Adenovirus mediated gene delivery of tissue inhibitor of metalloproteinases-3 induces death in retinal pigment epithelial cells

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Background: Sorsby’s fundus dystrophy (SFD) and age related macular degeneration (ARMD) are retinal diseases associated with a high level of accumulation of mutant and wild type TIMP-3, respectively, in Bruch’s membrane. The pathogenic role of TIMP-3 in these diseases is uncertain, but causative mutations have been identified in the TIMP-3 gene of patients with SFD. Recent reports that TIMP-3 causes apoptosis in certain cell types and not in others prompted the authors to investigate whether TIMP-3 causes apoptosis in cultured retinal pigment epithelium (RPE) cells.

Methods: RPE and MCF-7 cells (as a positive control) were initially infected with replication deficient adenovirus, to overexpress β-galactosidase (RadLacZ) or TIMP-3 (RadTIMP-3). TIMP-3 was detected by western blotting and ELISA. Cell viability was defined by cell counts. ISEL was used to investigate the mechanism of cell death.

Results: Cultured RPE cells produced small quantities of endogenous TIMP-3 and remained viable. However, overexpression of TIMP-3 caused a dose related death of RPE cells. The mechanism of cell death was apoptosis.

Conclusion: The previously unreported finding of TIMP-3 induced apoptosis of RPE cells may account for some of the early features seen in SFD and ARMD.

Materials and Methods

Reagents

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co (UK) and were of the highest grade available. Culture media (MEM and DMEM), fetal calf serum (FCS), and glutamine were obtained from Gibco BRL. A penicillin, streptomycin and amphotericin antibiotic/antimycotic solution, the fluorescent cytokeratin 8 and 18 antibodies, and the horseradish peroxidase conjugated secondary antibodies were all obtained from Sigma Diagnostics (UK). The polyclonal TIMP-3 antibody, raised against loop-1 of human TIMP-3 was obtained from Chemicon (UK).

Experimental tissues and cell lines

Retinal tissue was obtained from cadaver eyes, donated to the Bristol Eye Bank with permission for research. The age of the donors was under 40 years, and the RPE sheets were dissected from the ocular globes within 36 hours of death.

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

Human RPE cells

Following a simplified version of previously described methods,19–23 eyes were opened posterior to the ora serrata and the vitreous and retinal tissues were removed. The RPE sheets were dissected and fragmented using sterile forceps and a scalpel in a small quantity of MEM medium supplemented with 10% v/v FCS, glutamine, and the antibiotic/antimycotic mixture in 5 cm diameter petri dishes. The fragmented tissue was then transferred into 25 cm² culture flasks and left for a minimum period of 1 hour at 36°C in a 5% carbon dioxide incubator to allow the tissue to adhere to the flasks. Additional MEM, with 10% v/v FCS (2 ml), was then added to each flask. After 3 days’ incubation under the same conditions, the MEM was removed. As fibroblasts and choroidal cells fail to grow in low serum conditions, to inhibit their propagation, the medium was replaced with MEM containing 2.5% v/v FCS. This was subsequently replenished every 3–4 days. Immediately before infection, the RPE and MCF cells of three wells were trypsinised and counted with a haemacytometer. Cells were visualised microscopically at ×400 magnification using a cobalt blue filter (488 nm).

Recombinant adenoviruses

Recombinant replication defective adenovirus RAdlacZ (RAd35), which contains the *Escherichia coli* β-galactosidase (lacZ) gene under the control of cytomegalovirus major immediate early promoter (CMV IE), was kindly donated by Dr Gavin W G Wilkinson (University of Cardiff, Wales). The construction and characterisation of the replication deficient adenovirus containing the coding region of the human TIMP-3 (RAdTIMP-3) gene driven by CMV IE promoter has been described in detail previously.24

Adenoviral infection protocol

RPE and MCF7 cells were plated separately into six well plates at densities of 2.5 × 10⁵ cells/well and incubated in complete medium for 24 hours. Immediately before infection, the RPE and MCF cells of three wells were trypsinised and counted with a haemacytometer. Cells in the remaining wells were infected at the required adenoviral titre in 2 ml fresh medium and left for 18 hours. After removing the medium and washing, the cells were incubated in 2 ml fresh complete medium. Viable cell numbers were determined after a further 48 hours.

