Proliferative Response of Corneal Endothelial Cells from Young and Older Donors

Cheng Zhu and Nancy C. Joyce

Purpose. To compare the effect of epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor-BB (PDGF-BB), bovine pituitary extract, and fetal bovine serum (FBS), alone or in combination, on proliferation of human corneal endothelial cells (HCEC) cultured from young (<30 years old) and older donors (>50 years old).

Methods. Corneas from donors 2 to 79 years old were obtained from the National Disease Research Interchange. Descemet's membrane with intact endothelium was dissected. Cells were isolated by EDTA treatment and cultured to confluence. The HCEC marker, antibody 9.3.E, tested for pure endothelial populations. Antibody Ki67 and ZO-1 tested either before or after cultured cells reached confluence to indicate cell proliferation and cell–cell contact formation. Cell morphology was documented by inverted phase-contrast microscopy. Passages I through VII were used to test the effect of various factors on cell proliferation. For each study, equal numbers of cells were seeded, maintained overnight in 4% FBS to permit cell attachment, washed, and incubated for up to 3 weeks in one of the following: modified Eagle's Minimum Essential Medium (Opti-MEM-I) alone; Opti-MEM-I plus EGF, NGF, PDGF-BB, bovine pituitary extract, or FBS; or a combination of factors. At various times after seeding, cell numbers were determined by electronic cell counter. For each condition, three separate wells were tested and each sample was counted three times. Studies were repeated at least twice using cells from different donors and age groups. Within each study, a one-way ANOVA test was performed to analyze statistical significance.

Results. Cells stained positively with antibody 9.3.E, indicating isolation of HCEC and lack of contamination with epithelial cells or keratocytes. Positive staining of Ki67, indicating cycling cells, was found in subconfluent cultures. Plasma membrane-associated ZO-1 staining and lack of Ki67 staining indicated that cultured cells formed a contact-inhibited monolayer. Cultured cells decreased in density, increased in size, and became more heterogeneous depending on donor age and on the number of passages. Incubation in OptiMEM-I promoted attachment and induced a moderate proliferative response above that of MEM ($P < 0.001$). In general, proliferative responses to growth stimuli were relatively slow, with cell counts generally plateauing 10 to 14 days after exposure to growth-promoting agents. EGF yielded a broad, dose-dependent effect and, at 5–50 ng/mL, peak cell counts were significantly higher ($P < 0.001$) than basal levels. EGF consistently stimulated proliferation in cells from younger donors, but was less effective in stimulating growth of cells from older donors. NGF did not show a consistent significant stimulatory effect at any concentration tested. PDGF-BB (25 ng/mL) tended to stimulate growth to a greater extent than EGF ($P < 0.05$) in cultures from the same donor. Pituitary extract significantly increased counts at 1.0 ($P < 0.05$) to 100 ug/mL ($P < 0.001$). PDGF-BB plus pituitary extract demonstrated greater stimulation than pituitary extract ($P < 0.01$) or PDGF-BB alone ($P < 0.01$). FBS (1%–8%) increased cell numbers in a dose-dependent manner, and, at 4%–8%, yielded counts significantly higher ($P < 0.001$) than that of any single growth-promoting agent tested.

Conclusions. HCEC from both young and older donors can proliferate in vitro in response to growth-promoting agents. Proliferation in the presence of multiple mitogens ceased when confluence was reached, indicating the formation of a contact-inhibited monolayer. In general, cells cultured from young donors were more responsive to the agents tested, but the relative response of HCEC to these agents was similar, regardless of donor age. The relative difference in the extent of the response of the same cell population to different mitogens suggests that these mitogens induce different downstream signals. The relatively robust proliferative response of HCEC to FBS may involve stimulation of multiple downstream signaling pathways and/or induce more sustained downstream signaling than the other growth-promoting agents tested. (*Invest Ophthalmol Vis Sci.* 2004;45:1743–1751) DOI:10.1167/iovs.03-0814

Corneal endothelium is a fragile monolayer of cells with high metabolic activity mostly represented by Na^+/K^+-ATPase[1] and Mg^{2+}-ATPase ionic pumps.[2] The endothelium forms a leaky barrier between the aqueous humor and corneal stroma by the formation of focal tight junctions,[3] as well as gap[4] and adhesion junctions.[5] Together, the barrier and pump functions of the endothelium help maintain corneal transparency. Human corneal endothelial cells (HCEC) are considered to be nonproliferative in vivo, since the rate of proliferation does not keep pace with the rate of cell loss. Observation of endothelial wound healing indicates that cell enlargement and migration are the major means of endothelial repair.[6,7] Age-related changes[8,9] lead to decreased cell density and cell enlargement. Pathologic changes of corneal endothelium due to certain diseases[10,11] and physical damage from accidental or surgical trauma[12] will also cause an increased rate of cell loss. To a certain extent, neighboring cells can compensate for cell loss and maintain corneal clarity. Decompensation of the endothelium, resulting in the inability to maintain stromal detergescence and corneal clarity, can occur when cell density decreases below a critical level. Decompensation, once it occurs, is irreversible and corneal transplantation is required to restore visual acuity.

Studies by Wilson et al.[13,14] have demonstrated that HCEC in vivo retain proliferative capacity, while studies from this laboratory[15,16] have provided evidence that HCEC in vivo are

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arrested in G_1-phase of the cell cycle. HCEC have been successfully isolated and cultured using various techniques.[17–25] Results of these culture studies indicate that cells from younger donors are easier to establish in long-term culture than cells from older donors. In many cases in which cells from older donors were successfully cultured, results have been inconsistent, with cells often assuming a fibroblastic rather than polygonal morphology. This laboratory has reported the development of culture techniques and a medium formulation that promote consistent culture of untransformed corneal endothelial cells from older donors and yield normal polygonal morphology at confluence.[26]

Previous studies using ex vivo wound healing models have demonstrated mitotic changes in human corneal endothelium in response to stimulation by growth promoting agents, such as serum,[27–29] epidermal growth factor (EGF),[6,28,30] or a combination of the two.[29] Studies from this laboratory have used similar ex vivo wound models to demonstrate different cell cycle kinetics in HCEC from young and older donors.[29] Development of a method to culture HCEC consistently now permits comparative studies to be directly conducted to determine the relative response of HCEC from young and older donors to various growth stimuli. The goal of the current studies was to further refine our culture techniques for successful growth of HCEC and to test the effect of various growth-promoting agents on the relative proliferative response of cells from young (<30 years old) and older donors (>50 years old).

