Expression of E6/E7 or SV40 Large T Antigen-Coding Oncogenes in Human Corneal Endothelial Cells Indicates Regulated High-Proliferative Capacity

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Purpose. Human corneal endothelial cells are thought to have limited capacity for proliferation. Little is known about the mechanisms that regulate the proliferation of these cells. The authors introduced oncogenes into human corneal endothelial cells to modulate proliferation. In addition, they sought to establish cell lines to facilitate study of human corneal endothelial cells.

Methods. Early-passage human corneal endothelial cells were transduced with disabled retrovirus (pLXSN16E6/E7) coding for the human papilloma virus type 16 transforming oncoproteins E6 and E7. Early-passage cells were also stably transfected by electroporation with the pMTV-D305 plasmid vector, in which SV40 large T antigen (SV40 LTAg) mRNA expression is positively regulated by the mouse mammary tumor virus promoter. Expression of E6/E7 mRNA or SV40 LTAg mRNA in cell lines was monitored with the polymerase chain reaction. SV40 LTAg protein expression was detected by immunocytology and Western blot analysis.

Results. Human corneal endothelial cells were efficiently infected with disabled retrovirus (pLXSN16E6/E7), and seven strains of cells have continued active proliferation for more than 50 population doublings (PD) (<8 control PD). E6/E7 mRNA was expressed by each cell strain. E6/E7 transformed cells proliferate rapidly and form a monolayer of cells with a high degree of contact inhibition. Transfection with pMTV-D305 is less efficient, and only a single strain was developed. pMTV-D305-transfected endothelial cells (dexamethasone-induced) proliferated at a slower rate than E6/E7-transduced cells or cells transfected with a vector (pSV3neo) in which SV40 LTAg is constitutively regulated. In the absence of dexamethasone, the proliferation of pMTV-D305-transfected cells was even slower, but cells continued to produce SV40 LTAg mRNA and protein. The latter results indicated that SV40 LTAg mRNA continued to be synthesized at significant levels in pMTV-D305-transfected cells in the absence of the inducer dexamethasone.

Conclusions. This study suggests that human corneal endothelial cells have a high capacity for proliferation. Thus, cell division is normally controlled in human corneal endothelial cells by poorly characterized, but efficient, mechanisms. Because the E6 and E7 proteins, as well as the SV40 large T antigen, specifically bind to and interfere with the activity of the retinoblastoma (RB) and p53 tumor suppressor proteins, our results suggest that these proteins have critical roles in regulating the proliferation of human corneal endothelial cells. Invest Ophthalmol Vis Sci. 1995;36:32-40.

Normal human corneal endothelial cells are thought to have limited proliferative potential. Although studies have demonstrated that human corneal endothelial cells have some capacity for cell division in vivo, mitosis appears to make only a minor contribution to wound healing. Thus, healing of the human corneal endothelium appears to occur primarily by enlargement and migration. Normal human corneal endothelial cells also have limited capacity to proliferate in vitro. In general, the capacity that is present decreases with increasing donor age.

We have previously demonstrated that the capac-
ity of human corneal endothelial cells for proliferation in vitro can be increased by transfection with nucleic acid sequences coding for oncoproteins, such as the SV40 large T antigen. We have extended these observations in the present study through the transduction of genes coding for the E6 and E7 human papilloma virus type-16 oncogenes into human corneal endothelial cells. We have also introduced SV40 large T antigen-coding sequences that are under the control of an inducible promoter into human corneal endothelial cells and demonstrated that the proliferation of the transfected cells can be partially regulated.

METHODS

Human corneal endothelial cells were grown from donors, who were younger than 5 years of age using previously described methods in Eagles' minimum essential medium culture medium (JRI, Lenexa, KS) containing 10% fetal bovine serum and 5% calf serum (complete medium). A Coulter counter (Hialeah, FL) was used to count cells, and PD were determined using a published method.

pMTV-D305 is a hybrid plasmid that contains the bacterial neomycin–kanamycin resistance gene neo and an altered SV40 early region coding for the SV40 large T antigen, but not the SV40 small T antigen, under the control of the mouse mammary tumor virus promoter that is positively regulated by corticosteroids (dexamethasone). The neo gene confers resistance to the antibiotic G418 to transfected mammalian cells. This resistance allows for selection of SV40 large T antigen-transfected cells. Electroporation was performed as previously reported, with second-passage cells using the Gene Pulser electroporation unit (BioRad, Hercules, CA) equipped with a capacitance extender using 210 volts at a capacitance of 250 μF. Control cells from the same donors were prepared without electroporation or were electroporated with carrier sheared salmon sperm DNA alone. Cells were passed at confluence.

