Modulation of hydrogen peroxide induced injury to corneal endothelium by virus mediated catalase gene transfer

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Aim: To examine the effect of catalase gene transfer on survival of corneal endothelial cells (EC) following challenge with hydrogen peroxide (H$_2$O$_2$) in an ex vivo model of oxidative stress.

Methods: A recombinant adenovirus vector (AdCL) was used to transfer human catalase cDNA into EC of whole thickness rabbit corneas ex vivo. The resulting catalase protein concentration was measured in corneal lysates by ELISA; catalase functional activity in lysates was determined using a H$_2$O$_2$ activity assay. To examine the morphological effects of catalase gene transfer in modulation of H$_2$O$_2$ induced injury, transduced corneas were maintained in ex vivo culture and challenged with H$_2$O$_2$. Laser scanning confocal microscopy was used to image EC injury. Cell density, cell morphology, and ratios of viable to necrotic cells were determined.

Results: Following incubation with AdCL, catalase expression reached maximum at 5–7 days. Corneas transduced with AdCL showed increased EC cell survival following challenge with H$_2$O$_2$ on day 3 when compared to null vector control or mock infected corneas.

Conclusions: Ex vivo catalase gene transfer can protect EC from death mediated by H$_2$O$_2$. This gene based approach to the protection of corneal endothelium from oxidative stress may have application in prevention of EC loss in pathological conditions in which H$_2$O$_2$ is involved and in ex vivo donor corneal storage before transplantation.

Death of corneal endothelial cells (EC), by necrosis and possibly apoptosis, plays a major part in many corneal diseases and in corneal graft failure. Human EC do not undergo mitosis to any significant extent and respond to loss of neighbouring cells by enlargement and spreading. In consequence, EC death results in diminished cell density. If density is reduced below critical levels, the endothelial deturgescence pump fails and aqueous enters the stroma, resulting in oedema and loss of corneal transparency.

Hydrogen peroxide (H$_2$O$_2$) is produced by the cornea and neighbouring tissues. Aqueous humour itself generates H$_2$O$_2$, with reported maximum levels of approximately 0.09 mM in bovine aqueous. High intraocular concentrations of reactive oxygen species such as H$_2$O$_2$ have been found in inflammatory disorders of the eye and are associated with tissue damage. A significant role has been attributed to oxidants in the loss of EC in ageing and in some disease states.

In addition to the significance of EC loss in disease, death of these cells is also a major limiting factor in maintenance of donor corneas ex vivo before transplantation. Falling EC density during storage accounts for about 30% of corneas being discarded from eye banks, the endothelium being considered of insufficient quality for transplantation. Both necrosis and apoptosis in endothelium of stored corneas have recently been described, indicating that intervention strategies would be required to protect EC from death by both pathways. Oxidative damage to EC by free radicals is one possible cause of cell loss during ex vivo storage. Protection of human vascular endothelium from oxidant injury by adenovirus mediated transfer of the gene encoding human catalase has been shown. This construct has also been used by Guy et al in suppression of experimental optic neuritis.

The feasibility of gene transfer to endothelium of corneas ex vivo having been demonstrated in previous reports, in order to examine possible use of gene based approaches to protect EC both during ex vivo storage and following transplantation, we tested the effect of catalase gene transfer on H$_2$O$_2$ induced cell death.

METHODS

Recombinant adenovirus vectors

The recombinant adenovirus containing a cDNA sequence encoding human catalase (AdCL) has been previously described. It is a replication defective recombinant type 5 adenovirus (Ad5) containing the 2.3 kb human catalase cDNA (a kind gift of Dr R Crystal, Cornell University, New York, USA). The vector control used was an E1 deleted Ad5 with no cDNA insert (Ad0, kindly provided by Dr Matthew Wood, University of Oxford, UK).

