

Corneal Endothelial Cell Proliferation: A Function of Cell Density

Sangita P. Patel¹ and William M. Bourne²

PURPOSE. To determine how stimuli that increase corneal endothelial cell proliferation of human corneas in culture relate to changes in endothelial cell density in the central and peripheral cornea.

METHODS. Human donor cadaver corneas not suitable for transplantation were divided into four pie-shaped wedges and incubated at 37°C in medium supplemented with fetal bovine serum, epidermal growth factor, fibroblast growth factor, and gentamicin. To promote a proliferative response, samples were treated with EDTA at concentrations of 0, 0.5, 2.5, and 5.0 mM for 1 hour and then returned to culture medium. Endothelial cell proliferation was assayed with Ki-67 immunolocalization 48 and 96 hours after EDTA treatment. Samples were mounted with propidium iodide or DAPI. The total number of cells and the number of Ki-67-positive cells were counted in three regions, defined as central, mid, and peripheral cornea, to determine endothelial cell density and percentage of proliferation.

RESULTS. A proliferative response to EDTA was not found. However, increased proliferation was noted in the central compared with the peripheral corneal region. Unexpectedly, the increased proliferation in the central region corresponded to a trend of lower endothelial cell density in the central region compared with the peripheral region. Corneal endothelial cell proliferation under our culture conditions is noted primarily when cell density is less than 2000 cells/mm².

CONCLUSIONS. Corneal endothelial cell proliferation under our culture conditions does not lead to supranormal endothelial cell density. Rather, cell proliferation is noted in those regions that may be experiencing a greater burden of cell loss. (*Invest Ophthalmol Vis Sci.* 2009;50:2742-2746) DOI:10.1167/iov.08-3002

The clear cornea is composed of multiple cell layers that form the primary refractive surface of the eye. The corneal endothelial cell layer lines the internal surface with a single layer of cells that functions to maintain corneal clarity by regulating corneal hydration.¹ Unlike epithelial cells that divide to repair defects, human corneal endothelial cells do not proliferate in response to injury.² When corneal endothelial cell loss is severe, as may occur from corneal trauma, surgery, or endothelial dystrophy, the precise control of corneal hydration is lost, the cornea becomes edematous, and visual acuity declines. Corneal transplantation with replacement of the endo-

thelial monolayer is the only treatment option to restore a clear cornea in these circumstances. However, even with uncomplicated corneal transplantation surgery, endothelial cells are continually lost at an accelerated rate.³ When cells are lost beyond a critical level for maintaining a clear corneal graft, late endothelial failure ensues and remains the primary cause for graft failure 5 years after transplantation.⁴

In recent years, there has been tremendous interest in understanding the proliferative potential of the corneal endothelium to arrive at better solutions for the management of endothelial cell loss.⁵ Corneal endothelial cells maintain proliferative potential because they are not terminally differentiated but remain arrested in the G1 phase of the cell cycle.⁵ This has allowed investigators to manipulate regulators of cell cycle arrest in endothelial cells to enhance proliferation. For example, corneal endothelial cell proliferation has been noted in response to inhibition of the cyclin-dependent kinase inhibitor p27Kip1 by siRNA treatment.⁶ Similarly, the release of corneal endothelial cell contact inhibition by EDTA treatment promotes proliferation.⁷ In addition, the endothelial cell monolayer is not a homogenous structure. A greater endothelial proliferative response has been noted in the peripheral cornea than in the central cornea.^{8,9} Cell density is also greater in the peripheral than in the central cornea.¹⁰

How do these variations in corneal endothelial proliferative response and endothelial cell density correlate? The natural assumption would be that an increase in cell proliferation would correspond to an increase in cell density. However, this has not been examined in studies looking at proliferative responses on intact corneal tissue. This would be the key factor if the goal were to increase cell density on corneal buttons to prolong graft survival. In this study, we examine corneal endothelial cell proliferation and cell density in central and peripheral corneal regions of human corneas in culture. We use EDTA as a stimulus to promote endothelial cell proliferation⁷ to compare the proliferative capacity of the central and peripheral areas of the cornea and to determine whether an increase in endothelial cell proliferation corresponds to an increase in endothelial cell density.