Selection of stable transfectants was performed by plating cells at low density in 24-well cluster plates (Primaria, Falcon, Gaithersburg, MD) and incubating in complete medium with 200 to 400 μg/ml G418 (Gibco, Grand Island, NY) for 2 weeks. Individual colonies were selected using a cloning cylinder and were maintained in separate flasks with complete medium containing 1 μM dexamethasone. Serum was stripped by 40 cycles of annealing for 1 minute at 55°C, extension for 2 minutes at 72°C, and denaturation for 1 minute at 94°C. Unless otherwise specified, all reagents were obtained from Sigma (St. Louis, MO). Horizotal 1.5% agarose (US Biochemical, Cleveland, OH) gel electrophoresis was performed according to a previously described technique using 27 μl of each PCR product and 3 μl of 10X loading buffer per lane with a 150 ml gel run in a wide Mini-Sub cell electrophoresis apparatus (Bio Rad). A 100-bp DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used for molecular size standards. Nucleic acid sequencing was performed by cloning PCR products in the TA Cloning Vector (Invitrogen, San Diego, CA) and sequencing with Sequenase 2.0 (United States Biochemical, Cleveland, OH).

Immunofluorescent staining for SV40 large T antigen protein was performed as previously described in 4-well tissue culture chamber slides (Lab Tek, Naperville, IL). Untransformed human corneal endothelial cells were used as controls. A 1:1 mixture of mouse anti-SV40 large T antigen monoclonal antibodies pab416 and pab419 clone supernatant was used as the primary antibody. The cells were photographed with an E. Leitz (Rockleigh, NJ) fluorescent microscope. Immunocytologic staining for the Howell, et al. corneal endothelial-specific antigen was performed by growing cells on 4-well tissue culture chamber slides (Lab Tek). Cells were fixed for 10 minutes with 70% ethanol at room temperature. Staining was performed with the DAKO (Carpinteria, CA) Quick Staining Peroxidase Kit according to the manufacturer's instructions. No counterstain was used. While photographing each cell strain, the positive and the

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was performed using a previously reported method. The pab416 monoclonal antibody was used as the primary antibody. Briefly, T-75 flasks of the pMTV-D305-transfected human endothelial cell strain that were approximately 80% confluent were used. Cultures of proliferating cells were incubated in medium with serum stripped of corticosteroids (control, three flasks) or with an additional 1 μM of dexamethasone (experimental, three flasks) for 10 days, with changes of medium at 3-day intervals. Cells from each group were combined and extracted with 25 mM Tris-hydrochloride (pH 7.6), 0.6 M KCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml antipain, and 5 μg/ml pepstatin. The supernatant was collected after centrifugation at 15,000 rpm for 10 minutes at 4°C. Proteins (20 μg/lane) were resolved with low-range prestained SDS–PAGE standards (BioRad, Hercules, CA) by SDS–PAGE and immunoblotted by a modification of a previously described method. Briefly, protein samples were resolved on 10% polyacrylamide gels using a Mini-Protean II Cell (BioRad) and transferred to Immobilon-P membrane (Millipore, Milford, MA) with a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). After blocking with 5% nonfat dried milk in Tris-buffered saline (TBS) and washing with 0.1% Tween 20 in TBS (TTBS), the membrane was incubated with pab416 mouse monoclonal antibody diluted 100-fold in TTBS. The membrane was washed with TTBS and incubated with TTBS containing anti-mouse IgG peroxidase linked species-specific F(ab’2) fragment from sheep (Amersham, Arlington Heights, IL). Target proteins were visualized using the ECL Western Blotting Detection System (Amersham). Identical lanes from the same gels were stained with Coomassie blue and photographed to demonstrate similar protein levels in each sample.

The tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted for this study.