Materials and Methods

Materials

OptiMEM-I, Minimum Essential Medium (MEM), Hanks' Balanced Salt Solution (HBSS), Medium 199 (M199), Dulbecco's Phosphate-Buffered Saline (PBS), gentamicin, and trypsin/EDTA were purchased from Gibco BRL/Life Technologies (Rockville, MD). Nerve growth factor (NGF; from mouse submaxillary glands), and bovine pituitary extract (also known as Keratinocyte Growth Supplement) were from Biomedical Technologies (Stoughton, MA). Epidermal growth factor (EGF; from mouse submaxillary glands) was obtained from Upstate Biotechnologies (Lake Placid, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Human recombinant platelet-derived growth factor-BB (PDGF-BB) was obtained from Cell Signaling Technology, Inc. (Beverly, MA). Ascorbic acid, chondroitin sulfate, calcium chloride, 0.02% EDTA solution (EDTA disodium salt), antibiotic/antimycotic solution, and Dextran D4876 (MWt 144,000) were purchased from Sigma (St. Louis, MO). FNC Coating Mix was obtained from Biological Research Faculty & Facility, Inc. (Ijamsville, MD). Monoclonal antibody, 9.3.E, was a kind gift of Jurgen Bednarz (Department of Ophthalmology, University of Hamburg, Hamburg, Germany). Mouse anti-Ki67 and rabbit anti-zonula occludins-1 (ZO-1) antibodies are purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Fluorescein (FITC) conjugated donkey antimouse IgG and rhodamine- and fluorescein-conjugated donkey antirabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Vectashield mounting medium with propidium iodide or DAPI was from Vector Laboratories, Inc. (Burlingame, CA).

Isolation and Growth of Human Corneal Endothelial Cells

HCEC were isolated and cultured according to published protocols,[26] but with some technical modifications. Donor corneas were obtained from National Disease Research Interchange (NDRI), Philadelphia, PA, and stored in Optisol-GS (Bausch & Lomb, Rochester, NY) at 4°C. Tables 1 and 2 provide a breakout of information regarding corneas used for endothelial culture. Corneas were obtained from donors whose ages ranged from 2 to 79 years. All corneas received from NDRI were considered to be unsuitable for transplantation, due to lack of a blood sample from the donor to conduct serology tests, defects of the epithelium or stroma within the optical zone, stromal infiltrates, or guttata. In accepting corneas from NDRI, the overall health of the donor before death was considered and tissue was rejected from donors with previous history or treatment that might damage the corneal endothelium. These criteria also include too long a period (>24 hours) between time of death and time of preservation, low endothelial cell densities, corneas from donors with diabetes, glaucoma, sepsis, or ocular infection, or from donors who were on large doses of chemotherapeutic agents.

Table 1. Donor Information for the Younger (<30 years old) Group

Age	Days	Cell Counts (OS/OD)	COD
2	7	5000	Gunshot wound
5	3	3891/3663	Lung diseases
6	8	3449/3023	Gunshot wound
8	3	3508/3906	Motor vehicle accident
9	3	N/A	Bronchial asthma
14	3	3300	Spinal atrophy
14	5	3703/3663	Viral endocarditis
15	2	3174	Motor vehicle accident
15	8	N/A	Motor vehicle accident
16	8	N/A	Head trauma
16	3	N/A	Motor vehicle accident
17	2	2300/2833	Motor vehicle accident
18	3	2610/2463	Motor vehicle accident
18	5	N/A	Motor vehicle accident
19	3	N/A	Head trauma
20	3	2264/2038	Motor vehicle accident
20	7	2932	Gunshot wound
20	7	2866/2933	Acute cardiac event
20	7	N/A	Head trauma
20	13	N/A	Motor vehicle accident
22	2	N/A	Head trauma
23	6	2875/2891	Heart attack
24	4	3000/2900	Subarachnoid hemorrhage
26	3	2600/2600	Chronic heart failure
27	4	2332/2338	CNS cancer
28	4	2415/2475	Suicide
30	5	2674/2682	Unknown

Days: Days from death to culture
COD: Cause of death
N/A: Not available

In general, primary cultures of endothelial cells were initiated within 1 week of preservation in Optisol-GS. Corneas were removed from the Optisol and washed several times with M199 containing 50 ug/mL gentamicin before being placed in a Petri dish. Descemet's membrane with intact endothelium was carefully dissected in small strips and then incubated in OptiMEM-I supplemented with 8% FBS overnight to stabilize the cells before culture. After centrifugation, the strips were incubated in 0.02% EDTA solution at 37°C for 1 hour to loosen cell–cell junctions. Cell junctions were disrupted by forcing the tissue and medium multiple times through the narrow opening of a flame-polished pipette. Cells were pelleted and resuspended in culture medium containing OptiMEM-I, 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL pituitary extract, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 50 μg/mL gentamicin, and antibiotic/antimycotic solution diluted 1/100. Isolated cells and pieces of Descemet's membrane that still contained attached cells were plated in 6-well tissue culture plates that had been precoated with undiluted FNC Coating Mix. Cultures were then incubated at 37°C in a 5% carbon dioxide, humidified atmosphere. Medium was changed every other day. After primary cultures reached confluence, cells were subcultured at a 1:2–1:4 split ratio. Immunocytochemistry (see below) using the human corneal endothelial cell marker, monoclonal antibody 9.3.E,[31] tested for the isolation of endothelial populations. A Nikon TS100 microscope (Nikon, Melville, NY) with a Nikon Coolpix 995 digital camera was used to take phase contrast images at frequent intervals during growth and at confluence to document cell morphology.

