EDTA: A Promoter of Proliferation in Human Corneal Endothelium

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Purpose. To determine whether it is possible to induce proliferation in the endothelium of older donor corneas by treatment of the intact monolayer with EDTA.

Methods. Corneas from donors 52 to 75 years of age were obtained from an eye bank and were usually cut in quarters to increase sample size. The effect of EDTA dose (0.02–2.0 mg/ml) and incubation time (6, 30, and 60 minutes) on endothelial cell–cell contacts was evaluated by staining for ZO-1, a cell junction marker. Cell death was tested by a commercial live–dead assay. Corneal pieces were incubated for 0, 24, 48, or 60 hours in culture medium (M-199, 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factor) before EDTA treatment. After treatment, pieces were incubated in the same medium for 24, 48, 72, or 96 hours to permit cell cycle entry. Tissue was fixed, stained for Ki67 (a marker for late G1-phase through the M-phase), and mounted in medium containing propidium iodide to visualize all nuclei. Confocal images were evaluated by computer (Image software; NIH, Bethesda, MD) to count Ki67-positive and propidium iodide–stained cells.

Results. EDTA released corneal endothelial cell–cell contacts in a dose- and time-dependent manner. At doses and incubation times tested, EDTA did not induce significant cell death. Preincubation in culture medium for 24 hours was needed for endothelial cells to efficiently initiate proliferation in response to EDTA. The endothelium of corneas incubated in mitogen-containing medium for up to 108 hours without EDTA treatment did not stain for Ki67. EDTA at 2.0 mg/ml for 60 minutes appeared optimal and stimulated 16% to 18% of the cells to proliferate. Ki67-positive mitotic figures were visible 48 hours after exposure to EDTA. Formation of daughter cells was visible after double-staining for Ki67 and ZO-1.

Conclusions. EDTA released cells from contact inhibition and promoted proliferation in corneal endothelium from older donors. The authors hypothesize that corneal endothelium from older individuals divide in situ when exposed to positive growth factors under conditions in which cells have been transiently released from contact inhibition. (*Invest Ophthalmol Vis Sci.* 2000;41: 2930–2935)

Human corneal endothelial cell density decreases with age,[1,2] indicating that these cells do not replicate sufficiently to replace dead or injured cells. Transfection studies using viral oncoproteins have demonstrated that human corneal endothelium has an intrinsic high proliferative capacity.[3,4] The fact that endothelial cells possess the capability to divide but do not normally replicate in vivo suggests that they are actively maintained in a nonreplicative state. Cell cycle studies indicate that human corneal endothelial cells in vivo are arrested in the G1-phase of the cell cycle.[5,6] Among the factors that may be responsible for maintaining these cells in G1-phase arrest are the nonavailability of positive growth factors[7] and the presence in aqueous humor of transforming growth factor (TGF)-β2, which suppresses S-phase entry in cultured corneal endothelial cells.[8,9]

In addition, studies of the developing cornea in neonatal rats suggest that a contact-inhibition–like mechanism may actively suppress replication in the mature endothelial monolayer.[10] Other evidence for cell-contact–mediated regulation of proliferation is that corneal endothelial cells divide in response to wounding.[11,12] In tissue culture and in organ cultured corneas, only endothelial cells adjacent to the wound edge or cells that have migrated into the wound bed proliferate, demonstrating the importance of releasing cell–cell contacts to promote proliferation. Formation of cell–cell contacts is mediated by a number of proteins that are associated with different types of junctional complexes, including cadherins (adhering junctions),[13] ZO-1 (tight junctions), [14] and connexin-43 (gap junctions).[15] These proteins all require calcium for maintenance of their adhesion function. In low-calcium environments, junctional complexes mediated by these proteins disassemble, and cell–cell contact is broken. Exposure of the corneal endothelium to calcium-free medium causes disruption of apical junctional complexes, increased transendothelial perfusion, and corneal edema.[16–18] These changes can be reversed by replacing calcium in the medium[17,18] or by exposing cells to ionophores that release intracellular calcium stores.[18]