RESULTS

Transfection with the pMTV-D305 vector was inefficient, and only a single colony of stably transfected human corneal endothelial cells was isolated. Cells in the pMTV-D305-transfected clone tended to be large, elongated, and irregular (Fig. 1). Figure 2 demonstrates that genomic (probably derived from unspliced heterogeneous nuclear RNA because we have not detected genomic amplifications for any other genes using this RT-PCR method) and messenger RNA PCR products of the expected sizes for the SV40 large T antigen sequence in pMTV-D305 were detectable at 6, 9, 10, and 25 PD. The PCR products were determined by nucleic acid sequencing to be identical to those expected from the amplified regions of SV40 large T antigen genomic and mRNA sequences derived from the pMTV-D305 vector. Cells transfected with the pMTV-D305 vector continued to proliferate for more than 60 PD before senescence compared with 4 PD for the untransfected or mock-transfected control cells from the same donors.

Proliferation of pMTV-D305-transfected cells was monitored in the presence or absence of dexamethasone. Figure 3 shows that cells grown in the presence of dexamethasone (the inducer for the mouse mammary tumor virus promoter) proliferated at a faster rate than cells grown in the absence of dexamethasone. Cells grown in the absence of dexamethasone increased their rates of proliferation when dexamethasone was subsequently added (Fig. 3). Conversely, cells grown in the presence of dexamethasone proliferated at a lower rate when dexamethasone was subsequently removed from the medium (Fig. 3). Similar results were obtained in triplicate trials of this experiment. Dexamethasone had no effect on the proliferation of untransfected normal human corneal endothelial cells. Figure 2 demonstrates that SV40 mRNA could be detected using PCR even if dexamethasone was absent from the medium. The PCR method used was qualitative, and even large differences in expression of the SV 40 large T antigen mRNA between cultures with and without dexamethasone would not have been detected. In Figure 4, it can be seen that SV40 large T antigen protein could be detected by immunofluorescence when the pMTV-D305-transfected cells were grown with or without dexamethasone. In the absence of dexamethasone, however, the intensity of the signal was diminished compared to parallel cells grown in the presence of dexamethasone. In Figure 5, quantitative Western blot analysis demonstrates more conclusively that the expression of the SV40 large T antigen protein was increased in the pMTV-D305-transfected cells in the presence of dexamethasone, compared with the control. These results demonstrate that an increase in SV40 large T antigen protein synthesis occurred in pMTV-D305-transfected cells in the presence of the inducer dexamethasone. Also, they demonstrate that lower level expression of SV40 large T antigen protein in pMTV-D305-transfected cells continues in the absence of the inducer dexamethasone.

Transduction with the disabled retroviral vector pLXS6N166E6/E7 was efficient. A large number of clones of transduced cells emerged from the second-passage cells that were infected with the disabled retrovirus. We isolated seven clones of pLXS6N166E6/E7-transduced cells and monitored proliferation in each clone for more than 50 PD (range, 53 to 65 PD) before submission of this article. Each cell line continued rapid proliferation. These cells tended to have a more
FIGURE 1. (A) Normal untransfected human, primary, corneal endothelial cells. A Descem- et’s-endothelial explant is present within the field. (B) Untransfected first passage human corneal endothelial cells. (C) Human corneal endothelial cells stably transfected with the pMTV-D305 vector. Cells transfected with pMTV-D305 were larger and more irregular than untransfected cells. (D, E, F) Three strains of cells stably transduced with LXSN10E6/E7 (strains E6/E7 2, 4, and 7 at population doublings 49.6, 47.1, and 55.5, respectively). Cells transduced with LXSN10E6/E7 tended to be smaller and more regular in shape than cells transfected with pMTV-D305, but they were similar in morphology to first-passage untransfected cells. Original magnification, ×200
D305-neo-TRANSFECTED
HUMAN CORNEAL ENDOTHELIAL CELLS

FIGURE 2. Polymerase chain reaction amplification products of the SV40 large T antigen mRNA from control and pMTV-D305 transfected human corneal endothelial cells. Lane M contains marker DNA, with the sizes of the fragments in base pairs indicated to the left. Lane C is an amplification from cDNA prepared from control corneal endothelial cells that were not electroporated with pMTV-D305. Amplification products from cDNA samples prepared from the 6, 9, 10, and 25 population doubling cells are provided. + and – indicate that cells were cultured in the presence and absence of dexamethasone, respectively. In each amplification from the stably transfected cells, products of the expected size for SV40 large T antigen mRNA (302 bp, arrowhead) and products of the expected size for SV40 large T antigen genomic sequence (probably derived from unspliced RNA rather than contaminating DNA) in pMTV-D305 (470 bp, arrow) were detected.