Virus propagation and purification

AdCL and Ad0 viruses were propagated in transcomplementing 293 cells as previously described. In brief, the 293 cells were cultured in DMEM 10% (v/v) fetal calf serum (FCS, Gibco, Paisley, UK), 2 mM l-glutamine (Flow Labs, Irvine, UK) 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) in 5% carbon dioxide at 37°C. At approximately 80% cell confluence, the culture medium was replaced with medium based on 2% (v/v) FCS and inoculated with adenovirus at a multiplicity of infection of approximately 10. When cytopathic effect occurred after 36–48 hours the infected cells were harvested and resuspended in 1/50th of volume of culture supernatant. Virus was released from the infected cells by three freeze-thaw cycles. The virus was purified by two rounds of caesium chloride (Boehringer Mannheim, Lewes, UK) density gradient centrifugation. The virus preparation was passed through a 0.2 µm filter and stored at −70°C. Virus titres were determined by a plaque assay to be 5×10$^5$ plaque forming units (pfu)/ml and 10$^9$ pfu/ml for AdCL and Ad0, respectively.
Corneal culture and transduction conditions

Corneas of New Zealand white rabbits were excised with a thin scleral rim and were cut into quarters, particular care being taken to minimise instrumental damage to the endothelium. For transduction, corneal specimens were placed into a 96 well plate, and incubated at 37°C in 5% carbon dioxide in 250 μl medium (MEM supplemented with 2% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing adenoviruses at different concentrations or no virus (mock infected negative controls). After 3 hours the samples were washed three times in PBS, transferred into a 24 well plate and 2 ml medium (MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) added. Medium was replaced on day 1 and subsequently every second day.

Exposure to H2O2

Rabbit corneal specimens were washed three times in PBS and placed endothelial side up in wells of a 24 well plate and covered with 995 μl of medium (MEM without FCS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Five μl of 100 mM H2O2 stock solution was added and mixed by pipetting up and down three times with a 1 ml pipette (final concentration 0.5 mM H2O2). The corneas were incubated for 16 hours at 37°C and 5% carbon dioxide and then examined by laser scanning confocal microscopy.

Preparation of corneal lysates

Transduced quarter corneas were washed five times with PBS before incubation with 1 ml of 40 mM TRIS-HCl pH 7.5, 1 mM NaCl for 5 minutes at 4°C. After centrifugation at 14 000 rpm (Jouan microfuge, Cedex, France) at 4°C for 10 minutes, the supernatant was discarded and 120 μl of 0.25 TRIS-HCl (pH 8.0) added. Corneal cells including epithelium, kerocytes and endothelium were lysed by three freeze-thaw cycles and the supernatants containing the cytoplasmic contents of the cells were aliquoted and stored at −20°C.

Competitive ELISA for catalase

A competitive ELISA was used as previously described to determine catalase levels in transduced cells. Briefly, a 96 well plate was coated with 1 μg of human erythrocyte catalase (Sigma, Poole, UK) and the substrate blocked with 1% BSA in PBS. Cell lysates obtained from either untransduced cells or cells transduced with catalase cDNA were mixed with rabbit anti-human erythrocyte catalase antibody (Calbiochem, San Diego, USA) at 1/10 000 dilution, and after 30 minutes of incubation at 37°C, Ag-Ab mixtures were added to wells of the ELISA plate and incubated for further 1 hour. After washing, biotinylated goat anti-rabbit IgG antibody (Dako, Denmark) was added at 1/20 000 dilution. After 1 hour’s incubation, extrAVIDIN (Sigma) at 1/1000 dilution was added to each well and incubated for 30 minutes. Following each step the wells were washed four times with PBS/0.1% Tween-20. Reactions were developed by using OPD substrate and stopped by adding 0.1 M H2SO4. The reaction was stopped by adding 100 μl of 6 mM sodium azide (Sigma) before incubation with 1 ml of o-DD solution (5% FCS, 100 mg/ml gentamicin sulphate (Sigma) 80 mg/ml o-DD in Eagle’s MEM without phenol red) and 10 μl of 1000 U horseradish peroxidase type IV (Sigma)/ml dH2O for 1 hour at 37°C. Finally, 150 μl of each reaction was transferred to individual wells of a flat bottomed 96 well ELISA plate. Absorbance was read at 470 nm using a microplate reader. Catalase activities were determined by reference to a standard curve constructed with serial dilutions of human erythrocyte catalase.