TABLE 2. Donor Information for the Older (>50 years old) Group

Age	Days	Cell Count (OS/OD)	COD
51	5	2457/2673	Lung cancer
51	4	3050	Heart failure
52	3	2578/2519	Intracranial bleeding
52	7	2945	Intracranial bleeding
53	4	2783	Cardiac arrest
54	6	N/A	Cerebrovascular accident
55	7	2474/2646	Gunshot wound
55	4	N/A	Myocardial infarction
56	4	2500/2600	Heart attack
57	5	N/A	Motor vehicle accident
58	3	3115/3048	ASCVD
58	3	2960	Lung cancer
59	4	2500/2500	COPD
59	4	N/A	Anoxia
60	2	1925/2398	Renal failure
60	4	2881/2800	Breast cancer
60	4	3003/3076	Pneumonia
61	3	2245/2182	Cardiac arrest
61	2	1800	Stroke
62	4	2583/2710	Lung cancer
62	3	2322/2166	Cardiopulmonary arrest
63	6	2600/2732	Angina
63	7	N/A	Pancreatic cancer
63	7	3300	Lung cancer
64	7	2683/2697	Unknown
64	6	2974/2708	Anoxic injury
64	7	2659/2531	Lung cancer
64	3	2770/2857	Cardiopulmonary arrest
64	1	2549	Renal failure
65	6	2303/2049	Cardiovascular accident
65	3	2314/2469	Aortic aneurism
66	2	2900/3050	Probable MI
66	8	3071/3017	SAB
66	8	3246	Lung cancer
66	3	2640/2680	Adrenal corticoid insufficiency
66	4	2712	COPD
66	6	2475/2500	COPD
67	5	2552/2448	Respiratory failure
68	5	3014	Neoplastic disease
68	7	2500	Interstitial pulmonary fibrosis
68	4	2850	Heart failure
68	6	2900/3050	Heart failure
69	4	2412/2459	Cardiac arrest
69	10	2700/3250	Heart failure
70	3	2632	Abdominal aortic aneurism
71	2	1150/1013	Respiratory failure
71	2	3048/3174	Heart failure
71	3	3278/2237	Myocardial infarction
71	10	2743/2686	Renal failure
71	9	N/A	Congestive heart failure
72	3	2500/2500	Respiratory failure
73	12	2666/2747	Cerebrovascular accident
76	1	2272/1831	Cardiac failure
76	3	2262/1972	Heart failure
76	3	2450	COPD
79	2	2369	Stroke

Days: Days from death to culture
COD: Cause of death
N/A: Not available

Immunocytochemical Localization

Cultured cells were plated in 2- or 4-well chamber slides that had been precoated with FNC and were allowed to attach overnight or grown to confluence, depending on the experiment. Established protocols were used for the fixation, blocking, and antibody incubation steps.[15,31,32] For immunolocalization using monoclonal antibody, 9.3.E, the lyophilized antibody was reconstituted in PBS with 10% FBS according to the protocol provided by J. Bednarz. FITC-conjugated donkey antimouse IgG (diluted 1:50) was used as secondary antibody. Ki67 was used undiluted and ZO-1 was used at a 1:100 dilution. Secondary antibody alone acted as a negative control for all immunolocalization studies. After washing in PBS, slides were mounted in medium containing propidium iodide or DAPI to stain all nuclei. Positive staining of cultured cells was visualized on a Nikon Eclipse E-800 fluorescence microscope equipped with a spot digital camera. For immunolocalization of the endothelium in situ, corneas were incubated overnight at 4°C in OptiMEM-I with 5% dextran to decrease stromal edema after immunostaining using established protocols.[15] Fluorescence was visualized using a confocal microscope (model TCS 4D; Leica, Deerfield, IL) equipped with a laser (model DMRBE; Leitz Lasertechnik, Heidelberg, Germany) and SCANware ver. 4.2 software (Leica).

Effect of Growth-Promoting Agents on Proliferation

For most studies, passages I through IV of HCEC were used to test the effect of various growth-promoting factors on cell proliferation. Later passage cells were used only when cultures exhibited morphologic and growth characteristics similar to those of early passage cells. Cells were harvested, pelleted, and equal numbers of cells were seeded into individual wells of 24-well culture plates that had been precoated with undiluted FNC Coating Mix. Cells were maintained overnight in 4% FBS to permit cell attachment, then washed once with HBSS. Test medium was added and changed every other day. The basal medium used for all growth studies consisted of OptiMEM-I, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 50 μg/mL gentamicin, and antibiotic/antimycotic solution diluted 1/100. Cells were incubated in test medium for up to 3 weeks. At various times after medium addition, cultures were trypsinized and cell numbers determined using a Coulter Counter (Coulter Electronics, Miami, FL). At least three separate wells were counted per time-point and condition. Cells from each well were counted three times. Results were averaged and SD was calculated. Each study was repeated using cells from two to three different donors per age group, that is, younger donors (<30 years old) and older donors (>50 years old). Within each study, a one-way ANOVA test was performed to analyze statistical significance, with $P < 0.05$ considered to be significant.

RESULTS

Primary Culture of Human Corneal Endothelial Cells

Methods developed in our laboratory for culture of HCEC from donor corneas have already been described.[26] In the current studies, they were modified somewhat to optimize the yield of healthy cells. Experience with over 100 corneal pairs from donors ranging in age from 2 to 79 years indicates that, with these methods, endothelial cells can be cultured with a high success rate. For example, over a one-year period, fifteen pairs of corneas were received from young donors (<30 years old) and yielded a culture success rate of 93.3%. Culture of endothelium from twenty-three older donors (>50 years old) yielded a success rate of 86.9%. Cultures were most successful when corneas were received in transplantation medium within 7 days after death. Prolonged corneal storage decreased the ability of cells to attach and grow. Careful dissection of Descemet's membrane without disturbing the attached endothelial cells or stroma was important to ensure that a maximum number of cells were harvested free from contaminating stromal keratocytes or corneal epithelial cells. Cell attachment was improved by coating tissue culture wells with FNC Coating Mix. In addition, preincubation of the Descemet's membrane/endothelial cell strips in 8% FBS permitted endothelial cells to stabilize before isolation and culture. Micrographs in Figure 1 demonstrate that cultured HCEC stained positively with anti-

