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# Gene transfer of cyto-protective molecules in corneal endothelial cells and cultured corneas: Analysis of protective effects in vitro and in vivo

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**Abstract**

The loss of corneal endothelial cells plays a critical role in many corneal diseases and is a common phenomenon following cornea transplantation. In addition, the non-regenerative capacity of human corneal endothelial cells (HCEC) ultimately requires appropriate protection of corneal tissues during ex vivo storage to ensure vitality of the cells. However, only 70% of donor corneas can be used for grafting because of endothelial deficiencies. Corneal endothelial cell loss during storage is mainly induced by apoptotic cell death. This study was undertaken, for proof of principle, to investigate whether over-expression of cyto-protective molecules Bcl-$x_L$, Bag-1, and HO-1 prevents the loss of corneal endothelial cells both in vitro and in vivo.

We demonstrate that gene transfer of both Bcl-$x_L$ and HO-1 has cyto-protective effects on HCEC in vitro. However, gene transfer of a single cyto-protective molecule does not prevent its rejection upon transplantation in a MHC class I/II disparate rat model.


*Keywords:* Gene therapy; Anti-apoptotic genes; Cyto-protection; Bcl-$x_L$; Transplantation; Cornea; Adenovirus

The loss of corneal endothelial cells plays a critical role in many corneal diseases and is a common phenomenon following penetrating keratoplasty (cornea transplantation) [1–3]. In addition, the non-regenerative capacity of human corneal endothelial cells (HCEC) ultimately requires appropriate protection of corneal tissues during ex vivo storage to ensure vitality of the cells. However, despite improved storage conditions only 70% of the donor corneas can be used for grafting because of endothelial deficiencies [4]. Corneal endothelial cell loss during storage is mainly induced by apoptotic cell death [3]. Recently, it has been described that inflammatory cytokines can induce apoptosis of corneal endothelium [5].

Many intracellular proteins are involved either in the induction or prevention of apoptosis. Bcl-$x_L$ is a member of the Bcl-2-family, which is able to regulate the membrane permeability and the release of cytochrome *c* from mitochondria [6]. This protein also confers resistance to a wide variety of pro-apoptotic stimuli. Bag-1, another member of the family of anti-apoptotic genes, exerts its protective effects mainly by binding and stabilizing Bcl-2 [7]. In animal models of retinitis pigmentosa, gene transfer of Bcl-2 and Bag-1 is effective in the prevention of photoreceptor cell loss

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[8]. Heme oxygenase-1 (HO-1, hsp 32) is a member of the family of heat shock proteins and is induced after exposure to various stress-mediating stimuli. The cyto-protective effects of HO-1 are based on the products of the heme catabolism [9]. Previous studies demonstrated that expression of HO-1 in the eye might be used as a protective strategy against various pathogenic stimuli [10]. In addition, homeostasis of calcium ions ($Ca^{2+}$) linking with $Ca^{2+}$-permeable ion channel activities plays a crucial role in apoptosis [11,12].

Gene therapy could be helpful to protect endothelial cells using ex vivo transfer of cyto-protective genes. A major advantage of this strategy relies in the fact that the donor cornea can be modified ex vivo prior to transplantation. Therefore, side effects of systemic gene therapy could be avoided [13]. Adenoviral (Ad) vectors have been shown to be very efficient gene transfer vehicles for transducing corneal endothelial cells [14–16].

In the present study, for the proof of principal, we investigated whether over-expression of the cyto-protective genes Bcl-$x_L$, Bag-1, and HO-1 protects corneal endothelial cells from apoptosis both in vitro and in vivo.

## Materials and methods

*Cell line.* A human corneal endothelial cell line (HCEC) was used in this study [17]. In brief, cells were cultured in DMEM supplemented with 2% FCS, 5% glutamine and 5% penicillin/streptomycin at 37 °C.