Laser scanning confocal microscopy

Rabbit corneas were cut in quarters, infected with AdCL, and either incubated with H2O2 or left untreated as controls. Corneal specimens were transferred to a vital staining solution containing propidium iodide (PI, 50 μg/ml, red in fluorescence light) and acridine orange (AO, 25 μg/ml, green in argon fluorescence light) and imaged by confocal microscopy (Bio-Rad MRC 600, fitted with an argon laser), as described by Wustman et al. Viable EC exclude PI and therefore appear green because of AO staining. Necrotic cells have a permeable cell membrane which cannot exclude PI: this results in red fluorescent nuclear staining on account of displacement of AO from DNA. Where necessary a z-plane series was carried out to adjust for the undulating endothelial surface.

Statistical analysis

Data are expressed as the mean (SD) of triplicate samples. The Mann-Whitney U test was used to determine the significance of differences between the means of treated and untreated groups.
RESULTS

Optimisation of virus concentrations for gene transfer

Catalase concentrations

Triplicate corneal specimens transduced with 0, $10^7$, $2.5 \times 10^7$, $5 \times 10^7$, $7.5 \times 10^7$, and $1.5 \times 10^8$ pfu per specimen AdCL were analysed for catalase production by ELISA after an interval of 3 days. The assay was repeated twice with consistent catalase activity results. Activity increased with the amount of AdCL virus applied, reaching 795 mU after incubation with $1.5 \times 10^7$ pfu. The dose-response curve reached a plateau at approximately $5 \times 10^7$ pfu of AdCL (Fig 1B). No increased expression of catalase was detected in lysates of Ad0 infected corneas compared to mock infected corneas (data not shown). The higher levels of endogenous catalase detected in the negative controls by the activity assay reflect the reduced sensitivity of the ELISA (specific for human catalase) to detect rabbit catalase when compared to the activity assay.

Catalase activity

Triplicate corneal specimens were transduced with up to $1.5 \times 10^8$ pfu AdCL as described above; corneal lysates were prepared and examined by the H$_2$O$_2$ activity assay. The assay was repeated twice with consistent catalase activity results. Activity increased with the amount of AdCL virus applied, reaching 795 mU after incubation with $1.5 \times 10^7$ pfu. The dose-response curve reached a plateau at approximately $5 \times 10^7$ pfu of AdCL (Fig 1B). No increased expression of catalase was detected in lysates of Ad0 infected corneas compared to mock infected corneas (data not shown). The higher levels of endogenous catalase detected in the negative controls by the activity assay reflect the reduced sensitivity of the ELISA (specific for human catalase) to detect rabbit catalase when compared to the activity assay.

Kinetics of catalase production

Concentration profile

Rabbit corneal specimens (quarter corneas) were transduced with $2.5 \times 10^7$ pfu AdCL as described above. This concentration of virus was chosen as it was just below that which produced maximal expression in the titration described above. From each cornea, three quarters were transduced by virus and one was incubated in virus-free medium. Triplicate specimens were incubated for up to 21 days, cell lysates prepared, and the concentration of catalase determined by ELISA. A peak concentration of catalase protein was reached on day 5 with 420 mU/quart cornea (Fig 2A).

Catalase activity assay

Corneal lysates prepared as above for the ELISA time course were used in the catalase activity assay. Corneal specimens were transduced with $2.5 \times 10^7$ pfu AdCL and cell lysates analysed from 1 to 21 days. Peak activity was detected at days 5–7, with 490 and 510 mU per sample respectively. At 14 days, no significant difference was found compared to negative controls (Fig 2B).

Imaging of EC and exposure to H$_2$O$_2$

Confocal microscopy was used to assess the number of viable and necrotic cells, as well as cell morphology, in intact corneas (Fig 3A–C). These were then used to optimise the dose and incubation time with H$_2$O$_2$. In most experiments we chose to contained catalase. The background levels of catalase are a consequence of endogenous enzyme levels in the cornea (including that contained in epithelial cells and keratocytes as well as EC).