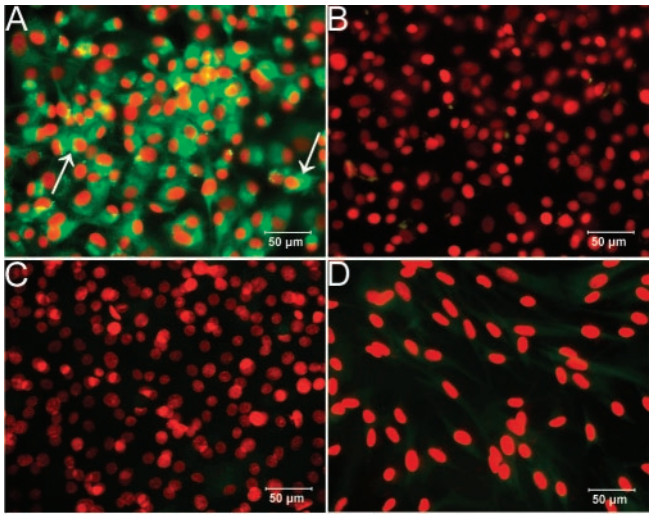

FIGURE 1. Immunostaining with antibody 9.3.E demonstrates successful isolation and culture of human corneal endothelial cells (HCEC). Micrographs in (**A**) and (**B**) are third passage cultures of HCEC from a 20-year-old donor. Positive 9.3.E staining (*green*) in (**A**) is visible particularly in the Golgi complex (*arrows*). Micrograph in (**B**) is the secondary antibody control. No positive 9.3.E staining was visible in cultures of human epithelial cells from a 50-year-old donor (**C**) or stromal keratocytes from a 41-year-old donor (**D**). Propidium iodide (*red*) was used to visualize all nuclei.

body 9.3.E.[31] This antibody specifically stains endothelial cells within the cornea; however, the specific antigen recognized by this antibody is not known. Corneal epithelial cells and stromal keratocytes did not stain positively with this antibody.

Although there was no statistical difference ($P < 0.166$) in the relative number of days it took for primary cultures of HCEC from young and older donors to reach confluence, there was a strong tendency for cells from young donors to grow faster than those from older donors (data not shown). Review of twenty-seven consecutive cultures indicates that HCEC from young donors ($n = 12$) reached confluence within an average of 14 ± 6 days, while those from older donors ($n = 15$) generally grew more slowly and reached confluence within an average of 18 ± 8 days. At confluence, primary HCEC from younger donors appeared more regular in shape and smaller in size than HCEC from older donors (Fig. 2A-D). The average endothelial cell density reported by the eyebank for corneas from younger donors was 2888 cells/mm^2 (range, 2023 to 3891 cells/mm^2), while that from older donors averaged 2619 cells/mm^2 (range, 1013 to 3174 cells/mm^2). The relative difference between the average densities in the two age groups was 10%—not a sufficient difference to account for the difference in the cell density of confluent primary cultured cells indicated by the phase-contrast micrographs. The apparent change in cell density in the confluent cultures may reflect a number of parameters that differ in cells isolated from young and older donors. One difference may be a lower rate of endothelial cell attachment in samples from older donors. Potentially lower cell densities in corneas from older donors could affect the original cell numbers available for culture. In addition, lower numbers in the confluent primary culture may reflect the relative ability of cells from young and older donors to respond to growth factors. Regardless of donor age, cell size tended to increase, cell shape became more heterogeneous, and the apparent number of multinucleated cells increased with increasing passage number (Figs. 2E, 2F). Staining for Ki67 indicated the presence of actively cycling cells[32] in subconfluent cultures (Fig. 3A). Lack of Ki67 staining (Fig. 3B) and positive staining for ZO-1, a tight junction-associated protein,[33] at the cell periphery (Fig. 3C) provided evidence that cells become contact inhibited at confluence, even in the presence of the multiple growth factors used in the primary culture medium.

Effects of Growth-Promoting Factors on Proliferation of HCEC

Dose–response studies were first conducted to determine that optimal concentrations of growth-promoting factors were used in the normal culture medium. Although a similar, but not identical, culture medium was previously reported by this laboratory,[26] no dose–response data was given to show that growth factor concentrations were optimal for growth of HCEC. In preliminary studies, basal media, including Opti-MEM-I, MEM, M199, and HBSS, were tested for their relative effect on HCEC attachment and growth using cells from the same donor to permit direct comparison. Incubation in MEM, M199 or HBSS did not support long-term cell attachment or growth. In contrast, OptiMEM-I promoted attachment and induced a moderate proliferative response ($P < 0.001$) above that of the other basal media (Zhu C, Joyce NC. *IOVS* 2002;43: ARVO E-Abstract 3184). As a result of these preliminary studies, the basal medium for all subsequent studies included Opti-MEM-I, as well as all the previously reported additives.[26] The dose-dependent effects of the following growth-promoting agents were tested on the proliferative response of HCEC: EGF (0.05–50 ng/mL), NGF (0.2–200 ng/mL), bovine pituitary extract (0.1–100 μg/mL), and FBS (1%, 2%, 4%, or 8%). HCEC cultured from a single donor were used for each dose–response study to assure internal consistency of the results. Each study was repeated two to three times using cells from different donors in the two age groups. Representative results are presented in Figure 4. EGF was tested because of its known positive effect on corneal endothelial wound healing in ex vivo models and in culture.[6,28,30] EGF induced proliferation in a dose-dependent manner in a range of 0.05–5 ng/mL (Fig. 4A). Peak cell counts were maximal and significantly higher ($P < 0.001$) than OptiMEM-I controls at 5 ng/mL. Lower cell counts were obtained when the dose of EGF was increased to 50 ng/mL. The ability of HCEC to proliferate in response to NGF was tested, because endothelial cells are considered to be of neural crest origin[34] and preliminary immunolocalization studies indicated that HCEC express TrkA, the high affinity receptor for NGF[35] (data not shown). NGF did not show a consistent, significant stimulatory effect on proliferation above basal levels, even in cells from young donors (Fig. 4B). Although NGF did not consistently stimulate proliferation in HCEC, it was retained as a constituent of the normal culture medium, because it appeared to have a positive effect on cell morphology (data not shown). Pituitary extract induced a dose-dependent response at concentrations of 0.1 μg/mL ($P < 0.05$) to 100 μg/mL ($P < 0.001$; Fig. 4C). Peak cell numbers were significantly ($P < 0.001$) higher than basal levels at a concentration of 100 μg/mL. FBS induced a dose-dependent increase in cell numbers in a range of 1% to 4%. Concentrations of 4% and 8% FBS consistently and significantly ($P < 0.001$) increased proliferation above OptiMEM-I controls (Fig. 4D). It should be noted that, although absolute cell numbers obtained with these growth-promoting factors differed somewhat from donor to donor, the same relative dose–response results were consistently obtained, increasing confidence that optimal concentrations of these factors were being used.

