Corneal Endothelial Cells Are Protected from Apoptosis by Gene Therapy

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Abstract

Corneal grafting is the most prevalent form of transplantation. Corneal endothelial cells (ECs), which form a monolayer of the cornea with minimal proliferative potential, are pivotal for maintenance of corneal clarity. Loss of EC viability and apoptosis leads to graft failure posttransplantation and reduces the quality of donor corneas in storage, such that up to 30% do not meet selection criteria and must be discarded. The current study investigates antiapoptotic effects of transduced mammalian Bcl-x_L and baculoviral p35 on human ECs. Multiple apoptotic cell features are observed while inducing apoptosis either via the extrinsic (death receptor) or intrinsic (mitochondrial) apoptotic pathway. Human ECs were studied under three experimental conditions: (1) as an immortalized cell line, (2) as primary cells, and (3) in an intact cornea. Interestingly, in primary EC suspensions, Bcl-x_L was protective against apoptosis mediated via both pathways. However, p35 was significantly more protective against apoptosis mediated via the intrinsic pathway compared with Bcl-x_L. Our results provide critical insight into the role of apoptotic pathways in the maintenance of EC viability and the efficacy with which these protective proteins exert their effect. These observations could form the basis for future applications of antiapoptotic gene therapy to corneal preservation aiming to reduce both graft failure after transplantation as well as donor corneal damage during storage.

Introduction

CORNEAL TRANSPLANTATION is the most common form of tissue transplantation. Although the 2-year allograft survival rate is more than 90% (Niederhorn, 1990), survival rates decline considerably over the years (Williams *et al.*, 2008). Corneal grafts in “high-risk” patients, who have a history of previous graft failure and have classic ocular surface inflammation, fail in almost 40% of cases even after 1 year, despite the use of topical and systemic immunosuppressive therapy (Williams *et al.*, 2008). Although at least 220,000 corneas are processed in eye banks each year worldwide to provide tissue for transplantation, there remains a shortage of corneal grafts suitable for transplantation (Goren, 2006; Jones *et al.*, 2009). Up to 30% of the processed donor tissue is currently discarded by eye banks because of impaired endothelial cell viability (Pels and Schuchard, 1983; Means *et al.*, 1995; Pels *et al.*, 2008), and at least one-fourth of all graft failures 15 years after transplantation are attributed to endothelial cell (EC) failure (Williams *et al.*, 2008). Therefore, understanding the mechanisms of EC loss can potentially lead to advances

that increase EC survival during corneal storage before transplantation and during ocular surgery, as well as increase long-term allograft survival after corneal transplantation.

The transparency of a corneal graft critically depends on the viability of its endothelial cells (Armitage *et al.*, 2003). Because of its minimal proliferative capacity, loss of ECs beyond a critical threshold leads to corneal edema and loss of vision (Carlson *et al.*, 1988). The density of ECs is the main criterion to evaluate the suitability of a donor cornea for transplantation during storage, and to assess graft quality after transplantation. A significant body of work has established the importance of apoptosis during storage of donor corneas in tissue banks (Armitage and Easty, 1997; Ehlers *et al.*, 1999; Albon *et al.*, 2000) and after transplantation of the corneal grafts (Bell *et al.*, 2000; Gain *et al.*, 2001; Rieck *et al.*, 2003; Bourges *et al.*, 2004, 2007; Sagoo *et al.*, 2004). Apoptosis, or programmed cell death, is an act of cellular self-destruction with distinctive morphological and biochemical features (Jacobson *et al.*, 1997). Two major apoptotic pathways have been identified in mammalian cells: the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Salvesen and Dixit, 1997).

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Because of the establishment of corneal storage for up to 5 weeks, the possibility of direct visualization of the effects of gene transfer, and the ready accessibility of the ECs (in direct contact with the storage medium), the cornea is particularly suitable for gene transfer approaches. The goal of our study, therefore, was to inhibit apoptosis *ex vivo* in corneal grafts by transfer of antiapoptotic genes. To study differences between two different types of proteins, we chose Bcl-x_L, a Bcl-2 family protein found in mammals. This protein functions as a communicator between the extrinsic, death receptor pathway, and the intrinsic, mitochondrial pathway (Hengartner, 2000). We compared the antiapoptotic potency of Bcl-x_L in ECs with that of p35, an antiapoptotic protein identified during genetic studies of baculoviruses (Xue and Horvitz, 1997). The p35 protein has broad specificity for members of the caspase family, effectively inhibiting apoptosis induced by a wide range of stimuli (Clem, 2007). To determine whether the efficacy of both antiapoptotic proteins depends on their action within specific apoptotic pathways, we investigated both the extrinsic and intrinsic pathways.