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Previous studies from this laboratory[19] have demonstrated that corneal endothelium from older donors (>50 years of age) is capable of proliferation in response to a mechanical wound. Cells from older donors responded in fewer numbers and entered the cell cycle more slowly than those from younger donors (<30 years of age) when corneas were cultured in medium containing 10% serum and 20 ng/ml fibroblast growth factor (FGF). Addition of 10 ng/ml epidermal growth factor (EGF) to this medium induced significantly more older cells to proliferate and increased the rate of cell cycle entry. As in previous studies, only cells within the wound bed or at the wound edge proliferated. Together, these data indicate that endothelial cells from older donors can proliferate if cell–cell contacts are broken by wounding and if given sufficient mitogenic stimulation. The present study was designed to determine whether it is possible to induce proliferation in the endothelium without mechanical wounding of the monolayer. Because EDTA, a known chelator of calcium and magnesium, has been used in many laboratories to release cultured cells from contact inhibition, it was tested for its ability to release endothelial cells from cell–cell contact and promote proliferation in an organ culture model.

Materials and Methods

Human Corneal Tissue

Donor human corneas were obtained from National Disease Research Interchange (Philadelphia, PA) and from the Central Florida Lions Eye Bank. All corneas were maintained at 4°C in preservation medium for 1 week or less before study. Endothelial cell counts were more than 2000 cells/mm^2. Criteria for exclusion of corneas from these studies included history of endothelial dystrophy, presence of central guttata, low endothelial cell density, and ocular inflammation or disease.

Ex Vivo Corneal Model

Twenty-one human corneas were obtained from donors 52 to 75 years of age (mean age, 65.1 years). Whole corneas were usually cut in quarters to increase sample size. In some cases, whole corneas were used as control samples. Corneal pieces were placed endothelial-side-up in individual wells of a 24-well tissue culture plate (Falcon, Lincoln Park, NJ). Pieces were incubated for 24 hours in medium-199 containing 10% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor (EGF; Upstate Biotechnologies, Lake Placid, NY), 20 ng/ml fibroblast growth factor (FGF; Biomedical Technologies, Stoughton, MA), and 50 mg/ml gentamicin to stabilize the endothelium before study. EDTA (di-sodium EDTA.2H$_2$0) was prepared in Hanks' balanced salt solution (HBSS; without calcium chloride, magnesium chloride, or magnesium sulfate; Life Technologies, Grand Island, NY), adjusted to pH7.4, and added to the culture medium at a final concentration of 0.02, 0.2, or 2.0 mg/ml. Corneas were treated with EDTA for 10, 30, or 60 minutes and then returned to culture medium for up to 96 hours. EDTA treatment controls included exposing corneal pieces to all manipulations and incubation conditions, including 1 hour in HBSS, but without EDTA. All corneas were maintained at 37°C in a 5% carbon dioxide, humidified atmosphere until removal for analysis of cell cycle progression.

Immunolocalization of ZO-1 and Ki67

Immunostaining for ZO-1 detected corneal endothelial cell boundaries, whereas Ki67 staining detected actively cycling cells. Immunolocalization was performed using the same antibodies and protocols as described previously for ZO-1[10] and Ki67.[19] Corneal pieces stained for Ki67 were mounted in medium containing propidium iodide (PI; Vector Laboratories, Burlingame, CA) to visualize all nuclei. In some cases, samples were double-stained for both Ki67 and ZO-1. Slides were viewed using a confocal microscope (model TCS 4D, equipped with a DMRBE laser; Leitz, Wetzlar, Germany; and SCANware ver. 4.2 software; Leica Lasertechnik, Heidelberg, Germany). Images were collected from the central region of the corneal specimens, away from the cut edges, by using a ×16, ×40, or ×100 oil-immersion lens. Laser power and gain controls were adjusted to achieve an optimal range of output signal intensity for each channel. Confocal images were collected, and micrographs were printed by computer (Photoshop ver. 4.0; Adobe, San Jose, CA). For some micrographs, the printing contrast was adjusted to provide a clearer image.

Evaluation and Quantification of Ki67-Positive Cells

Fluorescence confocal immunocytochemistry for Ki67 was used to evaluate corneal endothelial cells for their ability to enter and complete the cell cycle. All nuclei were stained with PI. Positive Ki67 staining patterns detected actively cycling cells and also acted as markers for specific phases of the cell cycle. Completion of the cell cycle was determined by observation of mitotic figures stained with Ki67. Three representative confocal micrographs were taken per corneal quarter with a ×40 objective lens. A software program (Image ver. 1.62; NIH) was used to count total PI-stained nuclei, total Ki67-positive cells, and cells in the G1, S/G2, and M phases of the cell cycle. Cells were counted in five 100 μm^2 areas of each micrograph (15 areas counted per corneal piece). Counts were averaged, and the percentage of actively cycling cells and of cells in each phase of the cell cycle was calculated. Each study was conducted using corneas from two to three donors. Statistical comparisons were made by computer, using a paired Student's *t*-test (StatView ver, 4.11; Abacus Concepts, Berkeley, CA). Results reported in Table 1 are expressed as percentages of Ki67-positive cells ± SD. Results reported in Figures 3B and 3C are expressed as percentages of Ki67-positive cells ± SEM.

