Corneal Endothelial Autocrine VIP Enhances Its Integrity in Stored Human Donor Corneoscleral Explant

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PURPOSE. To demonstrate corneal endothelial (CE) integrity enhanced during eye banking by a brief treatment of human donor corneoscleral explant (explant) with CE autocrine trophic factor vasoactive intestinal peptide (VIP).

METHODS. Paired explants were used as control versus VIP (10 nM)-treated before storage in corneal storage medium (4°C). CE ciliary neurotrophic factor receptor (CNTFR α) and CNTF (0.83 nM) responsiveness in connexin 43 upregulation were monitored (Western blot analysis). CE damage in CNTF-modulated explants and corneal buttons from explants was quantified by analysis of panoramic and microscopic images of the alizarin red-stained corneal endothelium. CE cells scraped from the Descemet's membrane were counted. CE VIP receptor was demonstrated (Western blot analysis).

RESULTS. CE cells in every VIP-treated, freshly dissected explant demonstrated higher CNTFR α levels than controls (100% vs. 142% $\pm$ 15%; $P = 0.014$; 7 pairs stored for 4 to 25 days). Nine days after VIP treatment of previously preserved explants, CNTF responsiveness was 174% $\pm$ 23% ($P = 0.023$; 4 pairs) of controls. Panoramic images of explants and corneal buttons revealed that VIP treatment reduced CE damage to 75% $\pm$ 6% ($P = 0.023$; 4 pairs) and 71% $\pm$ 11% ($P = 0.016$; 9 pairs) of controls, respectively, whereas CE damage to 39% (2 pairs) and 23% $\pm$ 4% ($P < 0.001$; 7 pairs), respectively, was revealed in microscopic images. Twenty-one days after VIP treatment of previously preserved explants, CE cell retention was 206% $\pm$ 38% ($P = 0.008$; 14 pairs) of the control. CE cells from human donor corneas expressed VIP receptor VPAC1 (not VPAC2).

CONCLUSIONS. CE integrity during eye banking was enhanced by a brief treatment of the explant with the CE autocrine VIP. (*Invest Ophthalmol Vis Sci.* 2011;52:5632-5640) DOI: 10.1167/iops.10-5983

A 90% success rate is expected in a first-time, uncomplicated corneal transplant without tissue typing or systemic immunosuppressive drugs.^{1,2} Nevertheless, graft failure, caused by corneal endothelial (CE) cell loss and immunologic rejection, does occur and results in the need for a second transplant,^{2,3} which has an expected 50% success rate.¹ Furthermore, after initial success, CE failure in the absence of rejection episodes can

occur as late as 5 to 10 years after transplantation.^{2,4-6} Grafts that develop late CE failure demonstrate a lower CE cell number immediately after transplantation than grafts that do not but not an increased rate of chronic postoperative CE cell loss.^{2,4,6}

Recently, the Cornea Donor Study Investigator Group has concluded that "the CE cell loss rates highlight the importance of continued research into ways to improve overall corneal health through advances in corneal preservation and postoperative management. This may become increasingly relevant with the advent of endothelial keratoplasty, which is potentially even more traumatic to the endothelium at the time of surgery than standard penetrating keratoplasty."⁷ As such, a 2009 report by the American Academy of Ophthalmology has concluded that "future research in corneal endothelial keratoplasty should be directed at enhancing endothelial cell survival."⁸ The corneal endothelium survives throughout life without a surplus of CE cells, indicating that the integrity of individual CE cells is maintained in the eye in vivo. Thus, the maintenance of CE cell integrity, including its differentiated state, is critical for the corneal endothelium to survive the procedures of dissection (from the cadaver), preservation, and keratoplasty.

Our extensive studies of CE cell physiology⁹⁻¹⁷ offer some insights into how CE cell integrity may be maintained in human donor corneoscleral explants preserved for transplantation. We find that, through the concerted actions of its autocrine trophic factors vasoactive intestinal peptide (VIP) and ciliary neurotrophic factor (CNTF), the corneal endothelium plays an active role in maintaining its own differentiated state and promoting self-survival.¹⁷ For example, CNTF is released in a complex with the CNTF-binding CNTF receptor α subunit (CNTFR α) by CE cells surviving oxidative stress.¹⁰ CNTF upregulates *VIP* gene and protein expressions in human donor corneoscleral explants, including those preserved in storage medium (Optisol-GS; Bausch & Lomb, Rochester, NY).¹³ *VIP* is a CE cell differentiation state-maintaining factor in that knocking down *VIP* gene expression results in a diminished level of the CE cell differentiation marker,^{18,19} the adhesion molecule N-cadherin and dramatically affects the morphology of the CE mosaic, in which hexagonal CE cells were replaced with irregularly shaped ones that were enlarged and had decreased cell densities.¹⁴ Exogenous *VIP* upregulates N-cadherin and the antiapoptotic protein Bcl-2¹⁶ and protects CE cells against the killing effect of oxidative stress.⁹ In CE cells, VIP stimulates glycogen breakdown; during the subsequent oxidative stress, VIP upholds the ATP level to allow switches of the death mode from acute necrosis to apoptosis while upregulating levels of the antiapoptotic Bcl-2 and N-cadherin in a kinase A-dependent manner.¹⁶ *VIP*, either endogenously¹⁴ or exogenously (data not shown), does not modulate the CE gap junctional protein connexin 43 level, whereas CNTF does in human donor corneoscleral explants.¹⁵

We hypothesized that brief treatment of human donor corneoscleral explants with the CE cell autocrine trophic factor

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VIP before their storage in storage medium (Optisol-GS; Bausch & Lomb) enhances CE preservation.