*Animals.* Inbred female rats of Dark Agouti (DA, RT.1$A^{av1}$) and Lewis (RT.1$A^{1}$) strains weighing 200–250 g were obtained from Charles River (Kisslegg, Germany). Lewis rats served as recipients of DA grafts. All animals were kept in wire-bottomed cages with controlled light/dark cycles. They are fed with a standard laboratory diet and free access to tap water was given. They were handled in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals" and German guidelines on the use of animals in research (Berliner Senatsverwaltung). Orthotopic corneal transplantation was performed as reported previously [18].

*Adenoviral constructs.* Three different E1/E3-deleted Ad-vectors encoding for the therapeutic proteins Bcl-$x_L$, Bag-1 or HO-1 were used in our experiments. The generation of Adbag-1 and AdHO-1 was described previously [19,20]. The Bcl-$x_L$ construct was generated as described before [15]. Titration of the virus concentration after elution from the column was performed by plaque assay. As control vectors, Ad-constructs expressing either *Escherichia coli* β-galactosidase (β-Gal) or Enhanced Green Fluorescent Protein (EGFP) have been used.

*Gene transfer in HCEC and cultured corneas.* Gene transfer in HCEC was carried out 24 h after plating using Ad-vectors at MOI 50 (multiplicity of infection) by adding the cationic polymer polybrene in the medium (8 μg/ml). The recombinant Ad encoding for Bcl-$x_L$, Bag-1 or HO-1 was incubated with the cells for 30 min at room temperature. Then medium was replaced and cells were further cultured under standard conditions. This protocol results in approx. 100% of transduced HCEC (Ritter, unpublished observation). For ex vivo transduction of rat corneas donor tissues were excised and cultured in 24 well plates and incubated at 37 °C and 5% $CO_2$ for 3 h with 500 μl DMEM containing $1.0 \times 10^8$ pfu of AdBcl-$x_L$, AdBag-1 or AdHO-1. After 3 h of transduction, supernatants were removed, corneas were carefully washed three times and incubated for additional 3 h in 500 μl DMEM at 37 °C before transplantation in MHC class I/II disparate recipients was carried out. For protein expression studies corneas were further incubated at 37 °C in 1 ml DMEM 2% FCS for 72 h.

*Detection of transgene expression by Western blot.* Six, 12, 24, and 48 h after transduction cells were harvested and solubilised in protein lysis buffer containing proteinase inhibitors. Fifty or 100 μg, respectively, of total protein were separated by 15% SDS gel electrophoresis and electroblotted onto a nitrocellulose membrane. Bcl-$x_L$, Bag-1, and HO-1 were detected using rabbit polyclonal IgG antibodies (Cell Signaling; Beverly, USA; Santa Cruz Biotechnology, Santa Cruz, USA; Stressgen Biotechnologies, Victoria, Canada), followed by the addition of donkey anti-rabbit Ig horseradish peroxidase linked $F(ab')_2$ fragment (Amersham Life Science, Freiburg, Germany). The membrane was developed with the ECL Detection Reagents Kit from Amersham Pharmacia Biotech (Freiburg, Germany).

Transgene expression in cultured corneas was analysed 72 h after transduction. Briefly, corneas were placed in protein lysis buffer and lysed mechanically using a mixer mill (Retsch, Duesseldorf, Germany) at a frequency of 30 Hz/s for 90 s, which was repeated three times. The protein concentration was measured with the Coomassie Protein Assay Reagent Kit (Pierce) corresponding to the manufacturer's protocol. Fifty microgram of total protein was precipitated with trichloric acid in order to concentrate the protein from the supernatant and for further analysis as described above.

*Induction of apoptosis.* Twenty-four hours after transduction with the adenovirus constructs, apoptosis was induced using different pro-apoptotic stimuli: TNF-α (100 ng/ml, R&D Systems, Wiesbaden-Nordenstadt, Germany)/cycloheximid (CHX, 20 μg/ml, Sigma–Aldrich, Taufkirchen, Germany); staurosporine (0,5 μM, Sigma–Aldrich, Taufkirchen, Germany), and camptothecin (5 μM, Serva, Heidelberg, Germany). Optimal concentrations and incubation times of the pro-apoptotic stimuli were determined in previous studies (data not shown). For the induction of apoptosis with TNF-α/CHX, cells were first incubated with the protein synthesis inhibitor CHX for 30 min at 37 °C before TNF-α was added. The treatment of HCEC with TNF-α/CHX and staurosporine was done for 6 h whereas the treatment with camptothecin was done for 24 h. An Ad-construct expressing the *E. coli* β-galactosidase gene (Adβ-Gal) was used as a control vector.