Results

EDTA Effects on Cell–Cell Contacts

Studies were first conducted to determine the effect of EDTA on the integrity of endothelial cell–cell contacts. Corneal pieces were incubated for 10, 30, or 60 minutes in 0.02, 0.2, or 2.0 mg/ml EDTA, then washed, and stained for ZO-1 to visualize cell boundaries. Representative micrographs in Figure 1 show the dose- and time-dependent effect of EDTA on cell–cell contacts. In the absence of EDTA, cells maintained normal lateral associations (Fig. 1A). With increasing EDTA concentration or incubation time, there was an increase in the lateral separation of cells, visualized as a double line of positive ZO-1 staining demarcating the two cell membranes (Figs. 1B through 1E). Incubation in the presence of 2.0 mg/ml for 60 minutes

TABLE 1. Effect of EDTA Concentration and Incubation Time on Percentage Ki67-Positive Cells

Condition	EDTA Concentration (mg/ml)	Incubation Time (min)	Ki67-Positive Cells* SD	*P*
1	0.2	30	8.67 ± 2.65	—
2	0.2	60	11.99 ± 2.63	0.13
3	2.0	30	14.28 ± 6.79	0.22
4	2.0	60	17.18 ± 1.21	0.007

n, 2–3 donor corneas per treatment condition.

* Five 100 μm^2 areas were counted per micrograph × three micrographs per corneal piece. Data are mean percentages ± SD $Ki67^+$ cells.

P is the comparison between mean percentage of Ki67-positive cells from condition 1 and the other three Ki67 values.

caused cells to become rounded with only thin cytoplasmic strands connecting adjacent cells. This treatment, however, did not cause cells to lose contact with Descemet's membrane. EDTA, at the concentrations and incubation times tested, did not significantly induce cell death as indicated by a commercially available live–dead staining assay (data not shown).

EDTA Effects on Cell Cycle Progression

Previous studies[19] revealed that corneal endothelial cells incubated in medium containing 10% serum, 20 ng/ml FGF, and 10 ng/ml EGF stained positively for Ki67 in response to wounding, but no positive staining was observed in unwounded areas of the endothelium. Similar absence of Ki67 staining is shown in Figure 2A which shows corneal quarters that were incubated in this medium for 108 hours. Thus, exposure of the