MATERIALS AND METHODS

Corneal Tissue

Human donor corneas not suitable for transplantation were obtained from the National Disease Research Interchange (Philadelphia, PA). Tissues were harvested and placed in solution (Optisol GS; Chiron Ophthalmics, Irvine, California), within 12 hours of death. Donor ages ranged from 15 to 68 years. Thirteen corneas from eight donors were used in these experiments. Endothelial cell densities of all corneas were greater than 1800 cells/mm². Experiments were started within 7 days of donor death.

Culture Conditions

Each cornea was divided into four pie-shaped wedges to increase sample size. Each sample was incubated endothelial side up in an individual well of a 24-well plate. Incubation medium consisted of tissue culture medium (Medium-199; Gibco, Grand Island, NY) supple-

From the ¹Massachusetts Eye and Ear Infirmary, Boston, Massachusetts; and the ²Mayo Clinic, Rochester, Minnesota.

Supported by National Eye Institute Grant EY02037, Research to Prevent Blindness, and the Mayo Foundation.

Submitted for publication October 13, 2008; revised December 7, 2008 and January 5, 2009; accepted April 3, 2009.

Disclosure: **S.P. Patel**, None; **W.M. Bourne**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Sangita P. Patel, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114; sangita_patel@meei.harvard.edu.

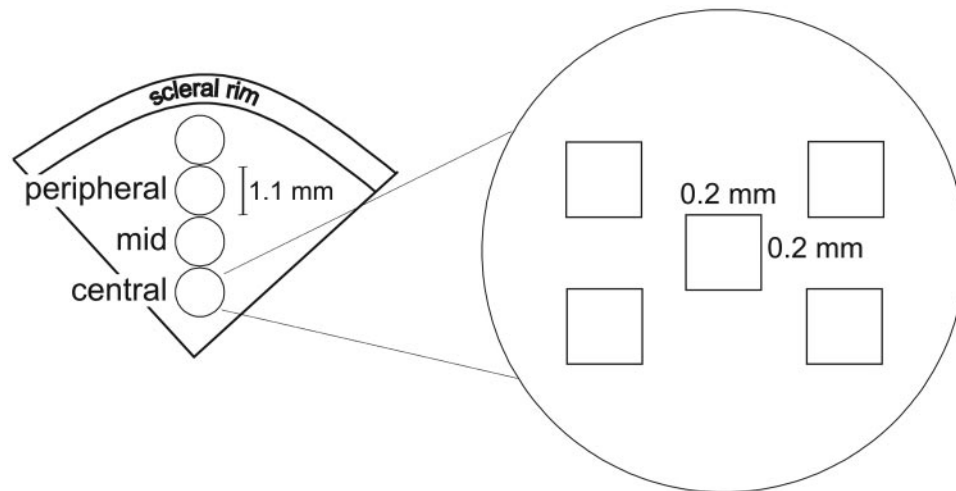


FIGURE 1. Schematic depicting areas of data analysis for each corneal sample.

mented with 10% fetal bovine serum (Gibco), 10 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA), 20 ng/mL fibroblast growth factor (Invitrogen), and 50 $\mu\text{g/mL}$ gentamicin (Gibco). Samples were preincubated at 37°C for 24 hours and then treated with EDTA. EDTA stock solution (0.5 M; Gibco) was diluted in tissue culture medium (Medium-199; Gibco) to concentrations of 0, 0.5, 2.5, and 5.0 mM (pH 7.4). The preincubation culture medium was removed and replaced with the EDTA solution, and corneas were incubated at 37°C for 1 hour. Samples were gently washed twice for 5 minutes each with tissue culture medium (Medium-199; Gibco). They were then returned to the supplemented culture medium and incubated at 37°C. Medium was changed every 24 hours. At 48 hours after EDTA treatment, some samples were removed for analysis, some remained in culture until 96 hours after EDTA treatment, and some were radially bisected, with one half analyzed at the 48-hour time point and the other returned to culture until 96 hours after EDTA treatment.