To date, no study has been performed to systematically determine the characteristic features of ECs undergoing death triggered via the extrinsic, death ligand-mediated apoptotic pathway or the intrinsic, mitochondria-mediated apoptotic pathway. Therefore, the contribution of intrinsic versus extrinsic apoptotic pathways in the evocation of apoptosis in corneal endothelial cells remains incompletely understood.

Our results clearly demonstrate the positive impact of this approach on EC survival even in the presence of strong apoptotic inducers. These findings suggest a basis for future gene therapy strategies to increase the availability of donor corneas, to give patients access to transplantations in countries suffering from a shortage of tissue, and to decrease graft failure after corneal surgery, thus reducing the risk of retransplantation.

Materials and Methods

Induction of apoptosis

Apoptosis was induced with the topoisomerase II inhibitor etoposide (intrinsic apoptotic pathway; Shawgo *et al.*, 2008) and the antineoplastic drug actinomycin D, provoking premature cell death via the extrinsic apoptotic pathway by specific stimuli (Kaiser and Bodey, 2000).

Detection and measurement of apoptosis

Apoptosis was studied by detection of phosphatidylserine translocation to the external surface of the cell (Fadok *et al.*, 1992). Annexin V (AnnV) is a member of the phosphatidyl-binding protein family, with strong affinity for phosphatidylserine (Andree *et al.*, 1990). The counterstain, propidium iodide (PI), is used to assay for cell membrane permeability (lysis). Consequently, viable cell populations will be non-fluorescent, and cells in metabolically active stages of apoptosis will stain for annexin V but not for PI. Late apoptotic bodies may enter secondary necrosis unless deleted by phagocytosis. These populations will bind both annexin V and the DNA-binding dye PI, whereas staining with PI indicates only final necrosis.

Apoptosis in human ECs induced through the intrinsic or extrinsic apoptotic pathway was detected with annexin V–

FITC and PI by quantitative flow cytometry (according to the manufacturer's instructions) (ApopNexin FITC apoptosis detection kit; Millipore, Temecula, CA). Apoptosis in ECs expressing antiapoptotic proteins or IZsGreen only (IZsGreen: ZsGreen [Clontech, Palo Alto, CA] expressed by a vector containing an internal ribosomal entry site) was detected with annexin V–PE and 7-amino-actinomycin D (7-AAD) (annexin V–PE apoptosis detection kit I; BD Biosciences, San Diego, CA). This approach allowed simultaneous detection of IZsGreen-expressing ECs and AnnV/PI by flow cytometry.

In human corneas, annexin V–cyanine 5 (Cy5) (Biovision, Mountain View, CA) was used to detect apoptosis by laser scanning microscopy, and analysis of antibody expression was then performed by pixel quantification (Adobe Photoshop; Adobe, San Jose CA). In addition, a terminal deoxynucleotidyltransferase (TdT)-mediated digoxigenin-deoxyuridine-5'-triphosphate (dUTP) nick-end labeling (TUNEL) assay (*in situ* cell death detection kit; Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions to measure DNA breaks in ECs within human corneas. Vital nuclei were visualized with TO-PRO-3 iodide (Molecular Probes/Invitrogen, Eugene, OR). Cell borders were visualized by rabbit anti-ZO-1 (zonula occludens-1) labeling (N-term) (Invitrogen, Carlsbad, CA). Corneas were washed and mounted with Vectashield medium for fluorescence (Vector Laboratories, Burlingame, CA). IZsGreen expression and TUNEL positivity were detected with a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany) (magnification, $\times 40$).