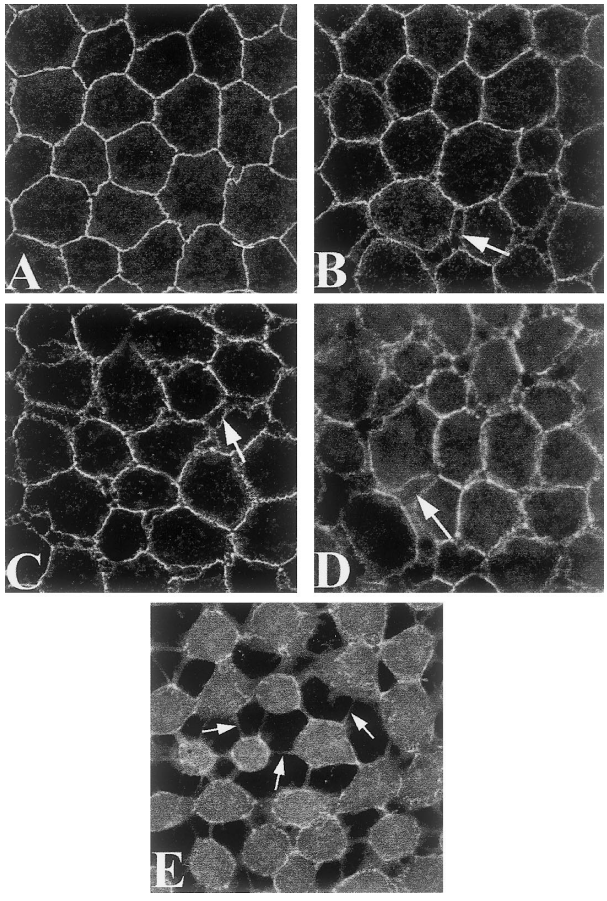

FIGURE 1. EDTA decreases the integrity of cell–cell contacts in a dose- and time-dependent manner. Corneal pieces from a 68-year-old donor were incubated in the absence of EDTA (**A**) or in 0.02 (**B**), 0.2 (**C**), or 2.0 mg/ml EDTA (**D**, **E**) for 30 (**B**, **C**, **D**) or 60 (**E**) minutes. Normal cell–cell contacts, visualized as an apparent single line of staining between cells, were observed in the absence of EDTA, but a gradual increase in lateral separation occurred with increasing EDTA concentration or incubation time. *Arrows*: Areas of cell–cell separation (**B**, **C**, and **D**); thin cytoplasmic strands retained between cells incubated for 60 minutes in 2.0 mg/ml EDTA (**E**). Original magnification, ×100.

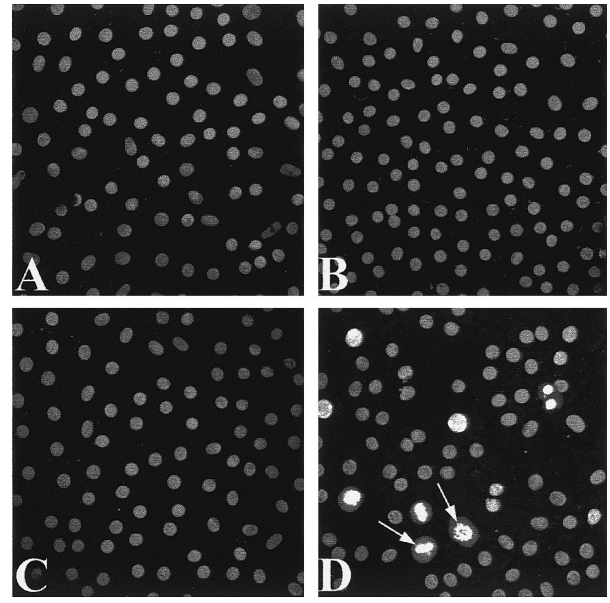

FIGURE 2. Stimulation of corneal endothelial cell proliferation requires both preincubation in mitogen-containing medium and treatment with EDTA. Corneal quarters from a 52-year-old donor were incubated under the following conditions: (**A**) for 108 hours in medium containing 10% serum, EGF, and FGF, but without exposure to EDTA; (**B**) no preincubation, 0.2 mg/ml EDTA for 30 minutes, postincubation in medium for 48 hours; (**C**) no preincubation, 2.0 mg/ml EDTA for 30 minutes, postincubation in medium for 48 hours; (**D**) preincubation for 60 hours in medium, 0.2 mg/ml EDTA for 30 minutes, postincubation for 48 hours. Positive Ki67 staining was visible only in endothelial cells that were both preincubated and exposed to EDTA. Note Ki67-positive mitotic figures (*arrows*) in (**D**). Original magnification, ×100.

intact endothelium to this mitogen-containing medium was not sufficient to induce cell cycle entry. Similarly, incubation in up to 2.0 mg/ml EDTA alone did not promote proliferation, even if cells were postincubated for 48 hours in mitogen-containing medium (Figs. 2B, 2C). In contrast, preincubation in mitogen-containing medium and subsequent exposure to EDTA followed by a 48-hour postincubation promoted cell cycle progression in the endothelium, revealed by the presence of Ki67-stained nuclei and mitotic figures (Fig. 2D). Negative control corneas were manipulated in a manner similar to EDTA-treated corneas. These corneas were incubated for 1 hour in HBSS alone to control for the EDTA incubation and then maintained for 48 hours in the culture medium described. Fluorescence microscopy showed only a very rare Ki67-positive cell (data not shown).