The relative proliferative response of HCEC from young and older donors was compared to EGF alone (5 ng/mL), FBS alone (8%), or the combination of EGF (5 ng/mL), NGF (20 ng/mL), pituitary extract (100 μg/mL), and 8% FBS used for primary culture. Cells cultured from a single donor were used to com-

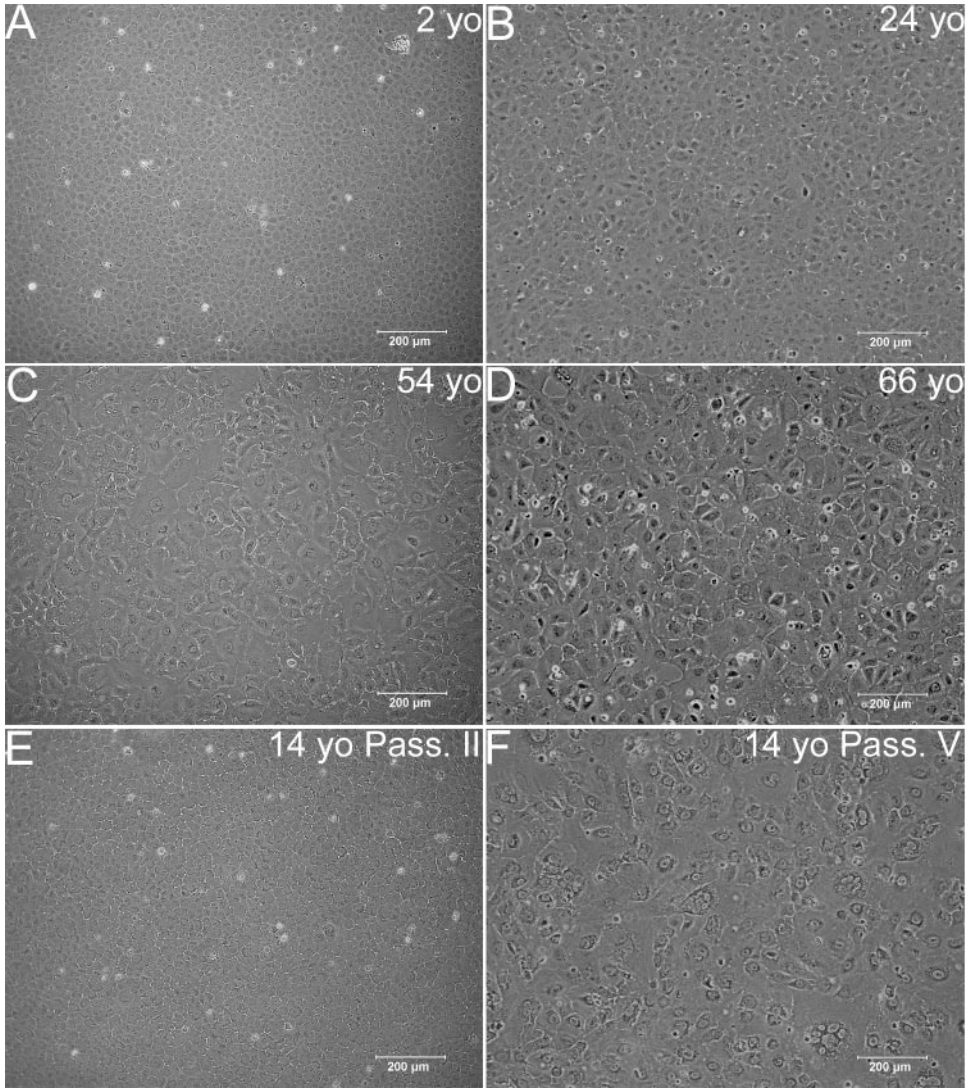

FIGURE 2. Phase-contrast images of confluent, primary cultures from donors of different ages and passage number illustrate morphologic differences. Micrographs in (**A–D**) show increased cell size, heterogeneity of cell shape, and an apparent lower cell density in confluent cultures with increasing donor age. Confluent cultures from a 14-year-old donor at passage 2 (**E**) and 5 (**F**) illustrate similar morphologic changes, plus an apparent increase in multinucleated cells, with increasing passage number.

pare the effect of the three treatments to assure internal consistency of the results. Responses were tested in HCEC cultured from at least two to three different donors in each of the two age groups. Figure 5 presents representative examples of the results. Note that the scale of the *y*-axis in this figure is greater than that in any graph presented in Figure 4. EGF (at 5 ng/mL) generally induced a moderate proliferative response in HCEC from younger donors (Figs. 4A, 5A). The response of HCEC from older donors to EGF was not as consistent. (Compare results in Figs. 5B, 5C, where there was little-to-no stimulation of proliferation, and in Fig. 6D, where EGF induced a moderate proliferative response.) In contrast, 8% FBS alone or in combination with EGF, NGF, and pituitary extract consistently stimulated proliferation in HCEC from both young and older donors. Regardless of age, the relative number of cells in cultures incubated with the combined growth-promoting agents or with FBS alone was significantly greater ($P < 0.001$) than that achieved with EGF alone. Although the combined factors generally yielded greater peak cell numbers than FBS, the relative difference was only marginally significant ($P < 0.05$). It should be noted that the overall response of HCEC from older donors to FBS or the combination of growth-promoting factors was consistently lower than in cultures from younger donors, but always greater than that achieved with EGF alone.

Studies from other laboratories have demonstrated that HCEC in vivo express both the α-and beta forms of the PDGF receptor. The beta form of the receptor, which preferentially binds the B-chain of PDGF, appears to be most abundant.[36] PDGF-BB also promotes healing in an ex vivo human corneal endothelial wound model[37] and enhances growth of corneal endothelial cells cultured from rabbit[38] and rat (Rawe I, personal communication, Schepens Eye Research Institute, 2003). Therefore the effect of PDGF-BB, alone and in combination with other growth-promoting agents, on proliferation of HCEC from young and older donors was determined. Within a single experiment, cells from the same donor were used to compare directly the effect of different growth factors on cell numbers. Responses were evaluated from at least two different donors per age group. The concentration of PDGF-BB used in these studies (25 ng/mL) was based on the concentration required for optimal stimulation of rat corneal endothelial cell growth (Rawe I, personal communication, Schepens Eye Research Institute). Representative results in Figures 6A–6D show that PDGF-BB stimulated proliferation to a level similar to that of EGF, regardless of donor age. Figures 6E–6H compare the relative effect of EGF, pituitary extract, PDGF-BB, PDGF-BB plus extract, and FBS on the same cultures as in Figures 6A–6D. Pituitary extract alone induced a significantly ($P < 0.01–0.001$) greater increase in cell numbers than did PDGF-BB alone.