Lentivirus expression system

The cDNAs of mammalian antiapoptotic protein Bcl-x_L and baculoviral antiapoptotic molecule p35 were each subcloned into the lentiviral vector pHAGE-CMV-MCS-IZsGreen by the Harvard Gene Therapy Initiative (Boston, MA). Both genes were cloned into the multiple cloning site that is preceded by an internal ribosomal entry site (IRES) and the coding sequence for the green fluorescent protein ZsGreen, which is derived from a reef coral. The IRES sequence allows for two open reading frames on one mRNA. lenti-IZsGreen (control virus), lenti-IZsGreen-BclxL, and lenti-IZsGreen-p35 are replication incompetent so that the infected cells do not produce virus (Gardlik *et al.*, 2005).

Gene transfer in primary and immortalized human endothelial cells and in cultured corneas

Gene transfer in primary and immortalized corneal ECs was carried out with pHAGE-CMV-MCS-IZsGreenW, pHAGE-CMV-IZsGreen-BclxLW, or pHAGE-CMV-IZsGreen-p35W (3×10^5 IU/ml) on confluency. To exclude donor-related variation, transduction of ECs within human corneas was carried out on single corneas cut into several pieces. One piece served as untreated control, another was transfected with pHAGE-CMV-MCS-IZsGreenW (parental vector, 3×10^5 IU/ml), a third with pHAGE-CMV-IZsGreen-BclxLW (3×10^5 IU/ml), and a fourth with pHAGE-CMV-IZsGreen-p35W (3×10^5 IU/ml). To detect the relevance of titer on protection of ECs against apoptosis, one corneal piece also was transfected with pHAGE-CMV-IZsGreen-BclxLW at 1.2×10^8 IU/ml.

Research-grade, human donor corneas were incubated with the gene of interest or the parental vector for 1 hr at 37°C in Biochrome cornea culture medium I (Biochrome, Berlin, Germany) containing Polybrene (8 µg/ml; Sigma-Aldrich, St. Louis, MO). To remove excess virus, corneas were washed before storage in the respective culture medium.

Detection of transgene expression

Transgene expression in cultured corneas was analyzed 24 hr after incubation of the respective ECs with the respective vectors for 0.5, 3, or 18 hr at 37°C. In human endothelial cell suspensions, IZsGreen expression was detected by quantitative flow cytometry. In human corneal fragments, expression of the reporter protein was detected by laser scanning microscopy (Leica TCS SP2).

Culture conditions for human endothelial cell suspensions and human corneas

The human corneal endothelial cell line was kindly provided by K. Engelmann (Klinikum Chemnitz, Chemnitz, Germany) and J. Bednarz (Universitätsklinikum Hamburg-Eppendorf [UKE], Hamburg, Germany). Cells (100,000) were seeded in each well of a 48-well plate (triplets each). Cells were maintained in complete minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and 0.5% gentamicin (G1272; Sigma-Aldrich). Before transfection, immortalized corneal ECs were treated with mitomycin C once confluent to halt cell proliferation and thoroughly washed with sterile phosphate-buffered saline (PBS). The cell number in each well of a 48-well plate was $0.154427 (\pm 0.29055) \times 10^6$ ECs.

Primary corneal endothelial cells were harvested from a donor cornea and cultured according to Joyce and Zhu in culture medium containing fetal bovine serum, epidermal growth factor (EGF), nerve growth factor (NGF), and bovine pituitary extract (Joyce and Zhu, 2004). All flow cytometric experiments with primary cells used passage 8 of the same cell type.

Twelve research-grade, human donor corneas with intact endothelium not suitable for transplantation (e.g., due to non-tissue-related exclusion criteria) were obtained from Tissue Banks International (Baltimore, MD) and from the Lions Eye Institute for Transplant and Research (Tampa, FL). Corneas were cultivated at 37°C in Biochrome cornea culture medium I containing 2% FBS.

Statistical analysis

Statistical analyses determined the mean for three replicates from different corneas; error bars displayed in the figures indicate the standard deviation (SD). Unless stated otherwise, comparisons between two groups were performed by unequal variance, two-tailed, Student *t* test. Data were considered statistically significant when $p \leq 0.05$.

Results

Our specific aims in this study were to compare the relative efficacy of the mammalian antiapoptotic protein Bcl-x_L with the baculoviral antiapoptotic protein p35. In addition, we wanted to determine whether these antiapoptotic gene therapy-based strategies worked differen-

tially in extrinsically compared with intrinsically mediated apoptosis and whether there was a differential effect in primary EC culture compared with EC lines and human corneas.