We have demonstrated that CE cells in human donor corneoscleral explants in storage in storage medium (at 4°C; Optisol-GS; Bausch & Lomb) gradually lose their CNTFR α and that the recombinant CNTFR α places itself in the CE cell membrane and restores functional CNTFR α in transducing CNTF signal upregulating connexin 43.¹⁵ In the present study, the levels of CE cell CNTFR α and CNTF responsiveness were used as end points in the demonstration of the beneficial effects of VIP treatment of human donor corneoscleral explants. In addition, the degree of CE damage was assessed after the corneal endothelium was stained with alizarin red S to reveal areas of the denuded Descemet's membrane and broken cell-cell adhesion. Finally, because it is well recognized that human donor corneoscleral explants gradually lose their CE cells during storage in storage medium, the present study also investigated whether VIP treatment of the human corneoscleral explants enhanced the long-term retention of CE cells. The presence of VIP receptor in CE cells of the human donor corneoscleral explants was also demonstrated.

MATERIALS AND METHODS

Media

The following media were used: medium A, Eagle's minimal essential medium with Earl's salts plus 20 mM HEPES and 2 mM glutamine; medium B, medium A supplemented with penicillin (200 U/mL) and streptomycin sulfate (200 μ g/mL); storage medium (Optisol-GS; Bausch & Lomb; Rochester, NY).

Human Donor Corneoscleral Explants

Fresh Human Donor Corneoscleral Explants. Using the same procedure as for the eye bank, fresh human donor corneoscleral explants were retrieved from cadavers (within 30 hours postmortem) in the Maryland State Anatomy Board and placed in Dulbecco's phosphate-buffered saline (DPBS) on ice. The cadavers were de-identified and not considered as human subjects by the Human Research Protection Office of the university.

Preserved Human Donor Corneoscleral Explants. Viable human corneoscleral explants that were without disease but were determined not suitable for transplantation because of the less than optimal CE cell densities and the advanced ages of the donors, stored in storage medium at 4°C, were obtained from the Lions Eye Institute for Transplant and Research, Inc. (Tampa, FL).

VIP Treatment of Human Donor Corneoscleral Explants

Fresh Human Corneoscleral Explants. Before their storage in storage medium at 4°C, human donor corneoscleral explants dissected from the right eyes were treated with VIP (V6130; Sigma, St. Louis, MO; 10^{-8} M in medium A), whereas those of the paired left eyes were used as controls and treated with medium A alone in 35-mm Petri dishes containing 3.5 mL medium A under 5% CO₂/95% air at 37°C for 30 minutes. Four days (five pairs), 10 days (one pair), and 25 days (one pair) after storage, corneoscleral explants in storage medium were warmed to room temperature (for 1 to 2 hours). One quarter of the cornea was stained with alizarin red S, and CE cells were scraped from the remaining three-fourths of the cornea for determination of CNTFR α levels by Western blot analysis.¹⁵

Preserved Human Donor Corneoscleral Explants. Explants previously stored in storage medium were removed from the vials for VIP treatment, which, except for CE cell retention studies, used the same procedure as that of the fresh ones, but the assignment of the left and right corneoscleral explants was reversed. For CE cell

retention studies, VIP treatment was performed in 2 mL medium A placed in each well of a 24-well plate.

CNTF Treatment of Human Donor Corneoscleral Explants

After VIP treatment and subsequent storage, human donor corneoscleral explants were removed from the storage medium-containing vials and were tested for the effectiveness of the CNTFR α preserved in CE cells in protecting the integrity of the corneal endothelium. Either whole corneoscleral explants or corneal buttons (8.5 mm in diameter, 56.7 mm² in area) trephined from the corneoscleral explants were incubated in 3.5 mL medium A in 35-mm Petri dishes for 1 hour at 37°C in 5% CO₂/95% air, followed by treatment with 0.83 nM rhCNTF (257-NT; R&D Systems, Minneapolis, MN) in medium B (37°C in 5% CO₂/95% air). In whole corneoscleral explants, the incubation times were 41 hours and 4 days for CE connexin 43 (Western blot analysis) and damaged area quantification, respectively, whereas the corneal buttons were incubated for 20 to 25 hours for quantification of areas of damaged corneal endothelium.

Alizarin Red S-Staining of the Damaged Corneal Endothelium

Saturated alizarin red S solution was prepared from 1% alizarin red S solution in calcium- and magnesium-free DPBS ([Ca⁺², Mg⁺²-free DPBS]; Gibco; 14190). After removal of the undissolved dye by centrifugation, the saturated alizarin red S solution was used without pH adjustment. Human donor corneoscleral explants in storage medium at 4°C were warmed to room temperature (1–2 hours), and those in organ cultures for CNTF treatment were processed for alizarin red S staining. They were rinsed twice with DPBS before the CE cells in corneoscleral explants and corneal buttons were covered with the saturated alizarin red S solution for 90 to 240 seconds, and then rinsed in DPBS for two or three times.