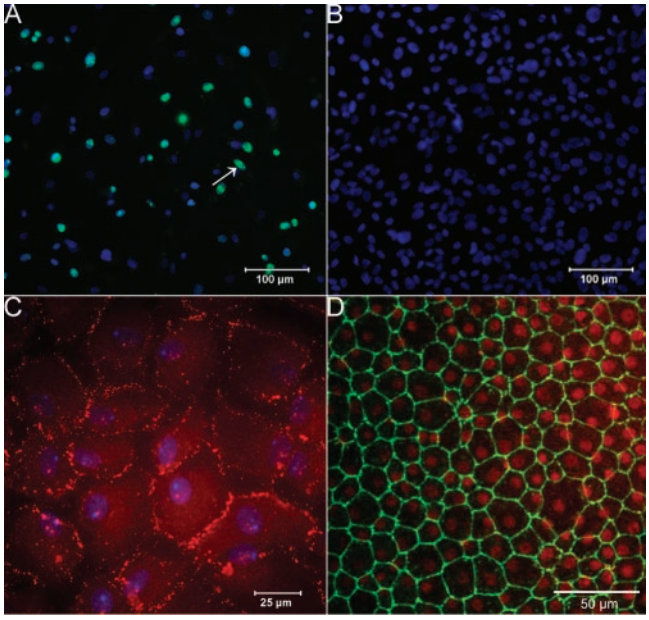

FIGURE 3. Immunostaining for Ki67 and ZO-1 illustrates that HCEC become contact inhibited at confluence. Micrographs in (**A**) and (**B**) are subconfluent and confluent cultures, respectively, from passage 4 HCEC from a 16-year-old donor. *Arrow* in (**A**) indicates a nucleus (*green*) that is positive for Ki67, a marker of actively cycling cells. Absence of Ki67-positive staining in (**B**) indicates the lack of proliferation in confluent cultures. In (**C**), formation of a confluent monolayer is illustrated by positive ZO-1 staining (*red*) in the cell periphery. HCEC in (C) are passage 3 from a 2-year-old donor. Confocal micrograph of in situ ZO-1 staining (*green*) in corneal endothelium from a 55-year-old donor is used for comparison. Nuclei in (**A**), (**B**), and (**C**) are stained with DAPI; nuclei in (**D**) are stained with PI.

PDGF-BB plus extract had an apparent additive effect ($P <$ 0.01–0.001), and this effect was seen regardless of donor age. Maximum cell numbers achieved with this combination were consistently and significantly higher ($P < 0.001$) than those achieved with EGF alone, even in cells from older donors; however, the increased cell numbers achieved by this combination were still significantly ($P < 0.001$) less than that that obtained when HCEC were incubated with 8% FBS alone.

DISCUSSION

Successful culture of untransformed HCEC has been reported from several laboratories.[17–26] Many methods for culture have been reported, including the use of specially prepared ECM[23,25] or ECM coating[19,21] and of selective medium to suppress stromal fibroblast growth.[19] A number of methods, although yielding successful harvest of HCEC, did not consistently result in confluent cultures with in vivo-like morphology. The culture technique originally reported by our laboratory[26] was modified as indicated above and has consistently yielded HCEC cultures from a wide donor age range that exhibit normal polygonal morphology. HCEC cultured in this laboratory have been successfully used for ex vivo transplantation to donor human corneas.[26] This culture method has also provided an ideal platform to compare systematically the relative proliferative response of endothelial cells from young and older donors to different mitogenic agents.

Donor corneas obtained from NDRI were originally rejected for transplantation, but with appropriate exclusion criteria, these corneas consistently yielded healthy endothelium that could be grown and passaged multiple times. The age-related differences observed in the morphology of the confluent monolayer were general phenomena and were quite similar to those reported by Miyata et al.,[25] who cultured HCEC on bovine corneal endothelial cell-derived extracellular matrix in the presence of 15% FBS and 2 ng/mL basic-FGF. Cells from older donors performed as well as or better than cells from young individuals. In some cases, cells from younger donors performed relatively poorly and resembled the response of cells from older donors. The duration between death, enucleation, and culture, as well as the relative health of the donor before death, appeared to affect the ability of these cells to grow and thrive in culture. In the present study, endothelial cells were isolated from the entire cornea and represented the average proliferative capacity of cells from individual donors. No comparison was made concerning the relative proliferative capacity of endothelial cells obtained from peripheral versus central cornea.

Previous studies examined the effect of various growth factors using a number of methods, including ex vivo cornea wound healing models,[6,27,37] growth factors added directly to the intact endothelium in ex vivo corneal culture,[28,30] and in cell culture.[19,39] In these studies, response to growth factors