Ki67 is expressed in cells from the mid-to-late G1 phase through mitosis, making it an excellent marker of actively cycling cells.[20,21] Immunostaining with antibody to Ki67 also permits semiquantitative analysis of the relative number of cells in late G1 phase through mitosis.[19,22] The graph in Figure 3A shows typical endothelial cell cycle kinetics in corneal specimens from a single donor preincubated for 24 hours in mitogen-containing medium, treated with 0.2 mg/ml EDTA for 60 minutes, and postincubated in medium for 0, 24, 48, or 72 hours. Cell proliferation began by 24 hours after EDTA treatment, and the cell cycle was completed in approximately 48 hours, as indicated by the presence of mitotic figures at that time point. By 72 hours after EDTA treatment, there was a mixed population of Ki67-stained cells with evidence of new cell cycle entry. With this method of analysis, it was not possible to determine whether this population of Ki67-positive cells represented a second round of proliferation or whether a subpopulation of cells responded more slowly to the EDTA+mitogen treatment. Double-staining of corneal pieces with Ki67 and ZO-1 revealed that in cells treated with EDTA and mitogens the cell cycle is completed and daughter cells formed (data not shown).

Determination of Optimal Incubation Conditions

Studies were conducted to determine incubation conditions that induce cell cycle entry in the largest number of endothelial cells, as determined by counting Ki67-positive cells. The requirement for preincubation of corneas in mitogen-containing medium was first determined. For these studies, preincubation times of 0, 24, 48, and 60 hours were used. EDTA concentration and treatment times were held constant, and corneal samples were postincubated in mitogen-containing medium for 48 hours. Figure 3B provides a representative example of the results, which indicate that, of the preincubation times tested, 24 hours was sufficient to yield the maximal percentage of Ki67-positive cells. This preincubation time was then used for all subsequent studies. In studies to determine the optimal postincubation time, corneal pieces were preincubated in medium for 24 hours, followed by EDTA treatment. Postincubation times tested included 0, 24, 48, 72, and 96 hours. Figure 3C provides a representative example, indicating that a maximum number of Ki67-positive cells was obtained 48 hours after incubation. Depending on the specific donor cornea, some Ki67-positive cells could be detected 72 hours after EDTA treatment; however, no samples showed actively cycling cells by 96 hours after incubation.