ZO-1 and Ki-67 Immunolocalization

For immunolocalization, samples were fixed in methanol at -20°C for 10 minutes. Samples were washed with phosphate-buffered saline (PBS) three times for 5 minutes each and then permeabilized with 1% Triton X-100 in PBS for 10 minutes. Samples were washed again with PBS three times for 5 minutes each and then blocked for 10 minutes in 4% bovine serum albumin. For ZO-1 immunolocalization, samples were incubated at room temperature for 2.5 hours with mouse anti-ZO-1 (FITC) antibody (diluted to 5 $\mu\text{g/mL}$ in 4% bovine serum albumin; Zymed Laboratories, South San Francisco, CA), washed with PBS three times for 10 minutes each, and mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) with DAPI. For Ki-67 immunolocalization, after the blocking step, samples were incubated for 2 hours at room temperature with mouse anti-Ki-67 antibody (prediluted preparation; Zymed), washed with PBS three times for 10 minutes each, and incubated for 2 hours at room temperature with FITC-goat-anti-mouse IgG (diluted to 25 $\mu\text{g/mL}$; Zymed), washed with PBS three times for 10 minutes each, then mounted with mounting medium (Vectashield; Vector Laboratories) with propidium iodide or DAPI.

Baseline Endothelial Cell Density

Baseline endothelial cell density of three corneas not maintained in culture was obtained by cutting the corneas into four pie-shaped wedges, as described, and processing for ZO-1 immunolocalization. Samples were mounted in mounting medium with DAPI (Vectashield; Vector Laboratories).

Statistical Analysis

For analysis of the percentage of Ki-67-positive cells, data were obtained from four adjacent 20 \times -objective fields from a fluorescence microscope (Fig. 1). Cut edges were avoided. Three regions—central, mid, and peripheral—were analyzed. In each region, the total number of cells and the number of Ki-67-positive cells were counted in five 0.2-mm \times 0.2-mm areas. These counts were used to determine the percentage of Ki-67-positive cells and endothelial cell density. Data were compared with paired *t*-tests or Wilcoxon signed-rank tests. Data from ZO-1 immunolocalization were acquired by confocal microscopy and analyzed qualitatively.

RESULTS

A robust proliferative response in areas of wounded endothelium has been noted.^{9,10} The presence of this response was used as a positive internal control for these experiments. The proliferative response was assayed by immunolocalization of Ki-67, a nuclear antigen found in the late G1, S, M, and G2 phases of the cell cycle. Ki-67-positive cells were consistently noted along the cut edges of the corneal samples in these experiments (Fig. 2). Cut edges were, therefore, avoided in the data analysis conducted here.

Promotion of corneal endothelial cell proliferation by EDTA is presumed to be secondary to disruption of cell contacts. We evaluated the distribution of ZO-1, a protein associated with tight junctions, to assess the degree of cell dissociation. Disruption of ZO-1 immunolocalization was noted with 2.5 mM EDTA treatment and was prominent at 5.0 mM (Fig. 3). There were no qualitative differences in cell dissociation from the central region toward the peripheral region.

Although EDTA disrupted corneal endothelial cell contacts, increased corneal endothelial cell proliferation assayed by Ki-67 immunolocalization was not noted in response to EDTA treatment in any of the three regions analyzed (Fig. 4). To assess whether there was a time-dependent effect after EDTA treatment, corneas were analyzed at 48 and 96 hours after EDTA treatment, but no time-dependent effect was observed. It did appear consistently that regardless of EDTA treatment, greater proliferation was noted in the central region than in the peripheral region.