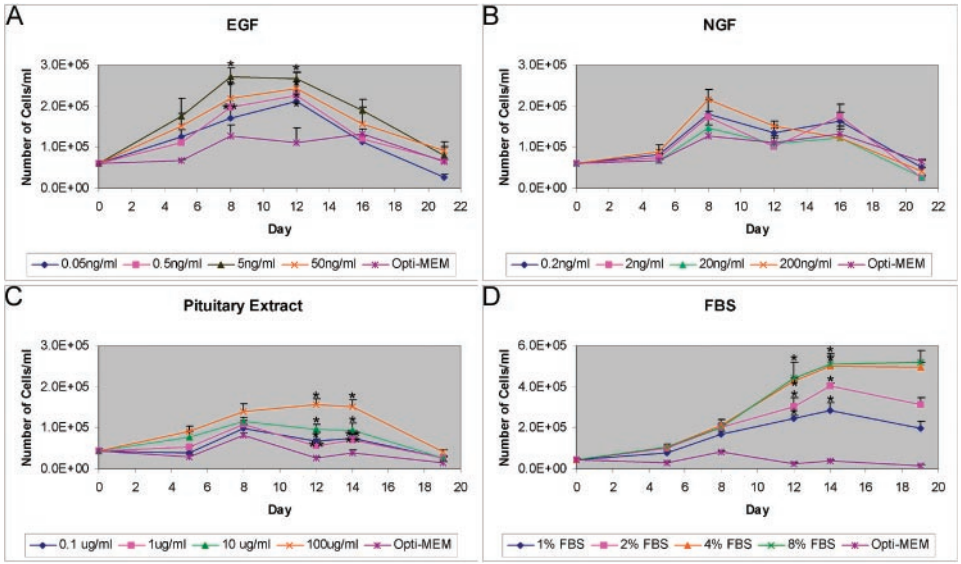

FIGURE 4. Representative graphs showing dose-dependent effects of growth-promoting agents used for the culture of HCEC. Results are shown for EGF (**A**: 0.05–50 ng/mL; 30-year-old donor, Passage 1), NGF (**B**: 0.2–200 ng/mL; 30-year-old donor, passage I), bovine pituitary extract (**C**: 0.1–100 μg/mL; combined cells from 65- and 71-year-old donors, passage II), and FBS (**D**: 1%, 2%, 4%, or 8%; combined cells from 65- and 71-year-old donors, passage II). Cells were counted over a period of 19–21 days and results were compared with OptiMEM-I alone. Note difference in scale of *y*-axis between graphs in (**A**–**C**) and graph in (**D**). Bars indicate SD $^*P < 0.001$, $^{**}P < 0.05$ compared with OptiMEM-I levels.

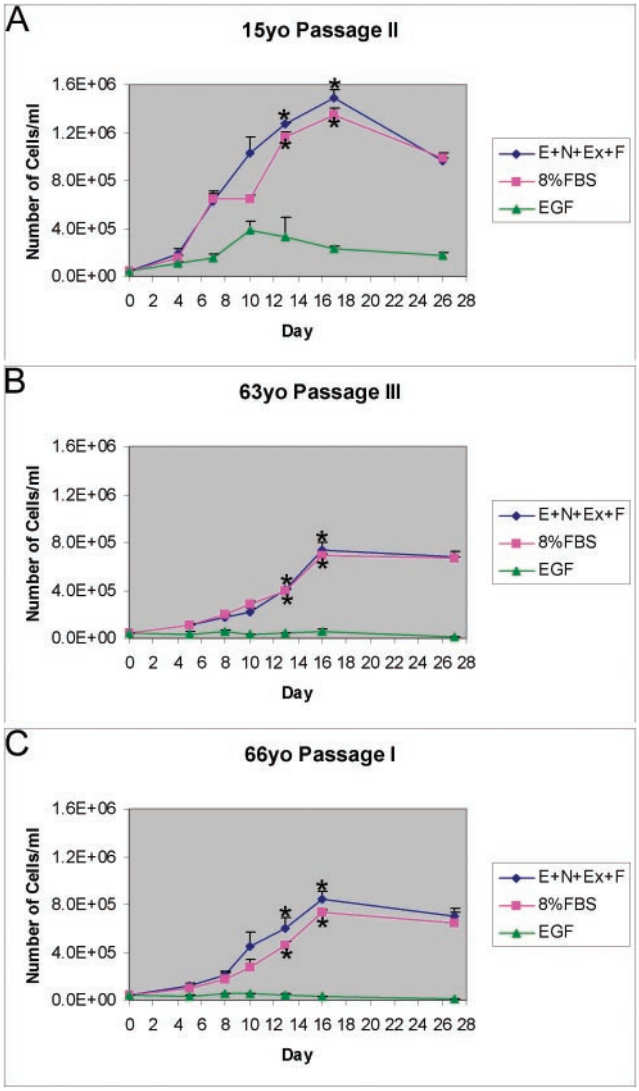

FIGURE 5. Representative results showing the relative effect of EGF, FBS, and a combination of growth-promoting agents on proliferation of HCEC from young (**A**) and older donors (**B** and **C**). Equal numbers of cells from a single donor were plated and cultures were maintained for up to 1 month in 5 ng/mL EGF, 8% FBS, or a combination of 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL pituitary extract, and 8% FBS (*E+N+Ex+F*). Bars indicate SD $^*P < 0.001$ compared with EGF levels.

was determined by autoradiography or counts of tritiated-thymidine incorporation to show DNA synthesis,[6,27,28,37,39] or by staining and counting of mitotic figures.[28,30] The present study used direct cell counts to determine the relative effect of various growth-promoting agents on proliferation of HCEC. EGF is a mitogen for corneal endothelium both in culture and in ex vivo wound healing models.[6,28,30] Previous studies from this laboratory using an ex vivo wound healing model[29] indicate that EGF has a positive effect on proliferation of human corneal endothelium when incubated in the presence of 10% FBS. The current studies evaluated the effect of EGF alone, but not the combination of EGF plus FBS. Cultured HCEC proliferated to only a limited extent when treated with EGF, although significant statistical differences were found in comparison with basal growth medium. Cells from younger donors were generally more responsive to EGF than those from older donors, although the specific response of cells appeared to be dependent on a number of factors, as indicated above and discussed below. Similar results were obtained by Hoppenreijs et al.,[6] who used an ex vivo wound model to study the effect of EGF on wound healing in human corneal endothelium. In those studies, the number of tritiated thymidine-labeled nuclei in corneas treated with EGF was significantly higher than in the untreated controls; however, it represented only 11 nuclei/mm^2—a number so small that it was concluded that stimulation of mitotic activity by EGF was very limited. Studies were not conducted to determine the effect on proliferation of alternating periods of growth factor withdrawal followed by EGF treatment. As observed by Woost et al.[40] in bovine corneal endothelial cells, NGF does not significantly stimulate proliferation of HCEC above basal levels at any concentration tested. It was retained in the culture medium formulation, because it appeared to have a trophic effect on the cells (data not shown). PDGF-BB alone induced a proliferative response similar to that of EGF. Bovine pituitary extract generally performed better than either EGF or PDGF-BB in stimulating proliferation and, in general, was able to maintain consistent cell numbers over time. Combination of PDGF-BB and pituitary extract produced an additive effect in HCEC obtained from both young and older donors. FBS at 4%–8% induced significantly more proliferation than EGF, PDGF-BB, pituitary extract, or the combination of PDGF-BB and extract. This stimulatory effect was observed in HCEC from both young and older donors.