Photography

Panoramic View. To take digital photographs of the whole CE sheet, the corneoscleral explants and corneal buttons were placed on a light box (CE side up).

Microscopic View. The corneoscleral explants and corneal buttons were mounted on coverslips (CE side down), and digital photomicrographs (3×10^{-3} mm²/field) of the CE sheet were taken under an inverted light microscope (Diaphot; Nikon, Tokyo, Japan).

Quantification of Areas of Damaged Corneal Endothelium

Panoramic View. The recently reported procedure by Saad et al.²⁰ was closely followed. Using three image editing tools (magnetic lasso, magic wand, and marquee of Adobe Photoshop, version 7.0; Adobe Systems, San Jose, CA), areas of damaged corneal endothelium revealed in photographs of the panoramic view of whole corneoscleral explants/corneal buttons (corneal endothelium side up) were quantified.

From the photograph of the whole corneoscleral explant, the panoramic view of the whole corneal endothelium was selected by dragging the magnetic lasso around the periphery of the cornea. The enclosure was formed automatically as the lasso was dragged. To finish selecting the cornea, the lasso was dragged back to the starting point. This selection was copied and pasted into a new image. These images and those of the panoramic view of the corneal buttons revealed alizarin red S-stained areas of damaged corneal endothelium, which were then quantified using the magic wand and marquee tools.

The magic wand tool selected areas with color similar to the spot clicked on by the wand. The size of the selected area was determined by the tolerance value set (range, 0–255). With a lower tolerance value, only areas with colors similar to those pointed at by the magic wand were selected. A high tolerance value allowed more color vari-

TABLE 1. Freshly Dissected Paired Human Donor Corneoscleral Explants from Seven Donors to Demonstrate the Beneficial Effect of VIP Treatment before Storage on Retaining CE Cell CNTFR α during Storage

Donor	Age/Sex	Cause of Death	Death-Excision (hours)	CNTFR α _{VIP} /CNTFR α _{Control}	Days in Storage Medium after VIP Treatment
1	87/M	Obstructive lung disease	22	1.22	4
2	62/F	Cardiac arrest	28	1.30	4
3	82/F	Stroke	18	1.44	4
4	83/M	Obstructive lung disease	16	1.24	4
5	76/M	Brain cancer	20	1.05	5
6	82/F	Lung cancer	12	1.44	10
7	53/F	Lung cancer	29	2.24	25

ations in a selection. With the right tolerance value set, the magic wand selected the damaged portions of the corneal endothelium while ignoring the healthy areas. Small imperfections were corrected with the use of the marquee tool for adding and subtracting small areas from the areas selected by the magic wand.

To figure out the percentage of damaged area in an image, the histogram function was used (Image-Histogram, which gave pixel counts for selected areas) to find the pixel count for the damaged area and the entire image. The damage was expressed as a percentage by dividing the amount of pixels in the damaged area by the amount of pixels in the entire image.

Microscopic View. Photographs of the microscopic fields (3×10^{-3} mm²/field) of the central cornea were taken and analyzed as described for those of the panoramic view of the corneal endothelium. Alizarin red S-stained corneal endothelium in flat-mounted corneoscleral explants/corneal buttons (endothelium side down) viewed under an inverted microscope ($\times 200$) revealed that there were three types of CE damage (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5983/-/DCSupplemental>).

CE Cell Extract

Corneal endothelium was scraped off the corneas using a razor blade and were homogenized in the RIPA buffer (25 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (one tablet of protease inhibitor cocktail [Complete Mini; Roche Diagnostics, Mannheim, Germany] per 10 mL). After centrifugation (12,000g, 10 minutes), the supernatant (cell extract) was collected, electrophoresed under reducing conditions, and electrophoretically transferred to nitrocellulose membranes for Western blot analysis.

Western Blot Analysis

For the detection of CNTFR α , the nitrocellulose membranes were immunostained with an affinity-purified goat anti-human CNTFR α primary antibody (AF-303-NA; R&D Systems), and an anti-goat IgG-alkaline phosphatase conjugate secondary antibody (401512; Calbiochem, La Jolla, CA). CNTFR α on nitrocellulose membranes was detected by a chromogenic method, using an alkaline phosphatase substrate solution made from tablets (Fast Red TR/Naphthol AS-MX; Sigma, St. Louis, MO). For the detection of connexin 43, actin, VPAC1, and VPAC2, the chemiluminescent method was used with affinity-purified rabbit-anti-connexin 43 (AB19012; Chemicon), a mouse monoclonal anti-actin (CP01, anti-actin [Ab-1; Calbiochem]), VPAC1 (20-272-191277; GenWay, San Diego, CA), and VPAC2 (MAB5470; Chemicon) primary antibodies, horseradish peroxidase-linked anti-mouse and anti-rabbit secondary antibodies, and horseradish peroxidase substrate (ECL kit; Amersham Pharmacia, Piscataway, NJ).