*Detection of apoptosis (caspases 3 and 7 activity).* The activation of the executioner caspases 3 and 7 during apoptosis was measured using the Apo1™ Homogenous Caspase 3/7 Assay (Promega, Mannheim, Germany), which provides a pro-fluorescent substrate for the caspases according to the manufacturers' instructions. Each experiment with all three Ad-constructs and each apoptosis-inducing stimulus was performed six times. Twenty-four hours after Ad-mediated gene transfer of Bcl-$x_L$, Bag-1 or HO-1, cells were treated with TNFα/CHX, staurosporine or camptothecin. To determine the effects of different transgenes on caspases recruitment, the percentage of the caspase activity of the treated cells versus untreated cells was calculated. The fluorescence of the media was subtracted from the fluorescence of the probe. This corrected fluorescence of the particular probe was related to the fluorescence of the TNF-α/CHX treated non-transduced control. To determine the effects of transgenes on caspases recruitment, the percentage of the caspase activity of the treated gene-modified cells from the treated non-transduced cells was calculated. To verify the significance of data passing the normality test the unpaired Student's *t*-test was used and for those, which did not fit a Gaussian distribution the Mann–Whitney test was applied. Data are presented as means ± SEM.

## Results

### *Expression of cyto-protective genes Bcl-$x_L$, Bag-1, and HO-1 in HCEC*

In order to study the effects of over-expressing cyto-protective genes by Ad gene transfer a number of constructs have been generated encoding for the cyto-protective genes Bcl-$x_L$, Bag-1, and HO-1. HCEC were transduced with Ad-constructs encoding either for Bcl-$x_L$, Bag-1 or HO-1, respectively. At different time points after gene transfer cells were harvested and transgene expression was confirmed by Western blotting. We could show that the thera-

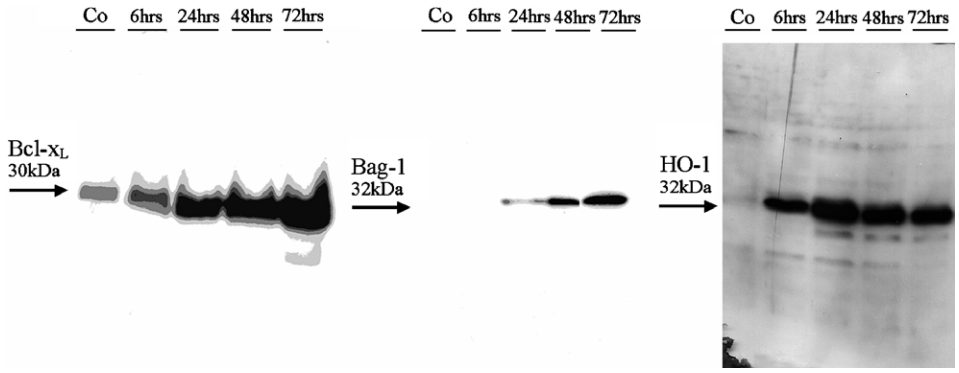


Fig. 1. Western blot analysis of transgene expression in HCEC at different time-points. Transgene expression in HCEC was analysed by Western blotting at different time-points (6, 24, 48, and 72 h) after transduction with the corresponding adenovirus construct. Untreated HCEC were used as control (Co).

peutic protein could be already detected 6 h after transduction which increased over time (72 h) (Fig. 1).