The general decreased responsiveness of HCEC from older donors to stimulation by growth-promoting agents may have multiple causes. Within the total endothelial population, there may be an increased number of senescent cells, which would be refractive to mitogenic stimulation and thus reduce the number of total cells capable of responding to mitogens. The relative number of specific growth factor receptors may be reduced in cells from older individuals, as indicated by the flow cytometric studies of EGF receptor numbers conducted by Lopez et al.[41] The overall response to growth factors could also be limited by receptor downregulation. Not surprisingly, FBS had the greatest effect on proliferation of HCEC compared with the other growth-promoting agents tested and this effect was observed in cells obtained from both young and older donors. This suggests that FBS may induce multiple downstream signaling pathways and/or induce a more sustained signaling response. The relative difference in the extent of the response of the same cell population to different mitogens suggests that these mitogens may induce different downstream signals. In future studies, HCEC from young and older donors will be used to identify specific differences in downstream signaling responses and/or in cell cycle kinetics between specific growth factors and FBS that could be responsible for the observed relative difference in the overall proliferative response.

In summary, improvements have been made in procedures for the consistent isolation and culture of untransformed HCEC. A normal monolayer of contact inhibited cells can be obtained and grown in sufficient quantities to permit the study of these important cells, even from older donors. The present study compared the effect of several growth-promoting agents on proliferation of HCEC from young and older donors. NGF did not induce proliferation above basal levels, regardless of donor age. EGF moderately stimulated proliferation in cells from younger donors, but did not consistently stimulate proliferation in HCEC from older donors. PDGF-BB and pituitary extract also moderately stimulated proliferation, generally above the level induced by EGF. The combination of pituitary extract and PDGF-BB had an additive effect, significantly increasing cell numbers above that achieved with EGF or either factor alone. Of the growth-promoting agents tested, FBS alone or in combination with EGF, NGF, and pituitary extract stimulated the greatest proliferation of HCEC, regardless of age. FBS

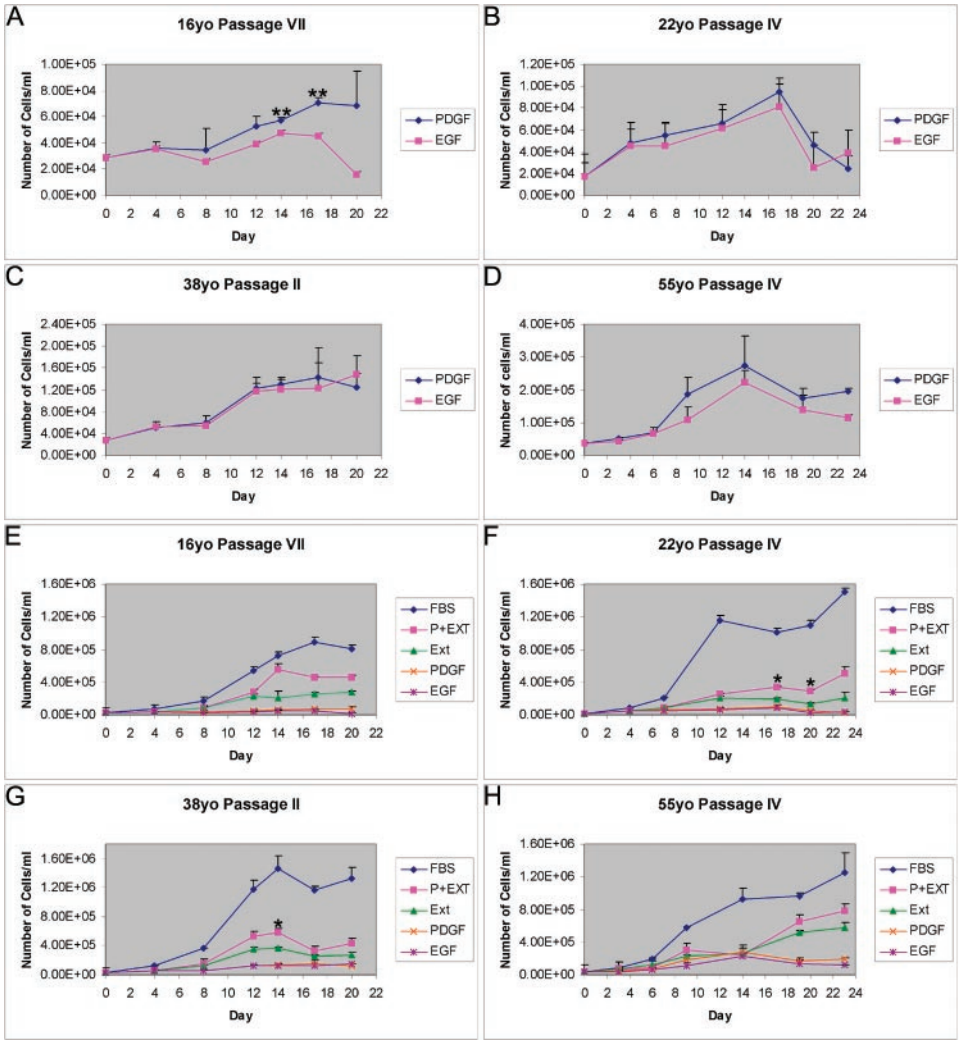

FIGURE 6. Relative effect of EGF, PDGF-BB, pituitary extract, combination of PDGF-BB and pituitary extract, and 8% FBS on proliferation of HCEC. Equal numbers of cells from a single donor were plated and cultures were treated with 5 ng/mL EGF, 25 ng/mL PDGF-BB, 100 μg/mL bovine pituitary extract, PDGF-BB plus extract, or 8% FBS for up to 3 weeks. Graphs **A–D** compare the effects of EGF and PDGF-BB (**$P <$ 0.05 compared with EGF levels). Graphs **E–H** compare the same data with that obtained when HCEC are treated with pituitary extract alone, extract plus PDGF-BB, or FBS. Note change in scale of *y*-axis. Bars indicate SD (*$P < 0.001$; ♦$P < 0.01$ compared with pituitary extract alone).

consistently yielded higher cell numbers in HCEC cultured from younger donors.

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