Catalase activity

Triplicate corneal specimens were transduced with up to $1.5 \times 10^8$ pfu AdCL as described above; corneal lysates were prepared and examined by the H$_2$O$_2$ activity assay. The assay was repeated twice with consistent catalase activity results. Activity increased with the amount of AdCL virus applied, reaching 795 mU after incubation with $1.5 \times 10^7$ pfu. The dose-response curve reached a plateau at approximately $5 \times 10^7$ pfu of AdCL (Fig 1B). No increased expression of catalase was detected in lysates of Ad0 infected corneas compared to mock infected corneas (data not shown). The higher levels of endogenous catalase detected in the negative controls by the activity assay reflect the reduced sensitivity of the ELISA (specific for human catalase) to detect rabbit catalase when compared to the activity assay.
adenoviral mediated transfer of a histochemical marker gene indicates that this vector transduces the endothelium only. This catalase expression was shown to protect EC from cytotoxic effects of H$_2$O$_2$. The H$_2$O$_2$ might be exerting its toxic effect by oxidative damage either intracellularly or on the cell surface. As catalase is expressed within the cell one might expect that it is more effective in protection against intracellular damage, and that surface structures of the endothelial cells might be degraded before H$_2$O$_2$ is degraded within the cell.

Previous work has indicated that low concentrations (250–500 µM) of H$_2$O$_2$ can induce apoptosis in some cell types. To investigate whether H$_2$O$_2$ induces apoptosis rather than oxidative injury and necrosis in corneal EC, we examined corneas with light, electron, and laser confocal microscopy combined with Annexin V and PI staining or with TUNEL assay for intervals up to 3 days following incubation with a range of H$_2$O$_2$ concentrations. By these methods, apoptosis could not be detected (data not shown). These findings suggest that the morphological changes found after H$_2$O$_2$ challenge are not a result of cell death by apoptosis.

Oxidative stress as a pathological feature in many corneal disorders might be treatable or preventable with gene based approaches. In the context of corneal transplantation, gene therapy strategies aimed at preventing corneal allograft failure have relied on the expression of immunomodulatory proteins. The approach outlined here has a different goal—namely, protection of the target tissue from death induced by the immune response or the stress of the grafting procedure. Similar strategies have been applied to hepatic transplantation, with the expression of both antioxidant and anti-apoptotic genes being used to reduce damage to grafts following transplantation. Damage to endothelium by oxidative stress may also be important in ex vivo donor corneal storage before transplantation. Prevention or even attenuation of endothelial injury in this setting would have major benefits in eye banking as it would result in (i) fewer donor corneas discarded on account of insufficient endothelial cell density following storage, and (ii) augmented endothelial function in donor corneas in vivo following transplantation. The causes of oxidative stress can include not only exogenous reactive oxygen intermediates, such as H$_2$O$_2$, but also agents that induce intracellular production of such molecules. For example, expression of catalase has been shown to protect cells from apoptosis induced by the cancer chemotherapy agent, cisplatin.

It is axiomatic that the benefit of a gene based approach to cell protection would depend on an acceptably low level of cytotoxicity and immunogenicity caused by the vector for gene transfer. In the studies described above, a null adenovirus vector control Ad0 was included in experiments to indicate any virus specific endothelial cell injury. However, it is clear that if gene based approaches to modulation of oxidative stress are to become useful in clinical practice, non-viral vectors or recombinant viruses with more extensive deletion of virus genes will be used, not least to minimise the immune response to virus infected EC following transplantation into a recipient eye. The kinetics of catalase production, with maximum levels detected at 5–7 days following exposure to virus vector, are similar to those reported in other studies of ex vivo adenovirus mediated gene transfer to the cornea.

For application of this approach in corneal storage before transplantation, longer term expression of catalase would also be desirable in order to allow EC protection during longer periods of ex vivo maintenance and in the earliest days in vivo following transplantation. This is likely to become feasible with further vector development.

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