To determine whether areas of greater proliferation corresponded to areas with increasing endothelial cell density, endothelial cell density was determined in all three regions 48 and 96 hours after EDTA treatment (Fig. 5). As anticipated,

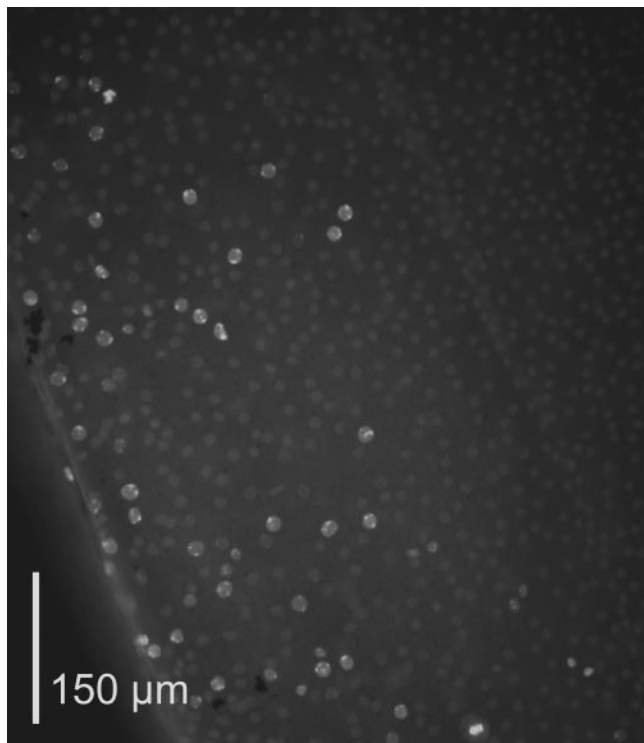


FIGURE 2. Robust proliferative response is noted at the cut margins of the samples, with cells noted to wrap around the cut edge. Faint nuclei, DAPI; bright nuclei, Ki-67⁺.

there was no change in endothelial cell density in relation to EDTA treatment. However, there was a trend toward greater cell density peripherally than centrally.

The percentage of proliferating endothelial cells and endothelial cell density showed no significant response to EDTA treatment, but there was a trend toward variation based on corneal region. To evaluate these trends, all data for each region were pooled, regardless of treatment or post-EDTA incubation time point (Fig. 6). This revealed a significant trend toward greater proliferation in the central cornea compared with the mid or peripheral cornea ($P < 0.001$). Endothelial cell density also showed a significant trend, with greater densities peripherally than centrally ($P < 0.001$). When all data were evaluated on a scatter plot of the percentage of proliferating cells in relation to endothelial cell density, there was a clear trend toward no proliferation at endothelial cell density greater than approximately 2000 cells/mm² (Fig. 7).

The difference in corneal endothelial cell density between central and peripheral regions observed in our study was similar to that reported previously.¹¹ In our corneal samples maintained in culture, endothelial cells were noted to be wrapping over the cut edges of the sample (Fig. 2). For our experiments, we suspected that the lower central endothelial cell density might be secondary to cell loss from closer proximity to the cut edges of the sample. Therefore, we analyzed three additional donor corneas to determine the central, mid, and peripheral endothelial cell density immediately after sectioning of the cornea into quarters. These samples were not subsequently maintained in culture and would, therefore, reflect baseline endothelial cell density. No significant differences were noted (mean \pm SD cells/mm²; $n = 3$; central 3138 ± 253 , mid 3308 ± 319 , peripheral 3038 ± 40), suggesting that the lower central endothelial cell density noted may be an artifact of cell loss during culture in our experimental design.

DISCUSSION

In our experiments, we expected to find increased corneal endothelial cell density in areas of increased cell proliferation.