FIGURE 3. Population doubling for pMTV-D305-transfected cells. When cells were cultured in the presence (solid triangles) or absence (solid circles) of dexamethasone, they proliferated at a faster rate than did cells cultured in the absence of dexamethasone (solid circles). When cells grown in the presence of dexamethasone were transferred to medium without dexamethasone (open triangles), the rate of proliferation decreased. Conversely, when cells grown in the absence of dexamethasone were transferred to medium containing dexamethasone (open circles), the rate of proliferation increased.

uniform morphology than pMTV-D305-transfected cells, and they proliferated at more rapid rates. Unless the cultures were maintained for several days beyond confluence, however, the pLXSN16E6/E7-transduced cells demonstrated a high degree of contact inhibition at confluence, with little tendency for cell piling. Figure 6 demonstrates that the cells expressed mRNA coding for the human papilloma virus E6 and E7 proteins. The PCR products were determined by nucleic acid sequencing to be identical to those expected from the amplified regions of the E6/E7 genomic and mRNA sequences derived from the pLXSN16E6/E7 vector.20 Chromosome analysis of the cell strain transfected with pMTV-D305 and three of the cell strains (2, 4, 7) transduced with pLXSN16E6/E7 indicated that the cells of each strain were mostly diploid. Each of the E6/E7-transduced cell strains (strains 1 to 7) and the pMTVD305 cell strain expressed the corneal endothelial specific antigen.21 Examples of staining for E6/E7-transduced strain 3 and the pMTVD305-transfected cell strain are shown in Figure 7. We noted that cells that were partially dissociated from the slide during fixation stained darker with the endothelial cell-specific antibody.

DISCUSSION
The results of this study extend our previous findings8 confirming that human corneal endothelial cells have
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were included, and sizes in kilodalton are provided to the left. The Coomassie blue-stained gel run in parallel with the immunoblotted gel is shown to the left (TP) and demonstrates that total protein loading was similar for the control (Dex −) and experimental (Dex +) cells on the blot. The Western blot (SV40) demonstrates that the SV40 large T antigen expression (band at 94 kd) was significantly increased in pMTV-D305-transfected cells cultured in the presence of 1 μM dexamethasone (Dex +) compared with cells maintained in medium without dexamethasone (Dex −). In the absence of dexamethasone, however, SV40 large T antigen is expressed in the cells at low levels.

a high capacity for proliferation. Expression of SV40 large T antigen or E6/E7 oncoproteins in human corneal endothelial cells extends the life of human corneal endothelial cells in vitro. Thus, cell division is controlled in human corneal endothelial cells in vitro and probably during normal in vivo function by poorly characterized, but efficient, mechanisms. Once a sufficient cell density is achieved during development, it is of obvious functional importance to the eye that further endothelial proliferation be tightly regulated.

E6 and E7 proteins have in common with the SV40 large T antigen modulation of the activity of the retinoblastoma (RB) and p53 tumor suppressor proteins. E6 interacts with p53, and E7 interacts with RB. The SV40 large T antigen interacts with both RB and p53. However, the mechanisms through which these oncoproteins function differ. SV40 large T antigen interferes with RB and p53 functions by sequestering the regulatory proteins into stable complexes. Conversely, the E6 protein has been shown to stimulate degradation of p53 through the ubiquitin-dependent proteolytic system. In either case, diminished active RB and p53 proteins with SV40 large T antigen or E6 and E7 expression leads to active and prolonged proliferation of cells in which the oncoproteins are expressed, including human corneal endothelial cells.

Similar roles for RB and p53 have been described in other cell types. Our results suggest that RB and p53 also have important roles in limiting the proliferation of human corneal endothelial cells and that investigations into the functions of these proteins could lead to a better understanding of the factors regulating corneal endothelial cell division. We cannot exclude the possibility that SV40 large T antigen and E6 and E7 promote corneal endothelial cell proliferation through the interaction with cell cycle controlling elements other than RB and p53. To date, however, there are no data to support other mechanisms of action of these oncoproteins.

