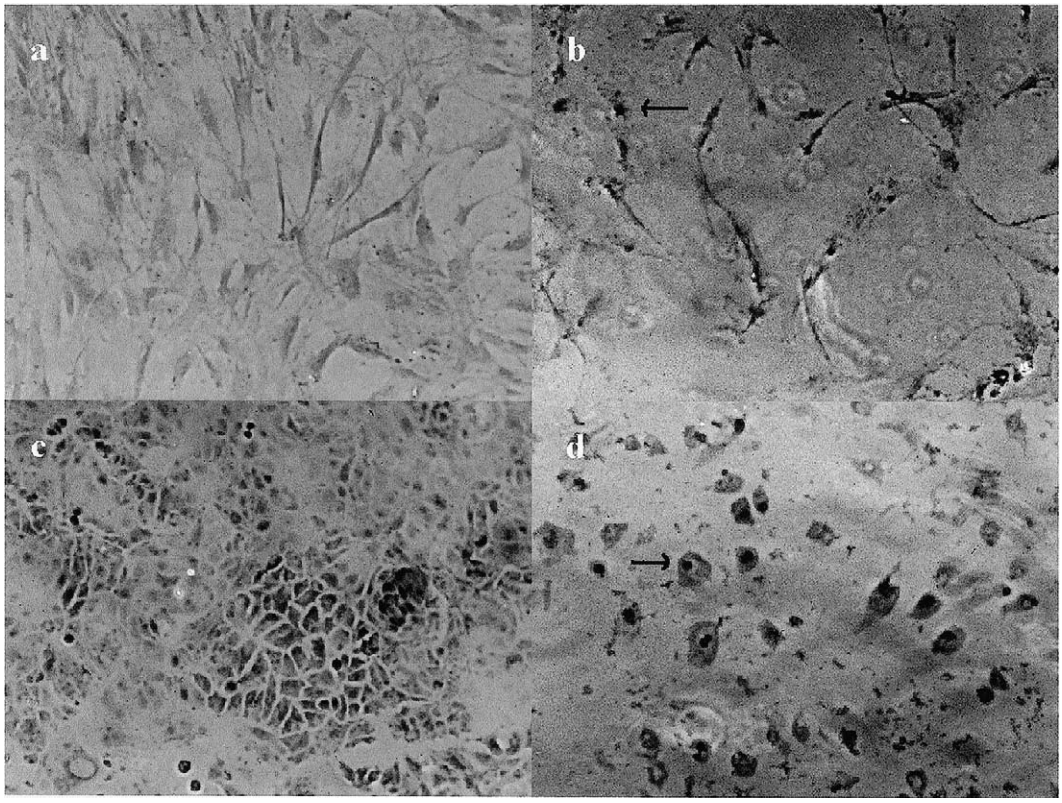

Fig. 3. Detection of apoptotic cells with ISEL. Representative photographs (×83 magnification) of cultured RPE and MCF-7 cells before (a and c, respectively) and after (b and d, respectively) transfection with wild-type TIMP-3. An arrow in panels b and d shows a typical ISEL-positive cell.

pression of wild-type TIMP-3 also causes apoptosis in RPE and cells MCF-7 [3,23]. To confirm the main findings with plasmid-mediated overexpression, we prepared an adenovirus that drove expression of the Gly-167 SFD mutant TIMP-3. Increasing doses of virus expressing wild-type and the Gly-167 SFD mutant caused increasing levels of death of RPE and MCF-7 cells. At each dose, however, slightly but significantly more cells died with the Gly-167 SFD mutant (Fig. 5A,B).

4. Discussion

The ability of SFD mutant TIMP-3 to induce apoptosis has not been investigated previously in any cell type. Among the TIMP proteins, TIMP-3 is unique in that it has a high affinity for cellular matrices and, when expressed at high concentration, induces apoptosis in some but not all cell types [2,3,24,25]. The apoptotic effect of TIMP-3 is not shared by TIMP-1 or TIMP-2 or by several broad spectrum synthetic MMP inhibitors [3,5]. Nevertheless, it does require an intact MMP inhibitory domain [5], and our demonstration here that each of the SFD mutants also promoted apoptosis is consistent with this finding. The most likely mechanism for TIMP-3-induced apoptosis is inhibition of a cell surface metalloproteinase that normally releases a death ligand from the surface of susceptible cells [2]. Consistent with this, recent observations demonstrate that TIMP-3-induced apoptosis is inhibited by dominant negative death-receptor domains and by inhibitors of caspases linked to death receptors [26]. The nature of the death ligand involved may, however, be different in different cell types, and the nature of the target MMP is unclear [26].

To investigate the apoptotic of SFD mutant TIMP-3, we used plasmid- and adenovirus-mediated transduction rather than the simple addition of proteins because it has proved extremely difficult to prepare the recombinant forms of the SFD mutants (unpublished results). However, previous studies with wild-type TIMP-3 demonstrate that similar results are obtained using gene transfer and addition of proteins [3]. In this study, plasmid-mediated TIMP-3 overexpression induced apoptosis of greater than 50% of RPE or MCF-7 cells, even though transfection efficiency was 20–25%. These results confirm that there is a bystander effect for TIMP-3-mediated apoptosis [3], and rule out that cells die as a result of internal accumulation of the TIMP-3.