Determination of infection efficiency of RPE and MCF7 cells

To determine the infection efficiency of RPE and MCF7 cells, plated cells were mixed, in triplicate with 100, 300, and 1000 pfu/cell of RAdlacZ, respectively, and incubated for 18 hours. After washing, these cells were incubated for a further 48 hours in fresh complete medium. Cells were stained with X-gal stain (5-bromo-4-chloro-3-indoyl-β-D-galactosidase) (100 mM sodium phosphate pH 7.3 (77 mM Na₂HPO₄, 23 mM NaH₂PO₄), 1.3 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, and 1 mg/ml X-gal). Positive (blue) cells were counted in three high power (×400) fields per section from triplicate cultures and the percentage infection efficiency calculated.

Estimation of viable cell density

To estimate the number of viable cells remaining after infection with RAdTIMP-3, cell cultures were trypsinised after 48 hours, mixed with an equal volume of trypan blue (0.2% w/v) in PBS, and counted with a haemacytometer. All counts were performed in triplicate.
Detection of TIMP-3 protein

After RA(t)TIMP-3 or RAdlacZ infection, the cells and their matrices were harvested in 0.05 M TRIS, pH 7.4 containing 2% w/v SDS (100 µl/well). Conditioned media were collected and 10-fold concentrated using Centricon microconcentrators (Amicon Inc, Stonehouse, UK). The samples were reduced with mercaptoethanol (2%), boiled for 10 minutes, and electrophoretically fractionated on SDS polyacrylamide (10% w/v) gels. The proteins in these gels were then western blotted onto PDVF (Millipore) membranes and immunostained using the polyclonal TIMP-3 primary antibody (1:200 with TRIS buffered saline + 5% FCS) and horseradish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody (1:1000). Diaminobenzidine (DAB) was used as the HRP substrate, following the manufacturer’s instructions.

TIMP-3 ELISA assays

To confirm that normal, healthy, uninfected RPE cells produce TIMP-3, confluent cultures of RPE cells and corneal keratocytes were washed with PBS and harvested in methanol. After homogenisation using a small glass homogeniser, aliquots of suspension were dried in air. Blocking solution (0.05M TRIS-HCl, pH 7.4 containing 150 mM NaCl, 5%v/v FCS, 2 mM mercaptoethanol, and 0.02% NaN3) was added for 2 hours, followed by primary TIMP-3 antibody (1:500 with TRIS buffered saline and 5% FCS) and secondary HRP conjugated rabbit IgG antibody (1:1000) with stringent washing, with TRIS buffered saline and TRIS buffered saline + 0.2% Tween 20, before and after incubation with each antibody. The HRP substrate, TMB (Sigma, UK) was added according to the supplier’s instructions and the kinetics of oxidation of TMB were followed at 370 nm at 30°C, using a Molecular Devices Spectromax Spectrophotometer.

In situ end labelling (ISEL) protocol

ISEL was used to determine whether the mechanism of RPE and MCF-7 cell death was apoptosis. The cells were plated out, in triplicate, on sterile glass coverslips and infected with RA(t)TIMP-3 at 1000 pfu/cell. ISEL was performed according to Baker et al.14 Incubation in cobalt-DAB was used to distinguish positive nicked DNA from negative DNA.

RESULTS

RPE cell cultures

Primary RPE cells isolated from the retinal explants (Fig 1A) were ready for experimentation in 21 days (passage 1 or 2). In concurrence with previous reports,23 the cultured RPE cells adopted several different morphological forms (Fig 1B, C, D). Although cells tended to become fusiform in shape (Fig 1D),...
pigmented polygonal RPE cells were still present in primary cultures maintained for 12 months (Fig 1C). All cells in the RPE cell cultures were positive for cytokeratin 8 and 18 (Fig 1F, G, H), indicating no significant contamination by fibroblasts or choroidal cells. The control corneal fibroblasts were negative for these cytoskeletal proteins (Fig 1E).

**Determination of transduction efficiency of RPE and MCF7 cells**

Photographs of RPE and MCF-7 cells infected with RadlacZ and stained with X-gal are shown in Figure 2. Histograms of the estimated numbers of infected cells in these cultures are given in Figure 3. The summed data indicate that 91% (SD 4%) of RPE cells and 92% (4%) of MCF7 cells were infected at 1000 pfu/cell. At 300 pfu/cell, 71% (5%) of RPE cells and 63% (5%) of MCF7 cells were infected and at 100 pfu/cell, 34% (3%) of RPE and 29% (4%) of MCF7 cells were infected. The numbers of RPE and MCF-7 cells infected were not significantly different (p >0.2, Student’s t test) and indicate that these cell lines are equally susceptible to infection with RadlacZ.