*Influence of pro-apoptotic stimuli on gene-modified HCEC expressing Bcl-$x_L$, Bag-1, and HO-1*

Next we examined whether gene transfer of Bcl-$x_L$, Bag-1, and HO-1 leads to protection of HCEC against various pro-apoptotic stimuli. HCEC were transduced with Ad-constructs expressing either Bcl-$x_L$, Bag-1 or HO-1 as described above and treated with various apoptosis inducing stimuli including TNF-α (100 ng/ml)/Cycloheximid (CHX, 20 μg/ml), staurosporine (0.5 μM) and camptothecin (5 μM). The activation of executioner caspases 3 and 7 was used to measure the induction of apoptosis. We could demonstrate that Bcl-$x_L$ gene transfer was able to prevent the activation of the executioner caspases 3 and 7 after treatment with both TNF-α/CHX and camptothecin with an inhibition of caspase activity around 63% and 40% compared to treated non-transduced control cells (Fig. 2, $n = 6$, $p \leqslant 0.05$), respectively. No beneficial effect of Bag-1 and HO-1 gene transfer on the activation of executioner caspases 3 and 7 over controls could be observed. The protective effect of Bcl-$x_L$ gene transfer after TNF-α/CHX treatment was confirmed by TUNEL-staining (data not shown). Moreover, gene transfer of Bcl-$x_L$ and HO-1 led to an inhibitory effect on pathophysiological high intracellular $Ca^{2+}$ concentration induced by oxidative stress ($H_2O_2$, 0.1 mM) associating with apoptotic cell death (Online Supplementary Material).

*Western blot analysis of transgene expression in cultured corneas and analysis of allogeneic graft survival after Bcl-$x_L$, Bag-1 or HO-1 gene transfer*

To further investigate the cyto-protective effects of anti-apoptotic proteins we investigated whether Bcl-$x_L$, Bag-1 or HO-1 proteins can be over-expressed in ex vivo cultured corneas. Fig. 3A shows that corneas transduced with Ad-vectors encoding either for Bcl-$x_L$, Bag-1 or HO-1 expressed the therapeutic gene 72 h after gene transfer (n = 2 for each construct).

Then we investigated whether cyto-protective effects observed in vitro could also be confirmed in the in vivo situation. We used a rat allogeneic MHC class I/II disparate transplant model. In this strain combination untreated or control-vector (*E. coli* β-galactosidase) treated corneas are rejected around day 13 (mean survival time (MST) ± SD 13.1 ± 1.1, $n = 16$; reporter gene (Adβ-Gal): MST ± SD 13.3 ± 0.8, $n = 6$, Fig. 3B) [21]. Fig. 3B clearly shows that transplantation of corneas over-expressing Bcl-$x_L$, Bag-1, and HO-1 did not lead to a significant prolongation of allogeneic graft survival (AdBcl-$x_L$: MST ± SD 13.8 ± 1.0, $n = 6$; AdBag-1: MST ± SD 13.6 ± 0.5, $n = 5$; AdHO-1: MST ± SD 14.0 ± 0.5, $n = 6$). In summary, cyto-protective effects of anti-apoptotic proteins could not be confirmed in an allogeneic corneal transplant model.

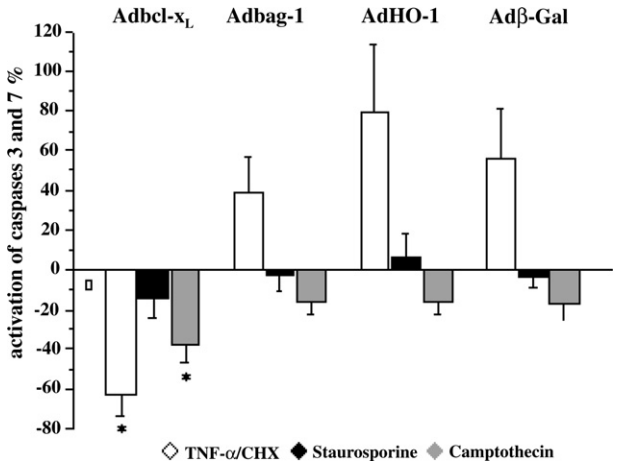


Fig. 2. Influence of pro-apoptotic stimuli on gene-modified HCEC. Activation of caspases 3 and 7 in Ad-transduced HCEC: 24 h after Ad-mediated gene transfer of Bcl-$x_L$, Bag-1 or HO-1, cells were treated with TNF-αCHX, staurosporine or camptothecin. Bcl-$x_L$ was able to significantly reduce the recruitment of the caspases 3 and 7 after induction of apoptosis with TNF-αCHX and camptothecin ($^*p \leqslant 0.05$).