CE Cell Retention in Corneoscleral Explants after Long-term Storage in Storage Medium

As previously reported,⁹ the numbers of CE cells retained in the corneal endothelium in each of the corneoscleral explants were determined by an uninformed observer using a cytotoxicity kit

(L-3224; Molecular Probes, Eugene, OR). One milliliter of the reaction mixture was added to cover and incubate the corneal endothelium in corneoscleral explants for 30 minutes at 37°C. It was first determined that nearly all CE cells retained in the corneal endothelium in a flat-mounted corneoscleral explant were alive. CE sheets with areas of attached Descemet's membrane were scraped from the corneoscleral explants using a razor blade and transferred to and incubated in microfuge tubes containing 0.4 mL of 10 mM EDTA in Mg²⁺- and Ca²⁺-free DPBS for 30 minutes at 37°C. After the tubes were vortexed to release Descemet's membranes, the CE cells in the supernatants were spun down in a microfuge (12,000g, 1 minute). CE cells were resuspended in 20 μ L reaction mixture. From each tube, duplicate 8- μ L cell suspensions were placed in each of the two wells (8-mm diameter; 50.2 mm²/well) of an eight-well slide (Erie Scientific, Portsmouth, NH), and each well was covered by a coverslip. Under an inverted microscope ($\times 200$ magnification) equipped with an epifluorescence attachment (Diaphot-TMD;

FIGURE 1. VIP pretreatment of fresh human donor corneoscleral explants before their storage in storage medium enhanced the preservation of CE cell CNTFR α . (A) Western blot analysis of CE cell extract from the paired corneoscleral explants (control vs. VIP treated) of a 62-year-old female donor (donor 2, Table 1) for CNTFR α and actin (as an internal standard) demonstrating increased CE cell CNTFR α level in the VIP-pretreated corneoscleral explant. (B) Relative CNTFR α levels in CE cells from seven pairs of human corneoscleral explants. CNTFR α densities from the Western blots were normalized against those of the actin internal standard. Within each pair of corneoscleral explants (Table 1), the normalized CNTFR α levels in CE cells of both the control and the VIP-treated corneoscleral explants were divided by the normalized CNTFR α level obtained from the control corneoscleral explant.

FIGURE 2. Increased CNTF responsiveness in CE cells (connexin 43 upregulation) resulted from a brief intermittent VIP treatment of human donor corneoscleral explants during their storage in storage medium. Corneoscleral explants from the left and right eyes previously preserved in storage medium (15–19 days; see Table 2) were treated with VIP (10^{-8} M) and vehicle at 37°C for 30 minutes, respectively, and returned to their respective vials for 9 additional days. The explants were then treated with 0.83 nM CNTF for 41 hours (at 37°C). (A) Western blot analysis for connexin 43 and actin (as internal standard) of CE cell extracts derived from donor 1 (Table 2). (B) Relative connexin 43 levels in CE cells from the control and VIP-treated paired human corneoscleral explants from four donors. Within each pair, the normalized (against actin) connexin 43 intensities of both explants were divided by those found in the control. Ratios $\times 100$ were expressed in the y-axis. Results were representative of four similar experiments and confirmed in freshly dissected human corneoscleral explants.

Nikon, Tokyo, Japan), the numbers of CE cells in the field (0.33-mm^2 area) defined by the photographic mask that was placed in the optical path of the microscope were counted. In each of the two wells, CE cells were counted in five masked fields, and the numbers were pooled to give a mean value for each corneoscleral explant. Within each corneoscleral explant pair, the mean values were normalized against those of the control corneoscleral explant.

RESULTS

VIP Treatment before Storage in Medium of Freshly Dissected Human Donor Corneoscleral Explants Prevented Loss of CNTFR α from CE Cells during Storage

We previously reported that CE cells in human donor corneoscleral explants gradually lose their CNTFR α during explant

storage in storage medium and that functional CNTFR α can be restored by the recombinant CNTFR α .¹⁵ Because VIP is responsible for the maintenance of the differentiated state of CE cells,¹⁴ VIP treatment of the CE cells may prevent CNTFR α loss. Before storage of freshly dissected human donor corneoscleral explants in storage medium (4°C), those of the right eyes were treated with VIP (10^{-8} M, which was determined to be the optimal concentration in our previous studies; 37°C , 30 minutes), whereas those of the paired left eyes were used as controls and treated with vehicle alone. Four (four pairs), 5 (one pair), 10 (one pair), and 25 (one pair) days after storage, CE cell CNTFR α levels were analyzed by Western blot analysis. Lindstrom²¹ has reported that CE cells in the human donor corneoscleral explants preserved in storage medium at 4°C are metabolically active. The brief VIP treatment resulted in a measurable increase in the retention of CE cell CNTFR α in every human donor corneoscleral explant from seven donors (Table 1). Relative CE cell CNTFR α levels found in the control and VIP-treated paired corneoscleral explants from all seven pairs were 1.00 and 1.42 ± 0.15 (mean $\pm$ SEM; $P = 0.014$), respectively. Of those stored for 4 days (four pairs), CNTFR α levels found in the control and VIP-treated paired corneoscleral explants were 1.00 and 1.30 ± 0.05 ($P = 0.005$), respectively. The effectiveness of VIP in preserving CNTFR α was also observed in corneoscleral explants that have been previously stored in storage medium (data not shown) (Fig. 1).