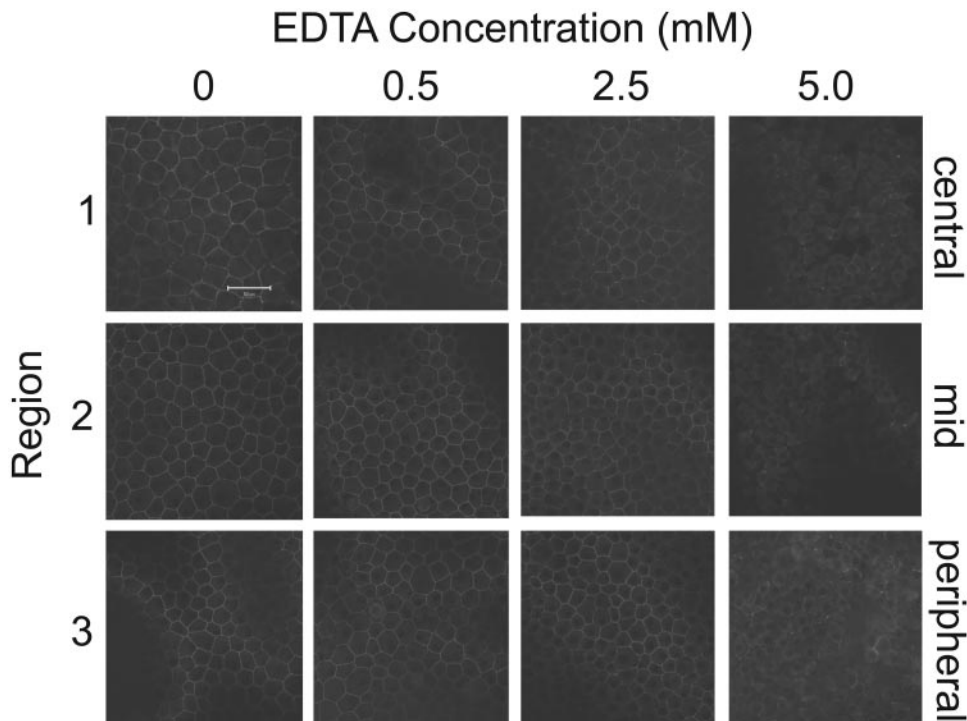
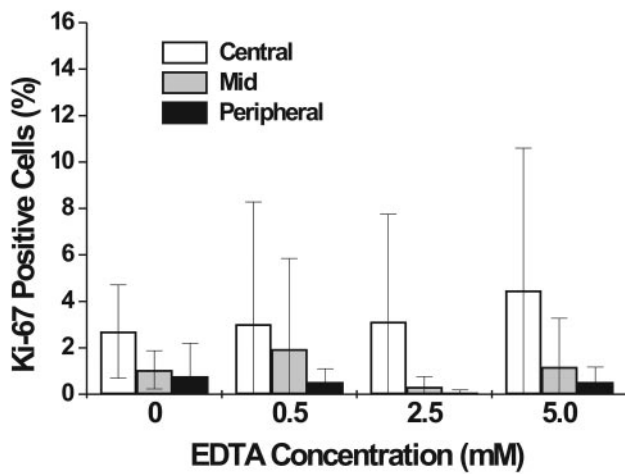


FIGURE 3. ZO-1 immunolocalization demonstrates disruption of cell contact in the endothelial monolayer with EDTA treatment. Treatment effect (lack of distinct ZO-1 immunolocalization at the cell membrane) is noted at EDTA concentrations of 2.5 and 5.0 mM. Scale bar, 50 μ m.