The rate of proliferation of human corneal endothelial cells transfected with pMTV-D305 can be partially regulated. In the presence of an inducer (dexamethasone) for the mouse mammary tumor virus promoter that regulates transcription of the SV40 large T antigen gene in pMTV-D305, there is increased expression of SV40 large T antigen protein and the rate of proliferation of the cells is higher. However, the mouse mammary tumor virus promoter is "leaky" because in human corneal endothelial cells grown in the absence of dexamethasone, SV40 large T antigen mRNA is detectable by PCR and protein is detectable by immunocytology and Western blot analysis.

Our results demonstrate that transduction using a disabled retroviral vector is an efficient method for introducing genes of interest into human corneal endothelial cells. This method provides a vehicle for expressing genes in the cells so that their effects on endothelial functions can be investigated. This technique is effective so long as the cells undergo a mini-

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**FIGURE 5.** Western blot for the SV40 large T antigen in pMTV-D305-transfected cells. Prestained size markers (m) were included, and sizes in kilodalton are provided to the left. The Coomassie blue-stained gel run in parallel with the immunoblotted gel is shown to the left (TP) and demonstrates that total protein loading was similar for the control (Dex −) and experimental (Dex +) cells on the blot. The Western blot (SV40) demonstrates that the SV40 large T antigen expression (band at 94 kd) was significantly increased in pMTV-D305-transfected cells cultured in the presence of 1 μM dexamethasone (Dex +) compared with cells maintained in medium without dexamethasone (Dex −). In the absence of dexamethasone, however, SV40 large T antigen is expressed in the cells at low levels.

**FIGURE 6.** Polymerase chain reaction amplification products of the E6/E7 mRNA from control and pLXSN16E6/E7-transduced human corneal endothelial cells. Lane M contains 100-bp marker DNA, with representative sizes of the fragments in base pairs indicated to the left. Transduced clones 1 to 7 are amplifications derived from clones 1 to 7 of the pLXSN16E6/E7-transduced cells. Lane HON is the amplification from the normal control human corneal endothelial cells that were not transduced. Lane CON is a control amplification with water as the target. Amplification of cDNA prepared from each transduced cell strain yielded products of the expected size for E6/E7 mRNA (312 bp) and E6/E7 genomic sequence (probably derived from unspliced, heterogeneous nuclear RNA rather than contaminating genomic DNA) in pLXSN16E6/E7 (500 bp).
FIGURE 7. Immunocytologic staining for corneal endothelial specific antigen. A, B) Cells from E6/E7-transduced cell strain 3 and the pMTV-D305-transfected cell strain, respectively, stained with the corneal endothelial cell-specific antibody ascites. Corresponding cells stained with control ascites are shown in a and b, respectively. Note that cells partially dissociated from the slide during fixation stained darker with the endothelial cell-specific antibody. Original magnification, ×200.

Because the SV40 large T antigen or E6/E7 proteins are continually expressed in these cells, and persistent cell proliferation occurs. There is strong evidence that biologically available hypophosphorylated RB protein is needed for differentiated functions to be expressed in many cell types. Thus, persistent expression of SV40 large T antigen and E7 proteins may divert the hypophosphorylated RB protein from its normal cellular functions of inhibiting cell proliferation on one hand and stimulating the expression of genes that are necessary for differentiated functions on the other. We are developing vectors in which the expression of SV40 large T antigen or E6/E7 can be tightly regulated by both positive and negative promoter elements so that expression of these genes can be better controlled by appropriate inducers. These vectors should allow a superior model for untransfected human corneal endothelial cells to be engineered, in which proliferation can be stimulated by inducers to provide sufficient numbers of cells for study. Subsequently, however, the differentiated functions of the cells could be investigated in the absence of oncogene expres-
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sion. It is also likely that the proliferation of corneal endothelial cells could be modulated in vivo if p53 and RB function could be modulated through the introduction of finely controlled regulatory elements that would respond to appropriate inducers that could be administered intracamerally or systemically. Regulatory genes coding for proteins that control RB and p53 functions could be introduced into cultured endothelial cells used to augment donor corneas for transplantation. Alternatively, the genetic elements may be introduced into cells in situ through the use of appropriate disabled viral vectors. Controlled proliferation of the engineered corneal endothelial cells in vivo could be modulated through the administration of the inducer until an appropriate endothelial cell density was attained. We are continuing efforts to develop such a regulatable system to control human corneal endothelial cell proliferation for use in treating corneal disorders in which decreased endothelial cell density is the underlying defect.

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Key Words
corneal endothelial cells, cell proliferation, SV40 large T antigen, E6/E7 proteins, cornea

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