Induction and detection of apoptosis in primary and immortalized human endothelial cells

Apoptosis was induced with the topoisomerase II inhibitor etoposide (intrinsic apoptotic pathway; Shawgo *et al.*, 2008), and the antineoplastic drug actinomycin D, which provokes premature cell death via the extrinsic apoptotic pathway through specific stimuli such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or Fas (CD95) (Kaiser and Bodey, 2000).

Use of the annexin V/propidium iodide assay in flow cytometric analysis allowed the study of viable (annexin V-negative, propidium iodide-negative), early apoptotic (AnnV⁺PI⁻), and late apoptotic (AnnV⁺PI⁺, AnnV⁻PI⁺) EC populations (Fig. 1A). Annexin V is a cellular protein competing for phosphatidyl-binding sites, and thus allowing the detection of phosphatidylserine translocating from the inner cell membrane to the cell surface during apoptosis. With further increase of the inducer concentration, the early apoptotic population decreased while the late apoptotic cell population significantly increased (data not shown).

To investigate the effect of both inducers on early and late apoptosis in immortalized human endothelial cells, the percentages of early and late apoptotic ECs were monitored during incubation for 48 hr with increasing times of incubation with both apoptotic inducers (Fig. 1B); similar data were obtained when the experiment was repeated with multiple concentrations of the inducers (data not shown). Interestingly, there was no significant difference in numbers of apoptotic, immortalized ECs when apoptosis was induced via the intrinsic or extrinsic apoptotic pathway. Early apoptosis in immortalized human ECs did not show a continuous increase with continuous incubation time; rather, several spikes were detected, creating a wavelike pattern (e.g., after 6, 24, or 30 hr of incubation) that suggested "bursts" of ECs undergoing death.

A significant difference in early apoptosis patterns was observed when the same experimental procedures were performed with primary corneal ECs and compared with immortalized human ECs. In primary human ECs, the percentage of early apoptotic ECs continuously increased with increasing time of incubation (Fig. 1C); similar data resulted with multiple concentrations of etoposide or actinomycin (data not shown). As with immortalized ECs, the percentages of early or late apoptotic cell populations were similar whether apoptosis was induced via the intrinsic or extrinsic apoptotic pathway.

Induction and detection of apoptosis in human corneal endothelial cells from human corneas

Both actinomycin and etoposide were used to induce apoptosis in ECs of human corneas (Fig. 2). Annexin V positivity, a measure of apoptotic changes on the cell membrane, was detected by laser scanning microscopy (Fig. 2A). Pixel quantification showed a significant increase in detected annexin V with increasing incubation with inducer (Fig. 2B). Interestingly, our data demonstrated that etoposide has a

significantly stronger apoptotic effect after a short incubation period with human corneal ECs compared with actinomycin. These data obtained in corneas validate the results found in cell suspensions when studying annexin V-positive apoptosis by flow cytometry (see Fig. 4).

Kinetics of IZsGreen expression in primary and immortalized corneal endothelial cells in suspensions or in human corneas

Studies were conducted to detect the expression of the green fluorescent reporter protein IZsGreenW cotransduced with the genes of interest and therefore a measure of the expected protein expression. At various time points after gene transfer IZsGreenW expression in primary and immortalized human ECs was evaluated by quantitative flow cytometric analysis. Both cell types showed increasing expression of IZsGreenW along with rising titers. However, primary human ECs consistently demonstrated up to 2.5 times higher expression of the reporter gene than immortalized human ECs at comparable titers (Fig. 3A).

In addition, IZsGreenW expression in ECs of human corneas was detected by laser scanning microscopy 24 hr after transfection with pHAGE-CMV-MCS-IZsGreenW at 3×10^5 IU/ml for 0.5, 3, or 18 hr in Biochrome cornea medium I in the presence of Polybrene (8 μ g/ml). Expression levels more than 90% relative to untreated controls were obtained. Apoptosis was measured by detection of DNA fragmentation TUNEL. The percentage of TUNEL-positive ECs steadily increased with elongation of incubation time (Fig. 3B), findings that were consistent with the respective FACS results. To prevent high levels of apoptosis induced by transduction, subsequent experiments in human corneas were performed with 1-hr transduction times.