A. 48 hrs



B. 96 hrs

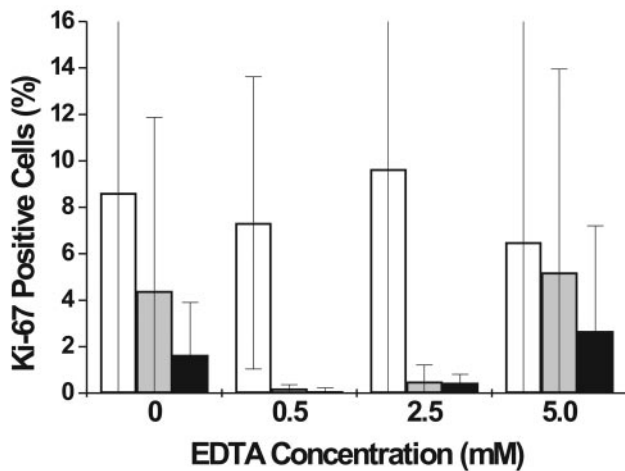


FIGURE 4. EDTA does not promote corneal endothelial cell proliferation 48 hours (A) or 96 hours (B) after treatment. For each data set $n = 4$, but at 48 hours, 2.5 mM EDTA, $n = 3$; at 96 hours, 2.5 mM EDTA, $n = 2$; and at 96 hours, 5.0 mM EDTA, $n = 3$.

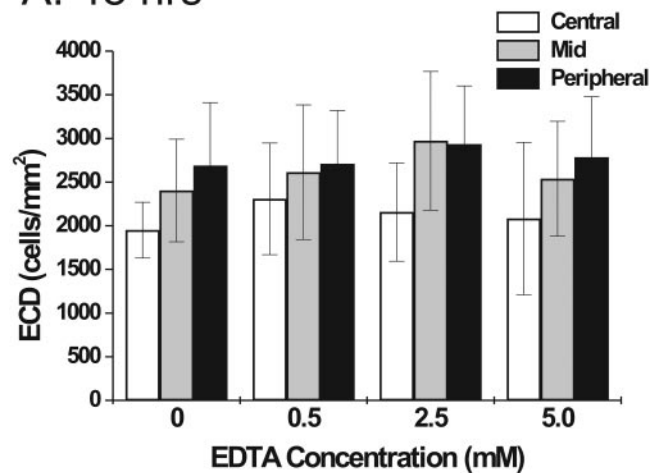
Instead we found the opposite—cells proliferated only in areas with cell densities less than approximately 2000 cells/mm². Intuitively, it seems logical that cells would not proliferate beyond a certain density, and perhaps this was the limit for corneas under our culture conditions. Our findings correspond with those of experiments of human corneal endothelial cell primary cultures in which cells from younger donors were confluent at a mean density of 2000 cells/mm² and those from older donors were confluent at a mean density of 754.6 cells/mm².¹² It would be interesting to determine whether corneas with endothelial cell density lower than 2000 cells/mm² can be induced to proliferate with an increase in cell density in culture conditions. The ability to reach a density of 2000 cells/mm² alone may make more corneas suitable for transplantation than are available today in some parts of the world. Each cornea used in our experiments had a starting endothelial cell density of approximately 2000 cells/mm² or greater. The lower density measured after culture was the result of cell loss from our corneal culture methods. Future experiments to evaluate corneas of lower starting endothelial cell density are best con-

ducted on whole corneas to avoid the problem of cell loss over the cut edges.

We noted a significant difference in corneal endothelial cell density between the peripheral and the central cornea in our samples maintained in culture. Previous studies have shown that a difference in corneal endothelial cell density between the central and the peripheral cornea exists.¹¹ However, in our experiments, this difference was an artifact of our corneal culture methods, as evidenced by lack of a difference in cell density when corneas were analyzed without being kept in culture. Previous studies have shown that corneal endothelial cells migrate over the cut edge.¹³ Although our data for the central region were taken away from the cut edges, the central region maintained greater proximity to the cut edges than the mid and peripheral areas analyzed. Cell migration over the cut edge in conjunction with cell loss at the cut margins from the damage of dissecting the corneas into quarters probably accounted for lower central compared with peripheral cell density. Cutting corneas into quarters to increase sample size for experiments is common in many experiments.^{7,9,10} Our data suggest caution in interpreting results in such experiments.