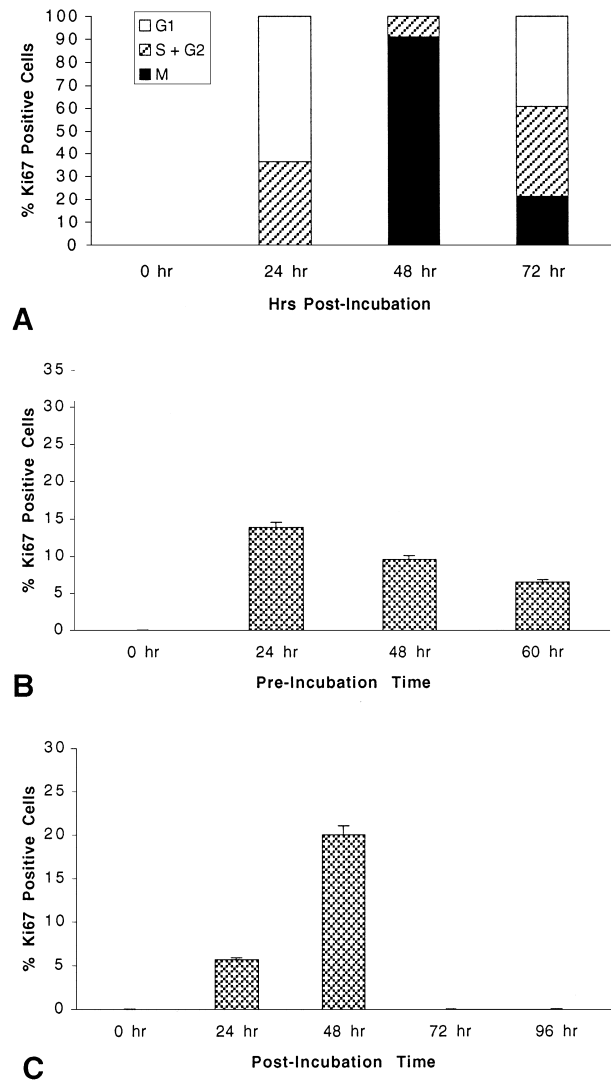

FIGURE 3. Effects of EDTA on cell cycle kinetics (**A**) and determination of optimal preincubation (**B**) and postincubation times (**C**). Corneal pieces from a 64-year-old donor were preincubated for 24 hours in medium containing 10% serum, EGF, and FGF and treated with 0.2 mg/ml EDTA for 60 minutes, followed by postincubation in the same medium for various periods. Results in (**A**) are expressed as the percentage of Ki67-positive cells exhibiting staining patterns for G1, S/G2, or mitosis. To determine optimal preincubation time, corneal quarters from a 54-year-old donor were preincubated in the same medium for different periods, followed by 30 minutes' treatment with 2.0 mg/ml EDTA and 48-hours' postincubation. To determine optimal postincubation time, corneal quarters from a 73-year-old donor were preincubated in the same medium for 24 hours, followed by 30 minutes' treatment with 2.0 mg/ml EDTA. Quarters were then postincubated in the same culture medium for different periods. Results in (**B**) and (**C**) are expressed as mean ± SEM.

As indicated in Table 1, EDTA at either 0.2 or 2.0 mg/ml was capable of promoting cell cycle progression. The relative percentage of Ki67-positive cells observed under the four EDTA incubation conditions was significantly higher than negative controls in which corneas were incubated in HBSS alone. Corneas treated with 2.0 mg/ml EDTA for 60 minutes yielded a significantly higher number of actively cycling cells (P =

0.007) than those treated with 0.2 mg/ml EDTA for 30 minutes. Repeated EDTA treatment cycles promoted proliferation over a longer period, but the treatment became gradually less effective (data not shown).

Discussion

The present study demonstrates that EDTA, a known chelator of calcium and magnesium, is able to disrupt lateral cell contacts within the corneal endothelial monolayer without apparent interference with cell–substrate associations or negative effects on viability. ZO-1-staining of endothelium treated for 60 minutes with 2.0 mg/ml EDTA (the highest concentration and longest treatment time tested) revealed thin cytoplasmic strands between rounded cells in a pattern very similar to that observed by Stern et al.,[18] who perfused the endothelium with calcium-free medium. Exposure of the intact endothelium to EDTA or to mitogens alone was not sufficient to induce proliferation. In contrast, a combination of the two, with appropriate timing, clearly stimulated a proliferative response.

Treatment of the monolayer with EDTA presumably interfered sufficiently with maintenance of junctional complexes to release cells from contact inhibition, making them sensitive to mitogenic stimulation. The reason preincubation of the endothelium in mitogen-containing medium was required is not clear. Pretreatment with mitogens may initiate cellular responses that prepare for cell cycle entry once cell–cell contacts have been released. Preincubation times shorter than the 24 hours used in this study may be even more effective, because receptor downregulation can occur with prolonged growth factor incubation. Future studies will test the effectiveness of preincubation times shorter than 24 hours and also identify any cell cycle–related changes that occur during this preincubation period.

The possibility that preincubation with mitogens prepares cells to enter the cycle is supported by the fact that cells appeared to initiate proliferation as an almost synchronous population. This response differed from cell cycle entry in wounded endothelium exposed to the same culture medium.[19] Under those conditions, cells continued to enter the cycle until the wound bed was completely repopulated.

An important finding from these studies was that, after treatment of the intact endothelial monolayer with EDTA and mitogens, approximately 17% of the cell population entered the cell cycle. It is expected that further refinement of the EDTA and/or mitogen treatment protocol will increase the relative percentage of proliferating cells. Because the relative percentage of Ki67-positive cells tended to differ with the specific treatment, it is possible that we may not have obtained optimal efficiency in stimulating proliferation. The ability to induce proliferation in endothelial cells does not appear to be restricted by the presence of a senescent population; we have not observed positive staining for β-galactosidase, a marker of cell senescence,[23] in the endothelium of older individuals (personal observation). Future studies will determine whether EDTA+mitogen treatment consistently increases cell density in donor corneas with low endothelial cell counts.

The induction of proliferation in these studies occurred without mechanical wounding and without apparent cell damage. This underscores the integral role of cell–cell contact in maintaining the corneal endothelium in a nonreplicative state and indicates that temporary interference with this important antiproliferative mechanism could induce transient proliferation in this physiologically important monolayer. These findings suggest that it may be possible to induce proliferation and thereby increase the density of endothelial cells. This treatment could be applied directly to the endothelium to increase endothelial cell density in corneas to be used for transplantation. In addition, it may be possible to stimulate endothelial cell division in situ by using EDTA (or similar reagents that temporarily interfere with cell–cell contact) plus mitogen to increase cell density in individuals at risk for vision loss due to low endothelial cell counts.

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