Bcl-x_L and p35 both protect ECs against apoptotic inducers, p35 being more efficient than Bcl-x_L against intrinsically mediated apoptosis

To test our hypothesis that ECs can be protected against apoptosis provoked by specific inducers, using antiapoptotic gene transfer, primary and immortalized ECs were transduced with the parental vector pHAGE-CMV-MCS-IZsGreenW, pHAGE-CMV-IZsGreenW-BclxL, or pHAGE-CMV-IZsGreenW-p35. In a second step, apoptosis was induced via the intrinsic apoptotic pathway (etoposide) or the extrinsic apoptotic pathway (actinomycin). Early apoptotic populations (annexin V⁺/PI⁻) were studied by quantitative

FIG. 1. Quantitative flow cytometric evaluation of apoptosis of human endothelial cells (ECs). **(A)** Quantitative flow cytometry for the detection of apoptosis in human ECs, using annexin V-FITC (AnnV) and propidium iodide (PI) to distinguish vital, early, and late apoptotic cell populations. A human EC line was incubated for 24 hr with actinomycin (3 ng/ml) and compared with untreated ECs (*left*). Vital cell populations (AnnV⁻/PI⁻, R1) decreased whereas early (AnnV⁺/PI⁻, R2) and late (AnnV⁺/PI⁺, R3) apoptotic cell populations increased (*right*). **(B)** In addition, early apoptotic cell populations in immortalized human ECs, studied after inducing apoptosis via either pathway, did not show a distinct difference in apoptotic cell populations (etoposide or actinomycin, 5 μ g/ml, up to 48 hr). **(C)** However, when performing this experiment in primary human ECs (etoposide, 5 μ g/ml), distinctly different apoptotic features compared with immortalized human ECs could be detected, especially in the AnnV⁺/PI⁻ (early apoptotic) cell population. Lines in **(B)** and **(C)** display percentages of apoptotic endothelial cells (means $\pm$ SD).

FIG. 2. Increasing annexin V positivity in ECs of human corneas after treatment with apoptotic inducers. Immunocytochemical detection of apoptosis in human donor corneal ECs, using annexin V (AnnV) and propidium iodide (PI). (A) Significant apoptotic alterations on the EC membrane could be identified with annexin V by laser scanning microscopy, after induction with actinomycin and etoposide (10 μ g/ml, for 3 or 18 hr, respectively). (B) Columns display pixel quantification of annexin V-only confocal images (means $\pm$ SD). Color images available online at www.liebertonline.com/hum

laser scanning microscopy and compared with transfected but untreated ECs.

Although cell death was provoked via the extrinsic, death ligand-mediated apoptotic pathway (actinomycin D), early apoptotic cell populations significantly decreased in primary ECs expressing either the mammalian antiapoptotic protein Bcl-x_L or the baculoviral protein p35. There was no significant difference in decrease in apoptosis whether primary ECs were transduced with p35 or Bcl-x_L (Fig. 4A, left). However, in immortalized ECs only p35 significantly protected against

this strong apoptotic inducer (Fig. 4A, right). Interestingly, when apoptosis was induced via the intrinsic apoptotic pathway (etoposide), primary ECs expressing either p35 or Bcl-x_L showed significantly less apoptosis relative to untreated ECs or those expressing the parental vector only (Fig. 4B, left). However, expression of p35 in both immortalized and primary ECs resulted in significantly less apoptosis compared with ECs expressing Bcl-x_L (Fig. 4B, right), suggesting that p35 is more efficient than Bcl-x_L in protecting ECs against apoptosis mediated via the intrinsic pathway.

Antiapoptotic protein expression in ECs of corneas leads to preserved cell morphology and protection against strong apoptotic inducers

To verify the findings with Bcl-x_L and p35 obtained from studying primary and immortalized human endothelial cells

in culture, we transduced corneal endothelium on human donor corneas with the parental vector IZsGreenW, Bcl-x_L, and p35 (3×10^5 IU/ml). To determine whether an increased titer of Bcl-x_L results in increased cell survival, ECs were transduced with a high titer (1.2×10^8 IU/ml). Immunohistochemical evidence of DNA strand breaks was obtained by determining TUNEL positivity after transduction of Bcl-x_L or p35 and measured by laser scanning microscopy after treatment with actinomycin D or etoposide at 3 ng/ml for 6 hr. ECs expressing either Bcl-x_L or p35 showed significantly less apoptosis compared with ECs expressing IZsGreen only (Fig. 5A, etoposide); similar data were obtained for actinomycin-treated ECs including consistent findings in different experimental settings (data not shown). The difference in TUNEL positivity detected after transduction of ECs with a low titer compared with a high titer of Bcl-x_L was not significant.