## Discussion

Several explanations have to be considered for the differences between the protective effects of Bcl-$x_L$ and HO-1 gene transfer in vitro and the in vivo situation (allogeneic transplantation). An important prerequisite of successful gene transfer is the sufficient expression of the therapeutic

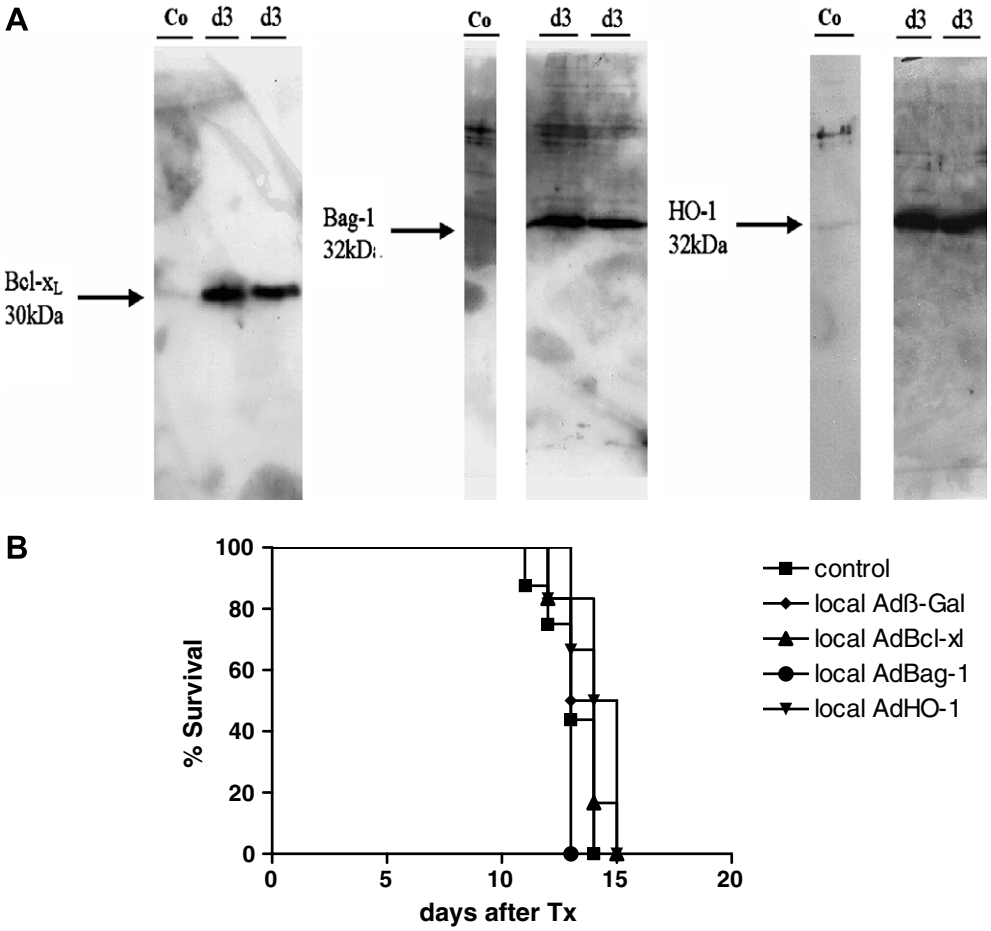


Fig. 3. Western blot analysis of transgene expression in cultured corneas and graft survival of allogeneic gene-modified corneal transplants. (A) Transgene expression in cultured corneas was analysed by Western blotting 72 h after transduction with the corresponding adenovirus construct. Untreated cultured corneas were used as control (Co). (B) Survival curves of gene-modified allogeneic corneal transplants expressing Bcl-$x_L$ ($n = 6$), Bag-1 ($n = 5$) or HO-1 ($n = 6$) are shown. Untreated ($n = 16$) or reporter gene treated Adβ-Gal, ($n = 6$) corneas served as control.

gene in target cells or tissues. As shown in Figs. 1 and 3A, Ad-mediated gene transfer lead to a significant increase of protein expression both in HCEC and in cultured corneas indicating the efficiency of this approach.