Our model for promoting corneal endothelial proliferation was based on the data of Senoo et al.⁷ using EDTA. However,

A. 48 hrs



B. 96 hrs

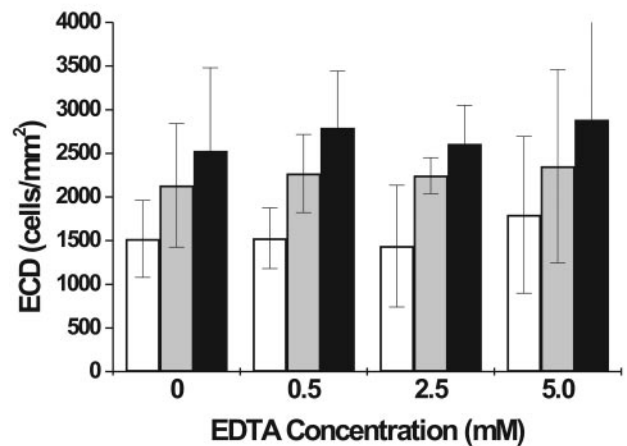
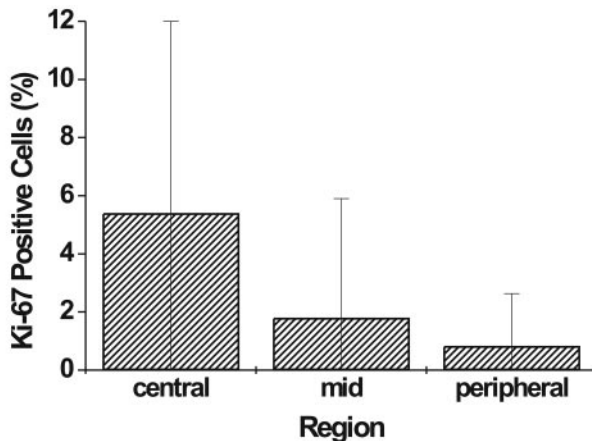


FIGURE 5. EDTA does not influence corneal endothelial cell density 48 hours (A) or 96 hours (B) after treatment. For each data set $n = 4$, but at 48 hours, 2.5 mM EDTA, $n = 3$; at 96 hours, 2.5 mM EDTA, $n = 2$; and at 96 hours, 5.0 mM EDTA, $n = 3$. ECD, endothelial cell density.

in contrast to their data, we did not note a corneal endothelial proliferative response to EDTA treatment despite the dissociation of tight junctions, as demonstrated by ZO-1 immunolocalization. Several possibilities account for these differences. The corneas we used for our experiments were, on average, from younger donors than were used in their study. Corneas from younger donors may correspond to a higher baseline endothelial cell density that may result in a less robust proliferative response based on our findings. In addition, we noted variations in corneal endothelial cell proliferation based on the region of cornea analyzed. This was not addressed in their data analysis. Finally, there may be subtle but significant differences in culture conditions, such as lot-to-lot variations in fetal bovine serum.

Our data emphasize the importance of assaying corneal endothelial cell density when evaluating the proliferative response of the endothelium. In these experiments, decreasing cell density below 2000 cells/mm² appeared to be an indirect trigger for corneal endothelial cell proliferation—that is, it

A.



B.

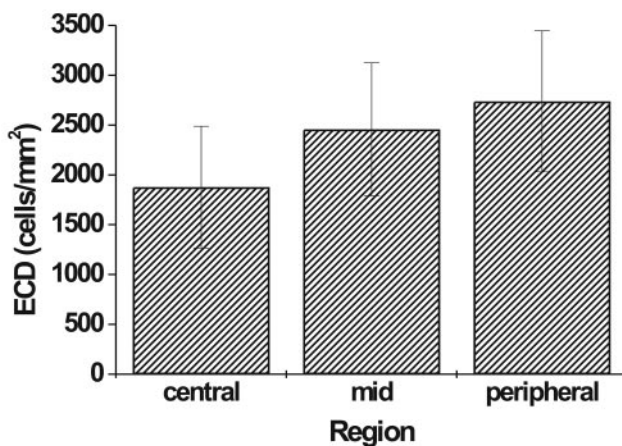


FIGURE 6. (A) Corneal endothelial cell proliferation is always greater in the central cornea than in the mid or peripheral cornea ($P < 0.001$). (B) Endothelial cell density shows the opposite trend (central compared with mid or peripheral cornea; $P < 0.001$). For both graphs, central $n = 29$, mid $n = 29$, and peripheral $n = 28$.