Discussion

Apoptosis has been identified as a principal underlying mechanism for EC loss during and after ocular surgery and in corneal grafts after transplantation, resulting in graft failure, and in causing the loss of donor corneal tissue viability during storage before transplantation (Komuro *et al.*, 1999; Albon *et al.*, 2000; Bourges *et al.*, 2004; Gong *et al.*, 2007). Given the nonproliferating characteristic of the EC monolayer, and its critical function in maintaining corneal transparency, a minimal EC density is crucial to ensure vision. In the current study, the role and function of mammalian (Bcl-x_L) and baculoviral (p35) antiapoptotic genes were investigated regarding the protection of human corneal endothelial cells against apoptosis induced via either the intrinsic or extrinsic apoptotic pathway. We systematically compared the apoptotic properties of a human corneal EC line with those of primary corneal ECs and cultured human corneas, identifying significant differences. The results demonstrate that antiapoptotic gene therapy of corneal endothelial cells consistently leads to a reduction of apoptosis in primary human corneal endothelial cell suspensions and in human donor corneas while maintaining physiological cell morphology. Application of this therapy therefore could prevent

FIG. 3. Kinetics of IZsGreenW expression in human ECs (cell suspensions and corneas). Shown is flow cytometric (A) and immunocytochemical (B) detection of IZsGreenW in immortalized and primary human EC suspensions or ECs of human corneas after transduction with increasing concentrations of pHAGE-CMV-MCS-IZsGreen (3×10^5 IU/ml, 24 hr). IZsGreenW expression was also visualized by epifluorescence microscopy 24 hr after transduction (A, inset). In addition, IZsGreenW expression and TUNEL positivity (apoptosis) in ECs of human corneas were detected by laser scanning microscopy 24 hr after the respective transduction time. (B) Columns display the percentage of corneal endothelial cells (means $\pm$ SD) expressing IZsGreenW; points (connected by black line) indicate the percentage of ECs (means $\pm$ SD) showing TUNEL positivity. At each time point, three corneas cut into several pieces were examined. Inset (left) shows blue nuclei and IZsGreen expression, a proxy for the expected protein expression (green); inset (right) demonstrates nuclear fragmentation (red) as a sign of apoptosis. Color images available online at www.liebertonline.com/hum

FIG. 4. Expression of antiapoptotic protein Bcl-x_L or p35 leads to significantly reduced apoptosis in primary, but not in immortalized, human ECs. Shown is a quantitative flow cytometric analysis of AnnV⁺/PI⁻ populations in primary and immortalized human ECs (untreated vs. expressing parental vector only [IZsGreenW], IZsGr-Bcl-x_L, or IZsGr-p35 [3×10^5 IU/ml for 24 hr]). **(A)** After transduction, apoptosis was induced via the extrinsic death ligand pathway (actinomycin, 3 ng/ml, 72 hr) or **(B)** the intrinsic, mitochondrial pathway (etoposide, 3 ng/ml, 72 hr). Primary human ECs expressing Bcl-x_L or p35 showed significantly less apoptosis after treatment, whereas there was no difference observed in immortalized cells. In primary ECs, Bcl-x_L was more protective against an apoptotic inducer triggering the extrinsic death ligand-mediated pathway. However, p35 was more efficient in preventing apoptosis mediated via the intrinsic, mitochondrial pathway. Columns display the percentage of AnnV⁺/PI⁻ EC populations (means $\pm$ SD).

EC apoptosis during storage of donor corneas and after their grafting posttransplantation.