Programmed cell death of endothelial cells seems to play an important role during ex vivo storage of the donor cornea and during the allogeneic immune response after transplantation. Although the triggered apoptotic pathways are not well understood, it has been previously shown that pro-inflammatory cytokines may induce apoptosis in corneal endothelial cells [5]. The Bcl-$x_L$ protein is able to interrupt the apoptotic program at a very early stage, prior to shrinking of the cells and DNA-fragmentation [22]. In different models of apoptosis Bcl-$x_L$ influences the mitochondria-dependent step [23–25]. However, the capacity of the protein to act as cyto-protective molecule during receptor-induced apoptosis remains controversial. There is convincing evidence that the members of the bcl-2 family can prevent TNF-α mediated cell death in endothelial cells [26,27]. We could demonstrate that gene transfer of Bcl-$x_L$ in HCEC followed by treatment with TNF-α/CHX led to a significant reduction in the recruitment of the executioner caspases 3 and 7 (Fig. 2).

Other strategies to protect the corneal endothelium by gene therapy using anti-oxidative genes have been described. Hudde et al. expressed the human catalase gene in human corneas by Ad-mediated gene transfer, which confers resistance to oxidative stress induced by hydrogen peroxide [28]. However, its efficacy in vivo has not been proven. In this context, a previous study demonstrated that hydrogen peroxide increases the influx of calcium–ions in HCEC [12]. This may finally mediate apoptotic insults by conducting $Ca^{2+}$ into cells as observed in other cell types [29,30]. Therefore, we have tested whether over-expression of cyto-protective molecules in HCEC is able to prevent harmful rises in intracellular free $Ca^{2+}$. Indeed, both Bcl-$x_L$ and HO-1 gene transfer inhibited this effect (Online Supplementary Material). In summary, there is convincing evidence that gene transfer of various cyto-protective genes is able to protect HCEC from apoptosis in vitro. However, this effect could not be observed in the in vivo situation, in particular in a model of allogeneic cornea transplantation. But we can not rule out the possibility that other apoptotic pathways independent from over-expression of Bcl-$x_L$, Bag-1, and HO-1 may contribute to endothelial cell loss in vivo [31].

Replication-deficient recombinant adenoviruses, as used in this study, are very effective vectors for the transduction of corneal endothelial cells however the immunogenic potential and the transient expression of the therapeutic gene restrict their application [13]. Despite the fact that Ad-vectors may induce both humoral and cellular immune responses they are currently being used as therapeutic vector for the treatment of eye specific diseases e.g. age-related macular degeneration [32]. We could recently show that liposomal (low-immunogenic) gene transfer did not alter rejection kinetics in an allogeneic corneal transplant model compared to adenoviral (high-immunogenic) gene transfer [21]. This may indicate that in vivo immune-mediated endothelial cell loss or transgene expression is unlikely to be involved in the failure of the proposed strategy using cyto-protective molecules. From these data we conclude that administration of adenovirus in an immuno-privileged area might not induce harmful immune responses leading to accelerated rejection of allogeneic transplants and could be considered as a therapeutic approach.

We suggest that combined gene transfer of cyto-protective molecules will be required for effective protection of corneal endothelium during long-term storage and upon transplantation. Effective protection due to combined application of cyto-protective molecules was demonstrated recently in a model of photoreceptor cell loss [8]. In addition, tissue specific factors in the anterior chamber of the eye may also contribute to the lack of function of gene therapeutic approaches in the cornea transplant model.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.146.

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