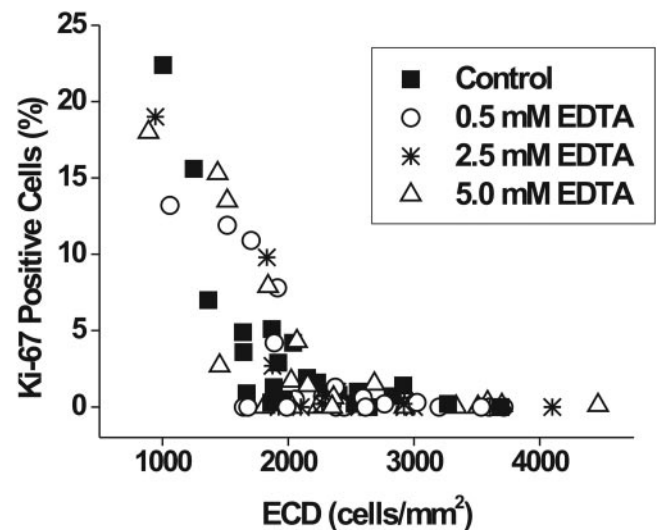


FIGURE 7. Corneal endothelial cells do not proliferate at densities greater than approximately 2000 cells/mm².

enabled a cellular response to mitogens in the culture medium. The mechanisms involved remain to be elucidated.

Acknowledgments

The authors thank Nancy Joyce for critical review of the experimental design and results.

References

- Fischbarg J, Maurice DM. An update on corneal hydration control. *Exp Eye Res.* 2004;78:537-541.
- Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res.* 2003;22:359-389.
- Bourne WM. Cellular changes in transplanted human corneas. *Cornea.* 2001;20:560-569.
- Patel SV, Hodge DO, Bourne WM. Corneal endothelium and post-operative outcomes 15 years after penetrating keratoplasty. *Am J Ophthalmol.* 2005;139:311-319.
- Joyce NC, Mehlir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci.* 1996;37:645-655.
- Kikuchi M, Zhu C, Senoo T, Obara Y, Joyce NC. p27kip1 siRNA induces proliferation in corneal endothelial cells from young but not older donors. *Invest Ophthalmol Vis Sci.* 2006;47:4803-4808.
- Senoo T, Obara Y, Joyce NC. EDTA: a promoter of proliferation in human corneal endothelium. *Invest Ophthalmol Vis Sci.* 2000;41:2930-2935.
- Mimura T, Yamagami S, Yokoo S, Araie M, Amano S. Comparison of rabbit corneal endothelial cell precursors in the central and peripheral cornea. *Invest Ophthalmol Vis Sci.* 2005;46:3645-3648.
- Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci.* 2000;41:660-667.
- Mimura T, Joyce NC. Replication competence and senescence in central and peripheral corneal endothelium. *Invest Ophthalmol Vis Sci.* 2006;47:1387-1396.
- Amann J, Holley GP, Lee S-B, Edelhauser HF. Increased endothelial cell density in the paracentral and peripheral regions of the human cornea. *Am J Ophthalmol.* 2003;135:584-590.
- Joyce NC, Zhu C. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. *Cornea.* 2004;23:S8-S19.
- Cameron JD, Flaxman BA, Yanoff M. In vitro studies of corneal wound healing: epithelial-endothelial interactions. *Invest Ophthalmol.* 1974;13:575-579.