In previous studies, use of lentiviral vehicles for gene transfer to corneal endothelial cells has been shown to result in the rapid onset of protein expression and preservation of cell viability in the transduced cells (Wang *et al.*, 2000; Barcia *et al.*, 2007; Parker *et al.*, 2007; Bertelmann, 2009). Lentivirus-mediated gene expression resulted in high IZsGreen expression after only 30 min of infection, while toxic effects were of minor relevance. Lentiviral vehicles are rendered replication incompetent to avoid detrimental effects on cells transduced with this HIV-based vector. To study a possible effect of an increase in viral titers on cell viability two different titers of Bcl-x_L were used to transduce ECs. No significant differences were detected regarding TUNEL positivity of ECs transduced with Bcl-x_L at various titers.

Protective effects of antiapoptotic genes have been shown in previous studies in the rat cornea (Parker *et al.*, 2007), in the mouse cornea (Barcia *et al.*, 2007), and in murine and human corneal endothelial cell lines (Beutelspacher *et al.*, 2005; Barcia *et al.*, 2007; Gong *et al.*, 2007). To prove our hypothesis that antiapoptotic gene transfer results in poten-

tially clinically relevant promotion of cell survival of this minimally proliferative cell type, corneal ECs of human origin were studied because of their relevance in clinical disease and transplantation. Our results obtained in primary ECs differed markedly from the currently preferred research model, the human corneal endothelial cell line. These differences were consistent at multiple levels as to detection of apoptosis, expression of the reporter gene, and finally during protection experiments with ECs expressing antiapoptotic proteins. With increasing induction time, primary ECs showed a continuous increase in apoptotic cells whereas EC lines demonstrated a wavelike pattern with several peaks of apoptosis. Moreover, levels of IZsGreen-positive ECs were up to 2.5-fold lower, showed altered kinetics and different characteristics after transduction with antiapoptotic proteins. Only those ECs of the cell line expressing p35 demonstrated significantly reduced apoptosis. In addition, the overall levels of apoptosis did not reach those observed in primary cells. This might be a result of the simian virus 40 (SV40) transfection of ECs employed in these studies (Bednarz *et al.*, 2000). In the aggregate, these data suggest that use of a corneal EC line may reflect *in vivo* cell apoptotic characteristics

FIG. 5. Expression of antiapoptotic protein Bcl-x_L or p35 equally protects ECs in human corneas against apoptosis regardless of the mediated pathway. Shown is the immunocytochemical detection of apoptosis in human donor corneal ECs by TUNEL assay (TO-PRO-3 [blue], nuclei; TUNEL, red; ZO-1, green). (A) ECs expressing antiapoptotic proteins showed significantly fewer TUNEL-positive ECs compared with untreated ECs when incubated with either actinomycin or etoposide (3 ng/ml, incubation for 6 hr, respectively). Etoposide-treated cells are depicted: *top left* shows untreated cells (healthy controls), *top right* depicts untreated ECs incubated with the apoptotic inducer, *bottom left* shows ECs transduced with Bcl-x_L and incubated with the apoptotic inducer, and *bottom right* shows ECs transduced with p35 and incubated with the apoptotic inducer. (B) Columns display the percentage of TUNEL-positive ECs (means $\pm$ SD). Color images available online at www.liebertonline.com/hum

only to a limited extent. Apoptosis induction and gene transduction experiments using human corneas validated the data obtained with primary human ECs.

In the current study, apoptotic EC populations could be significantly reduced by inhibition of apoptosis mediated via intrinsic or extrinsic pathways in human ECs expressing p35 or Bcl-x_L. It has long been known that FasL- and TNF-induced death can lead to apoptosis via the extrinsic apoptotic pathway (Laster *et al.*, 1988). However, the complexity of this death receptor-induced signaling network exceeds by far that of a simple linear pathway as demonstrated by the receptor-triggered caspase cascade, for example, considering the extensive mitochondrial control mechanisms of cell death regulation in the intrinsic pathway. The Bcl-2 family member Bcl-x_L inhibits proapoptotic signaling after caspase activation, primarily at the mitochondrial level (Hengartner, 2000), a characteristic that seems to be limited to the BH3-only subfamily of Bcl-2 proteins (Sattler *et al.*, 1997). p35, however, has been characterized as a pan-caspase inhibitor (Clem, 2007). According to our data, ECs similarly develop early or late apoptosis regardless of the apoptotic pathway. Protection against cell death could be achieved through either the expression of the mammalian Bcl-x_L or the baculoviral p35, whereas p35, because of its broader antiapoptotic spectrum, seemed to be more efficient against intrinsically mediated apoptosis compared with Bcl-x_L.