Enhanced CE Cell CNTF Responsiveness Found in Stored Human Donor Corneoscleral Explants with Previous VIP Treatment

CNTF upregulates the gene and protein expressions of connexin 43 in CE cells in human donor corneoscleral explants.¹⁵ Higher CE cell CNTFR α levels preserved in VIP-treated human donor corneoscleral explants may lead to enhanced responsiveness to CNTF modulation in the expression of connexin 43 in CE cells. After VIP treatment and preservation in storage medium (4°C) for 9 days, the explants were then treated with 0.83 nM CNTF for 41 hours (at 37°C). Western blot analysis for connexin 43 demonstrated that CE cell responsiveness to CNTF increased in every VIP-treated corneoscleral explant to an average of $174\% \pm 23\%$ ($n = 7$ pairs; $P = 0.023$) of their respective paired controls (Fig. 2, Table 2). The results presented were representative of four similar experiments using corneoscleral explants that have been preserved in storage medium previously and confirmed in freshly dissected human corneoscleral explants (data not shown).

Decreased CE Damage Observed in CNTF-Modulated Human Corneoscleral Explants That Experienced Brief Intermittent VIP Treatment during Storage

To investigate the possibility that the increased CNTF responsiveness in CE cells in VIP-treated corneoscleral explants (Fig.

TABLE 2. Paired Human Corneoscleral Explants from Four Donors in the Demonstration of Increased CE Cell CNTF Responsiveness (Connexin 43 Upregulation) 9 Days after a Brief Intermittent VIP Treatment during Storage

Donor	Age/Sex/Days in Storage Medium before VIP Treatment	Cause of Death	Death-Excision (hours)	Connexin 43 _{VIP} /Connexin 43 _{Control} $\times 100$
1	67/F/19	Brain cancer	7	138
2	63/F/19	Acute cardiac crisis, hypertension	8	239
3	75/F/17	Pneumonia, chronic obstructive pulmonary disease	7	148
4	69/M/15	Lung cancer, pneumonia	6	173

TABLE 3. Paired Human Donor Corneoscleral Explants from Four Donors in Storage in the Demonstration of the Beneficial Effect of a Brief Intermittent VIP Treatment on Reducing CE Damage

Donor	Age/Sex/Days in Storage before VIP Treatment	Cause of Death	Death–Excision (hours)	CE Damage Control vs. VIP (%)
1	64/F/23	N/A	15	22 vs. 15
2	64/M/23	Atherosclerotic cardiovascular disease	19	32 vs. 21
3	51/M/20	N/A	8	43 vs. 33
4	75/F/20	Congestive heartfailure, hypertension	10	40 vs. 37

2, Table 2) can lead to enhanced integrity of the corneal endothelium placed under the modulation of CNTF, the CE damage in both the whole corneoscleral explants and in corneal buttons (8.5-mm in diameter) trephined from corneoscleral explants was assessed.

Whole Corneoscleral Explants. Nine days after VIP treatment of previously storage medium-preserved explants (Table 3), they were transferred to and incubated in the minimal essential medium (0.83 nM CNTF; 37°C; 4 days), before the CE damage was quantified. The panoramic view of the whole corneoscleral explants shown in Figure 3A demonstrated that the alizarin red S-stained area in the

corneal endothelium of the control was larger than that of the VIP-treated corneoscleral explants. In the control and VIP-treated corneoscleral explants, the percentages of CE damage were $34.3\% \pm 4.7\%$ and $26.5\% \pm 5.1\%$, respectively ($P = 0.01$; $n = 4$ pairs). The beneficial effect of VIP treatment in reducing CE damage was observed in all four pairs (Table 3); the damaged areas observed in the corneal endothelium in VIP-treated corneoscleral explants were $75.0\% \pm 6.4\%$ of those in the respective controls ($P = 0.02$; $n = 4$ pairs) (Fig. 3B). The extensive damage observed in the periphery of the corneal endothelium of both the VIP-treated and the control corneoscleral explants was caused by the swelling of the stroma when the explants were transferred from storage medium (4°C) to minimal essential medium (37°C) (data not shown). These results are representative of 1 of the 2 experiments using human donor corneoscleral explants that have previously been preserved in the eye bank and confirmed using freshly dissected human corneoscleral explants. To ascertain the beneficial effect of VIP treatment on minimizing the CE damage, the center of two additional pairs of alizarin red S-stained corneoscleral explants was examined microscopically. As shown in Table 4, the beneficial effect of VIP in reducing the area of damage in the center of the corneal endothelium was observed in both pairs, to 31% and 46% (average, 39%) of the respective controls.