**Analysis of transgene production**

Evidence that TIMP-3 was produced by RadTIMP-3 infected RPE and MCF-7 cell cultures is given in Figure 4A, by a representative immunostained western blot of the SDS extracted proteins from cell matrix fractions and from 10-fold concentrated conditioned media. In the cell matrix fractions, the two characteristic TIMP-3 bands of Mr 24 000 and 28 000, corresponding to the unglycosylated and glycosylated forms, respectively, were both present. The glycosylated form of TIMP-3 was predominant (lanes 3 and 7). In addition, a band at Mr 50 000, consistent with dimerised TIMP-3 was seen. Tenfold concentrated media fractions showed similar but lower intensity bands (lanes 1 and 5). Similar extracts of RPE and MCF-7 cell matrices and 10-fold concentrated conditioned media, obtained after infection with RadlacZ contained little or no detectable TIMP-3 in RPE conditioned media or cell matrix fractions (lanes 2 and 4), or MCF7 fractions (lanes 6 and 8).

**Confirmation that low levels of TIMP-3 are produced by RPE cell cultures**

Despite, the failure to reliably detect TIMP-3 in normal RPE cell cultures by western blotting, evidence that cultured RPE cells normally produce TIMP-3, was obtained by ELISA. For comparative purposes, similarly obtained protein samples from corneal fibroblasts and their cell matrices were also assayed. The results obtained are shown in Figure 4B.

**Effect of TIMP-3 overexpression on RPE and MCF7 cell survival**

TIMP-3 overexpression resulted in a dose dependent reduction in cell number in both RPE and MCF7 cultures. RPE and MCF7 cells, 48 hours after infection with 300 pfu/cell of RadTIMP-3, are shown in Figure 5A and B, respectively. The percentage survival of RPE and MCF-7 cells at increasing RadTIMP-3 infection dosage is shown in Figure 5C. The averaged percentage of viable RPE cells remaining after infection with 100, 300, and 1000 pfu/cell RadTIMP-3 was 69% (3%), 52% (2%), and 24% (2%), respectively.

Given that the RPE and MCF-7 cells were equally susceptible to infection by RadlacZ, it follows that the RPE cells were more readily killed by infection with RadTIMP-3 than the MCF-7 cells (p values <0.001, Student’s t test).

**Evidence that TIMP-3 induces RPE cell apoptosis**

ISEL staining of the dead or dying RPE and MCF-7 cells in cultures infected with RadTIMP-3 at 1000 pfu/cell showed dark DAB stained nuclei, providing evidence that the mechanism of TIMP-3 induced RPE and MCF7 cell death was apoptosis. Control cells, infected with RadlacZ, showed no DAB staining. Representative photographs of these cells are given in Figure 6.

**DISCUSSION**

The pathological processes that occur in Sorsby’s fundus dystrophy and ARMD are similar and involve the excessive accumulation of mutant or wild type TIMP-3 in Bruch’s...
The micropathological sequence of events in these conditions, however, is largely unknown. 7-10

TIMP-3 is a matrix metalloproteinase inhibitor and as such its accumulation may limit the degradation and promote thickening of tissues rich in collagens, elastin, and basement membrane, such as Bruch's membrane. Diffuse thickening of Bruch's membrane is seen as a late pathological feature in these conditions and is thought to lead to suppression of nutrient delivery to RPE cells, resulting in RPE cell death. Despite this, pathological reports on SFD and ARMD nutrient delivery to RPE cells, resulting in RPE cell death.

In addition to MMP inhibition, recent reports have shown that TIMP-3 can induce apoptosis in certain cell types, but not in others. 7-10 Our present study confirmed that healthy RPE cell cultures produced small amounts of TIMP-3 and remained viable for long periods. However, infection of RPE cells with recombinant replication deficient adenoviruses resulted in high levels of transgene production. Specifically, high level TIMP-3 overexpression, dose dependently, induced apoptosis in RPE cells. These results were consistent with those of MCF7 cells.

Given that RPE cells are the origin of the majority of TIMP-3 in the eye and that TIMP-3 accumulates in Bruch's membrane, 7,10 it is likely that there is a concentration gradient of TIMP-3 between RPE cells and Bruch's membrane. We propose that early, localised accumulation of TIMP-3 around RPE cells accounts for the RPE cell loss seen in early SFD and ARM. This phenomenon was previously unrecognised. TIMP-3 may consequently bind to components of Bruch's membrane and interactions within Bruch's membrane and with MMPs are likely to play a part in the late pathology of these diseases. These interactions and the role of the SFD mutant proteins remain to be explored.

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