The functional importance of antiapoptotic gene transfer of the mammalian protein Bcl-x_L or the baculoviral molecule p35 is illustrated by the finding of improved physiological endothelial cell morphology during treatment with strong apoptotic inducers. This finding is of critical importance because of the demonstrated increase in cell viability in the human cornea. It has been established that cell loss during corneal preservation occurs by both apoptosis and necrosis, with apoptosis predominating (Komuro *et al.*, 1999). Reportedly, up to 30% of all donor corneas are discarded for excessive endothelium cell loss during storage (Means *et al.*, 1995; Jones *et al.*, 2009). Moreover, after corneal transplantation 48% of ECs die during the first year after corneal grafting (Ruusuvaara and Setälä, 1988), a rate that exceeds 60% after 20 years (Vasara *et al.*, 1999).

However, translation of this approach could face hurdles by regulatory agencies. As we used a replication-incompetent lentiviral vector, the plasmid is permanently integrated into the host DNA. To avoid permanent integration, a non-integrating viral vector (such as an adenovirus or an adeno-associated virus) could be chosen as a carrier. In addition, the promoter could be designed to be inducible, for example, using a tetracycline (on/off) switch. This may increase safety and manageability during application in human tissue. Moreover, an important question for translation into the clinic concerns whether or how many viral vector particles can be found on the cell membrane of ECs after the procedure of washing the endothelium with PBS at the end of the transduction period. Theoretically, viral vector residuals on the surface of the donor tissue could lead to transduction of other cell types in the anatomical neighborhood of the corneal endothelium (e.g., those with high proliferative capacity) and lead to some safety concerns. We do not believe this to be of high risk because corneal cells either have a low turnover (stromal cells) or they are entirely replaced by the host cells (epithelium) (Joyce and Zhu, 2004). There are no

reports of corneal EC tumors described in the literature; ECs do not even form a bilayer (not even in *in vitro* experiments). Therefore, the chance of inducing local tumors is nearly null. Second, approaches targeting ECs have been of two types, that is, (1) to induce proliferation of ECs or (2) to suppress EC death, with the present studies belonging to the latter category. Whereas by promoting proliferation there could be a viable concern about inducing certain pathologies, by suppression of EC death tumor induction of ECs is not a concern because ECs do not proliferate, unlike many epithelia.

There have been "proof of principle" experiments in the mouse model transplanting corneas with ECs treated with the same viral vector construct used in this study (lenti-IZsGreen-Bcl-xL). The data showed that corneal grafting with ECs expressing Bcl-x_L resulted in significantly enhanced graft survival compared with controls (Barcia *et al.*, 2007; routine follow-up time in this model is 8 weeks). Longer observation periods might be necessary to address important safety questions, such as the biodistribution of treated ECs.

In summary, these findings lend further and independent support to the concept that antiapoptotic gene transfer can increase EC survival by inhibiting apoptosis mediated via either the intrinsic or extrinsic pathway. The data illustrate the strength with which the protective proteins can exert their effect, even in the presence of strong apoptotic inducers, suggesting that the translation of these technologies to the clinical setting could potentially lead to strategies that potentially increase EC survival during preservation of donor tissue and after transplantation. Interestingly, this approach is applicable to developments in the transplantation of the corneal endothelial monolayer (e.g., Descemet's stripping endothelial keratoplasty [DSEK], Descemet's membrane endothelial keratoplasty [DMEK]), techniques currently being globally adopted by corneal surgeons (Lombardo *et al.*, 2009). Translation of this antiapoptotic approach toward storage of donor corneas and corneal transplantation could decrease the amount of discarded donor tissue and increase the quality of patient care, as grafts with a higher EC count are less likely to undergo graft failure, resulting in a decreased requirement for regrafting. Last, this approach could be applicable to other forms of cell transplantation procedures, such as use of engineered stem cell sheets (Hayashi *et al.*, 2009) or skeletal myocardial sheet transplantation to improve the damaged heart (Shimizu *et al.*, 2009).

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