Corneal Buttons. Five days after VIP treatment of corneoscleral explants (Table 5), corneal buttons (8.5-mm diameter) were trephined and were then transferred to and incubated in the minimal essential medium (0.83 nM CNTF; 37°C; 20–25 hours) before CE damage was quantified. The panoramic view of two whole corneal buttons from paired explants from donor 8 in Table 5 is shown in Figure 4A. The alizarin red S-stained area in the corneal button from the control was larger than that from the VIP-treated corneoscleral explant. In buttons from nine pairs of corneoscleral explants, the percentages of CE damage decreased from $26.3\% \pm 7.5\%$ in the control to $15.4\% \pm 4.3\%$ ($P = 0.027$) resulting from VIP treatment of the corneoscleral explants (Fig. 4B). The damaged areas observed in the corneal endothelium in the buttons from the VIP-treated corneoscleral explants were $71.0\% \pm 11.0\%$ of those in the respective controls ($P = 0.016$; $n = 9$ pairs) (Fig. 4B). A microscopic view of the center of the corneal buttons revealed consistently the beneficial effect of VIP treatment of the corneoscleral explants on reducing the CE damage in all corneal buttons examined ($n = 7$ pairs; Table 5). A microscopic view revealed a more pronounced reduction in CE damage resulting from the VIP treatment of the explants than the panoramic view (Figs. 4B, 4C). Under the microscopic view of the corneal buttons, VIP treatment of the corneoscleral explants reduced the CE damage to $23\% \pm 4\%$ of the control ($P = 6.8 \times 10^{-7}$; $n = 7$ pairs) (Fig. 4C).

FIGURE 3. A brief intermittent VIP treatment during storage of human donor corneoscleral explants reduced CE damage (demonstrated in whole corneoscleral explants). After the VIP treatment, the explants were kept in storage medium for 9 additional days and then incubated at 37°C in the presence of CNTF (0.83 nM) for 4 days before the CE damage was assessed in alizarin red S-stained corneal endothelium. (A) Panoramic view of the whole corneoscleral explant (donor 1, Table 3) after alizarin red S staining. (B) Quantification of damaged (alizarin red S-stained) areas in the whole corneal endothelium demonstrating the beneficial effect of the VIP treatment. Percentages of damage in the whole corneal endothelium (gray) in the control and VIP-treated corneoscleral explants were $34.3\% \pm 4.7\%$ and $26.5\% \pm 5.1\%$, respectively ($P = 0.01$; $n = 4$ pairs). Damaged CE areas (black) found in VIP-treated corneoscleral explants were $75.0\% \pm 6.4\%$ of those in the respective controls ($P = 0.02$; $n = 4$ pairs). The results presented represented 1 of the 2 experiments using human donor corneoscleral explants previously preserved in the eye bank and were confirmed using freshly dissected human corneoscleral explants.

TABLE 4. Paired Human Corneoscleral Explants from Two Donors in Storage for Microscopic Demonstration of the Beneficial Effect of a Brief Intermittent VIP Treatment on Reducing Central CE Damage

Donor	Age/Sex/Days in Storage Medium before VIP Treatment	Cause of Death	Death-Excision (h)	CE Damage/Field (<i>n</i> = 10 fields) Control vs. VIP (%)	Relatively Damaged Area Control vs. VIP
1	19/M/24	N/A	20	39 ± 5 vs. 12 ± 2 <i>P</i> = 0.00005	1.0 vs. 0.31
2	62/M/18	Coronary artery disease, hypertension	19	13 ± 2 vs. 6 ± 1 <i>P</i> = 0.002	1.0 vs. 0.46

Retention of CE Cells in Human Donor Corneoscleral Explants in Long-term Storage: Effect of VIP Treatment

To quantify the effect of VIP treatment on CE cell retention, human donor corneoscleral explants in storage medium for 7 to 21 days were removed from storage vials, and the paired corneoscleral explants were used as either control or treated with VIP (10^{-8} M, 30 minutes, 37°C). After the treatment, corneoscleral explants were returned to their original storage vials and stored for an additional 1, 2, and 3 weeks. CE cells that have remained attached to the Descemet's membrane in the corneoscleral explants were nearly all alive and were counted (see Materials and Methods). Although no significant improvement was seen on CE cell retention 1 and 2 weeks after VIP treatment, corneoscleral explants (14 pairs) in storage for 3 weeks after VIP treatment demonstrated significant effect of the VIP treatment on retaining more CE cells in the explants. The number of CE cells (per microscopic field) derived from the control and VIP-treated corneoscleral explants were 25 ± 4 and 45 ± 7 , respectively ($P = 0.001$) (Fig. 5). The relative numbers of CE cells in the control and VIP-treated corneoscleral explants were 1 and 2.06 ± 0.38 , respectively ($P = 0.008$; $n = 14$ pairs).

VIP Receptor in CE Cells

Western blot analysis demonstrated that CE cells isolated from freshly dissected human donor eyes expressed a 64-kDa VPAC1-immunoreactive molecule, but no VPAC2-immunoreac-

tive molecule was detected (Fig. 6). The VPAC1 was also expressed in CE cells of the human donor corneoscleral explants in storage (data not shown).