The data show that SFD mutations modestly increased the apoptotic effect of TIMP-3 on RPE cells. This was confirmed

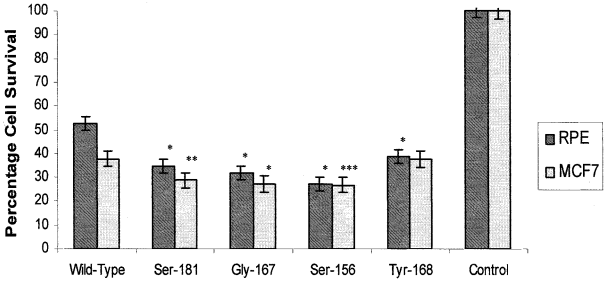

Fig. 4. Apoptosis quantified by in situ end labelling. The percentage of RPE and MCF-7 cells positive for ISEL was counted 48 h after transfection with wild-type or SFD mutant TIMP-3. For comparison with Fig. 2, the values are expressed as viability, i.e. 100-percentage stained. Statistical significance by Student's *t*-test as: $*P<0.001$, $**P<0.005$, $***P<0.01$ compared with wild-type TIMP-3 (n = three separate experiments).

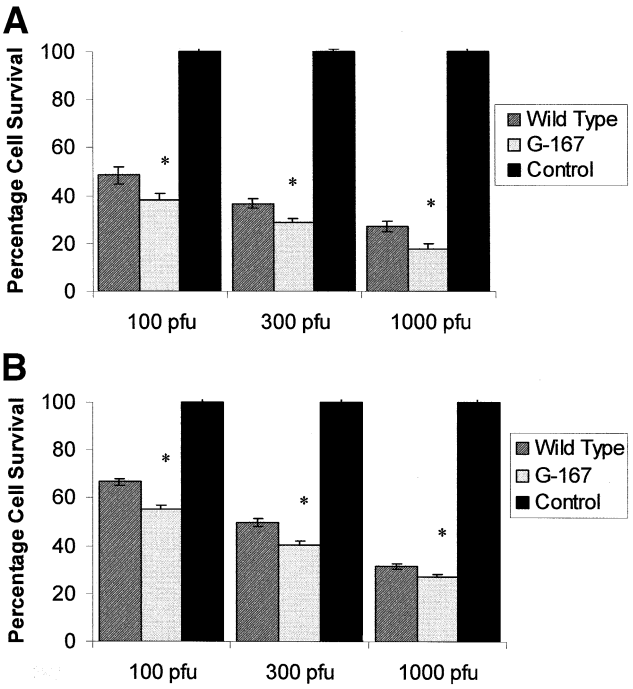

Fig. 5. Cell viability in adenovirus infected cultures. Relative numbers of RPE (A) and MCF-7 (B) cells that excluded trypan blue remaining 48 h after infection with increasing concentrations (plaque forming units/ml) of adenoviruses that drive expression of wild-type or Gly-167 SFD mutant TIMP-3 compared to control cells infected with adenovirus driving β-galactosidase expression. $*P<0.05$ compared with wild-type TIMP-3 (n = three separate experiments).

by three different measures of cell death using plasmid-mediated overexpression. Total cell counts include all methods of cell death, propidium iodide staining gives morphological evidence of apoptosis and in situ end labelling is also diagnostic for apoptosis. Furthermore, adenovirus-mediated transduction gave a similar conclusion for the Gly-167 mutant at three different doses. Given the limitations in quantifying TIMP-3 by dot blotting, the small differences in the sensitivity of RPE cells to wild-type and SFD TIMP-3 should not be overinterpreted. The apoptotic effect of the Tyr-168 mutant TIMP-3 may actually be considerably greater than that of the wild-type protein, but confirmation of this will require the development of a more sensitive assay for TIMP-3 levels. Nevertheless, we do not believe that, by itself, the small increase in apoptotic effect of SFD mutants is likely to fully account for the SFD phenotype.

The dominant nature of the pattern of inheritance of SFD suggests a gain of function mutation. Surprisingly, given the widespread distribution of TIMP-3 throughout the body, ocular disease is the only known consequence of the mutations. The sequence of events leading from the production of a mutant TIMP-3 protein to the formation of the characteristic neovascular complex in the macular region of the eye is presently unknown [16]. Choroidal neovascularisation is common to any condition that causes thickening of the Bruch's membrane, and histopathological studies have shown that excessive TIMP-3 deposition occurs in both ARMD and SFD eyes [16,21]. Mutant TIMP-3 accumulation could reduce the rate of turnover of the Bruch's membrane through MMP inhibition and the resultant pathological thickening could inhibit the flow of nutrients to RPE cells and cause their demise.

However, given our demonstration that wild-type and mutant TIMP-3 overexpression caused apoptosis of RPE cells, we suggest that RPE apoptosis triggered by accumulation of mutant TIMP-3 could also be a contributory mechanism.

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