DISCUSSION

The present study demonstrated for the first time the feasibility of using an autocrine of a tissue to enhance its integrity during preservation of the tissue for transplantation. The transplantation of human corneas is unique in that the survival of one particular cell type, CE cells, has been identified as the most critical for the success of the procedure.^{2,7,8} In the transplantation of other tissues, no particular cell type has been identified as the equivalent of the CE cell. Nevertheless, because both VIP and CNTF play trophic factor roles in the lung and heart, they may play similar roles in the enhancement of the integrity of these two organs during their preservation. In the heart, VIP, VPAC1, and VPAC2, are expressed in the myocytes and in the nonmyocytes,^{22,23} whereas CNTFR α is expressed in the myocytes and activation of the cardiac CNTFR α reverses left ventricular hypertrophy in obesity.²⁴ VIP, VPAC1, and VPAC2 are expressed in the lung, and the preservation of endogenous VIP level is linked to the maintenance of the function and integrity of the transplanted lung.^{25–27}

During human donor corneoscleral explant storage in storage medium, an increase in the retention of CE cells was observed 3 (but not 1 or 2) weeks after brief, intermittent VIP treatment. Corneoscleral explants used for transplantation

TABLE 5. Paired Human Donor Corneoscleral Explants in Storage Trephined for Corneal Buttons: A Brief Intermittent VIP Treatment of the Explants Enhanced CE Integrity in Buttons

Donor	Age/Sex/Days in Storage Medium before VIP Treatment	Cause of Death	Death-Excision (h)	CE Damage (%) Control vs. VIP	
				Panoramic View	Microscopic Fields
1	46/M/27	Acute cardiac crisis, hypertension	7	28 vs. 11	NA
2	63/F/22	Sepsis	16	58 vs. 47	4 ± 1 vs. 1 ± 0 <i>P</i> < 0.001, <i>n</i> = 19
3	66/M/21	Chronic obstructive pulmonary disease	9	67 vs. 22	1 ± 0 vs. 0 <i>P</i> < 0.001, <i>n</i> = 23
4	69/F/20	Congestive heart failure, coronary artery disease	6	16 vs. 15	NA
5	67/F/20	Acute cardiac crisis, congestive heart failure	6	10 vs. 8	24 ± 4 vs. 4 ± 1 <i>P</i> < 0.001, <i>n</i> = 13
6	73/M/19	Pneumonia	7	32 vs. 15	17 ± 3 vs. 4 ± 1 <i>P</i> = 0.001, <i>n</i> = 14
7	73/F/16	Lung cancer	12	13 vs. 10	6 ± 2 vs. 2 ± 0 <i>P</i> = 0.005, <i>n</i> = 21
8	92/F/0	Dementia	28	8 vs. 4	6 ± 1 vs. 0 <i>P</i> < 0.001, <i>n</i> = 20
9	82/M/0	Dementia, hypertension	18	5 vs. 7	30 ± 5 vs. 8 ± 2 <i>P</i> < 0.001, <i>n</i> = 14

n, number of fields; NA, not available.

FIGURE 4. Reduced CE damage in corneal buttons (8.5-mm diameter) trephined from human donor corneoscleral explants that experienced a brief, intermittent VIP treatment during their storage. After the VIP treatment, explants were kept in storage medium for 5 additional days before corneal buttons (8.5-mm diameter) were trephined from them. The buttons were incubated at 37°C for 1 hour and then in the presence of CNTF (0.83 nM) for 20 to 25 hours before the CE damage was assessed. (A) Panoramic view of the whole corneal buttons (donor 8, Table 5) after alizarin red S staining. (B) Quantification of damaged (alizarin red S-stained) areas in the panoramic photographs of the whole corneal buttons demonstrating the beneficial effect of the VIP treatment. The percentages of CE damage of the whole buttons (gray) were 26.3% ± 7.5% and 15.4% ± 4.3%, in buttons from the control and VIP-treated explants, respectively ($P = 0.027$; $n = 9$ pairs). The damaged CE areas (black) found in buttons from VIP-treated explants were 71.0% ± 11.0% of those in the respective controls ($P = 0.016$; $N = 9$ pairs). (C) Quantification of the microscopic damage (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5983/-/DCSupplemental>) in the corneal endothelium of flat-mounted buttons (corneal endothelium side down) revealed under an inverted microscope (magnification, ×200). The percentages of CE damage (gray) were 12.7% ± 4.2% and 2.8% ± 1.0% in buttons from the control and VIP-treated explants, respectively ($P = 0.011$; $n = 7$ pairs). The damaged CE areas (black) found in buttons from VIP-treated explants were 23.0% ± 4.0% of those in the respective controls ($P = 6.8 \times 10^{-7}$; $n = 7$ pairs). Both freshly dissected and preserved human donor corneoscleral explants from the eye banks were used (Table 5).

were stored in storage medium for much less than 3 weeks; thus, the direct clinical relevance of this observation was not clear. On the other hand, the result was indicative of the long-term effectiveness of the VIP treatment and, therefore, suggested the possibility that the CE sheets from the VIP-

FIGURE 5. The beneficial effect of VIP treatment on the retention of CE cells in human donor corneoscleral explants in storage medium. Fourteen pairs of human donor corneoscleral explants in storage for 7 to 21 days were removed from the medium-containing storage vials for the brief VIP treatment (10^{-8} M, 30 minutes, 37°C) and then returned to the same storage vials and stored at 4°C for 21 additional days. CE cells were scraped from the corneoscleral explants and counted under a microscope. The number of CE cells (per microscopic field) derived from the control and VIP-treated corneoscleral explants were 25 ± 4 and 45 ± 7 , respectively ($P = 0.001$).

treated corneoscleral explants may retain more CE cells than those from the untreated ones in the recipients' eyes. Nevertheless, the proof of the superior quality of corneoscleral explants that have been treated with VIP for transplantation awaits their application in corneal keratoplasty in in vitro model studies and, ultimately, in clinical trials.

Our strategy is mechanism based. First, CNTFR α is gradually lost during corneoscleral explant preservation.¹⁵ Second, CE cell CNTFR α in the donor corneas likely is under the modulation of the endogenous CNTF in the recipient eye. CNTF was discovered in an extract of ciliary body, iris, and choroid.^{28,29} CNTF, a cytokine that does not have a classic secretory signal peptide sequence, is released only after injury through an unknown mechanism.³⁰ We previously demonstrated that CNTF is released in a complex with the CNTF-binding CNTFR α by CE cells surviving the oxidative stress.¹⁰ We hypothesize

FIGURE 6. Western blot analysis showing VPAC1 (not VPAC2) expressed in CE cells from fresh human donor eyes. A 64-kD VPAC1-immunoreactive molecule was detected in CE cell extract. Std, molecular size standard.

that CNTF is released from the recipient's ciliary body and iris in response to transplant injury to modulate the donor CE cell CNTFR α . In addition to the fact that CNTF is present in the aqueous humor of the enucleated (injured) bovine¹⁷ and human (data not shown) eyes, our hypothesis is supported by studies of the role of CNTF in optic nerve injury. For example, CNTF not only promotes the survival of the retinal ganglion cells in intraorbital nerve crush injury³¹ but also is the mediator of lens injury-induced beneficial effects on retinal ganglion cell survival and regeneration in optic nerve injury.³² Thus, VIP treatment, while preserving the level of CNTFR α in CE cells in corneoscleral explant in storage for transplantation (Fig. 1), increased the CE cell responsiveness to CNTF modulation (Fig. 2), which might have led to enhanced preservation of the corneal endothelium. Indeed, results in Figures 3 and 4 and Tables 3 to 5 demonstrated, at both gross and microscopic levels, that after incubation at 37°C and in the presence of CNTF, the corneal endothelium of the corneoscleral explants treated with VIP was less damaged than that in their paired controls.

The beneficial effects of CNTF treatment observed in a variety of animal models of photoreceptor cell degeneration (see Ref. 33 for review) led to phase I, II, and III human clinical trials (a phase II study of implants of encapsulated human NTC-201 cells releasing ciliary neurotrophic factor [CNTF] in participants with visual acuity impairment associated with atrophic macular degeneration, ClinicalTrials.gov Identifier NCT00277134; a phase II study of an encapsulated cell technology [ECT] implant for patients with atrophic macular degeneration, ClinicalTrials.gov Identifier NCT00447954; a phase II/III study of an ECT implant for participants with early-stage retinitis pigmentosa, ClinicalTrials.gov Identifier NCT00447980; a phase II/III study of an ECT implant for patients with late-stage retinitis pigmentosa, Usher syndrome type 2 or 3, or choroideremia, ClinicalTrials.gov Identifier NCT00447993) in which encapsulated cells engineered to release CNTF were implanted into the vitreous of patients with retinitis pigmentosa and macular degeneration.^{34,35} The present study suggested a potential therapeutic use of CNTF intraoperatively in corneal transplantation.

VIP, a 28-amino acid neuropeptide, is widely distributed in the central and peripheral nervous systems, where its neuroprotective role is observed in a variety of in vitro and in vivo systems.^{36–38} VIP immunoreactivity is present in the aqueous humor of a variety of species, including human.^{12,39,40} VIP binds to two types of adenylyl cyclase stimulatory heterotrimeric G protein-coupled receptors (VPAC1 and VPAC2) and transduces signals through cAMP-dependent and -independent pathways.⁴¹ The VPAC1 (not VPAC2) receptor is expressed in bovine¹⁶ and human CE cells (Fig. 6). In CE cells, VIP stimulates the production of large amounts of cAMP,¹¹ phosphorylation of the cAMP-responsive element binding protein (CREB), and upregulation of the antiapoptotic protein Bcl-2.¹⁶ Although CREB phosphorylation mediates cell survival through the induction of antiapoptotic Bcl-2,^{42–44} apoptotic CE cells consistently represent <1% of the population throughout the 3 weeks of storage in storage medium.⁴⁵ On the other hand, CREB phosphorylation may play a role in cAMP-induced CNTFR α gene expression⁴⁶ and may mediate the VIP effect.

Because VIP upregulates the CE cell differentiation marker^{18,19} N-cadherin,¹⁴ higher CNTFR α levels observed in VIP-treated corneoscleral explants may result from the maintenance of the differentiated state. Regardless of the mechanism, the present study supported once again our notion that the two CE autocrine trophic factors, CNTF and VIP, work in concert to promote CE survival.¹⁷

In conclusion, this is the first mechanism-based strategy for the demonstration of the feasibility of using autocrines of CE

cells to enhance their integrity during the preservation of human donor corneoscleral explants for transplantation.

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