

Experimental Study of the Survival of Metastatic Cancer Cells in Corneal Organ Culture

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PURPOSE. Transmission of donor malignancy to the recipient could be one of the most disastrous complications of corneal grafting. Because of the scarcity of donor tissue and the lack of sufficient scientific evidence, the harvest of donor tissues from deaths due to systemic malignancy is permitted. This study was conducted to investigate the possible transmission of donor metastatic disease via corneal tissue preserved in organ culture (OC) conditions.

METHODS. The viability of four frequent human cancer cell lines (lung, breast, skin, and colon) was studied in OC. Various inoculums of cancer cells labeled with the membrane marker PKH67 were seeded on donor corneas and preserved in OC, followed by cell-tracking studies, histopathology, and immunohistochemistry. HLA matching of the dissected Descemet's membrane (DM) of preserved corneas was conducted, to demonstrate cell adherence. Primary cell culture was performed to confirm the viability of adherent tumor cells.

RESULTS. Viability tests showed a poor but persistent survival of cancer cells after 2 weeks in OC. Cell tracking, histopathology, and immunohistochemistry demonstrated cancer cell adherence to donor endothelium. HLA typing of the DM of preserved corneas revealed the presence of cancer cell alleles. Primary culture of the DM showed cell proliferation that was identical with the original cancer cell line, according to HLA studies.

CONCLUSIONS. The findings demonstrate that under laboratory conditions, metastatic cancer cells adhere to donor corneal tissue, survive, and retain proliferative capacity during storage in OC. Cell lines differ in their viability potential, as well as the pattern of adherence to donor endothelium. Further in vivo experimentation in laboratory animals is needed to determine the safety of such harvests. (*Invest Ophthalmol Vis Sci.* 2006;47:1339–1347) DOI:10.1167/iovs.05-1007

Cancer constitutes the second most important cause of death in industrialized nations, with more than 550,000 deaths annually in the United States.¹ It is therefore, one of the most common diagnoses in patients who donate their corneas and who may constitute between 30% and 40% of donor tissue

in eye banks.^{2,3} Transmission of donor malignancy to the recipient could be one of the most serious complications of corneal grafting. Various long-term studies performed to assess the effects of corneal transplantation with donor tissues derived from patients dying of malignancy reveal no evidence of transmission of donor disease to the recipient. There was also no increased incidence of malignancy or earlier mortality in the corneal recipients compared with a reference population.^{2–5} In contrast, three cases of transmission of donor cancer after corneal transplantation have been described. The first report in 1946 documented the transmission of a retinoblastoma after a fresh corneal graft and was validated by histopathology.⁶ The second case, reported in 2002, was of a donor-transmitted intestinal carcinoma that appeared as iris adenocarcinoma in the recipient 19 months after the graft. Proof of transmission in this case was demonstrated by histopathology and PCR for HLA II genes.⁷ The third case, reported in 2003, was that of probable transmission of a small cell carcinoma of the lung as suggested by identical histopathology and DNA typing of the tumor sample.⁸ These three cases demonstrate that metastatic cancer cells probably adhered to donor corneal tissue and were capable of overcoming the host and recipient immune mechanisms, which led to the subsequent transmission of donor disease to the recipient.

The presence of intraocular metastasis in patients who died of cancer was detected histopathologically in 12.6% of cases in an eye bank population.⁹ Aqueous cytology and immunophenotyping has shown the presence of tumor cells in the anterior chamber in patients with systemic malignancy metastasizing to the eye. Patel et al.¹⁰ reported positive aqueous cytology in a case of primary lung carcinoma metastasizing to the eye. The importance of cytologic examination of the aqueous humor or iris biopsy specimen was emphasized in a report of three cases of metastatic carcinoma of the iris and ciliary body simulating iridocyclitis.¹¹ In the largest study, which involved 19 patients presenting with intraocular malignancy masquerading as uveitis, Rothova et al.¹² demonstrated positive aqueous sampling in five (63%) of eight patients (two of three by cytology and three of five by immunophenotyping). Acute hypopyon uveitis with positive aqueous cytology has been reported frequently in hematogenous malignancies.^{13–15} Because of the scarcity of donor tissue and lack of sufficient scientific evidence to the contrary, eye bank associations in the United States and Europe permit harvest of donor tissues from donors who died of systemic solid malignancies, with certain exceptions such as like active leukemia, disseminated lymphomas, and malignant intraocular tumors.^{16,17} This practice is rather empiric since no experimental evidence concerning the possible transmission of malignancy exists in the literature. Corneal organ culture (OC) being the preferred mode of preservation in Europe and used in at least two of the cases reported, we sought to investigate experimentally the adherence of malignant cells to donor corneal endothelium and their survival during corneal storage—the two mechanisms required for an eventual transmission to the recipient.

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METHODS

Cell Lines

Four metastatic cancer cell lines responsible for most common human cancers (i.e., epidermoid lung carcinoma [Calu1], breast adenocarcinoma [Cal51], colon adenocarcinoma [HT29], and cutaneous melanoma [SKMel28]) were studied (lung, skin, and colon cell lines from American Type Culture Collection, Rockville, MD; breast cell line from DSMZ GmbH, Braunschweig, Germany). The cell lines were maintained in sterile T75 tissue culture flasks (BD Biosciences Labware, Le Pont De Claix, France) in respective recommended media (RPMI-1640 for SKMel28, McCoy's 5A for Calu1 and HT29, Dulbecco's modified minimum essential medium [DMEM] for Cal51, all from Sigma-Aldrich (St. Quentin-Fallavier, France), supplemented with 10% fetal calf serum, 400 U/mL penicillin, 200 U/mL streptomycin, and 2 mM L-glutamine at 37°C in the presence of 5% CO₂ and passaged on confluence. HLA class I allele (A and B) typing was performed for each cell line by the PCR-sequence-specific oligonucleotide (SSO) technique (described later) for future identification purposes.

Viability Tests In Vitro

From a subconfluent culture maintained in optimal conditions, 10⁶ cells of each line were plated in sterile 25-cm² tissue culture flasks (BD Biosciences Labware) in recommended media. Parallel cultures were maintained, one in the recommended cell culture condition, the other in the OC condition (i.e., in the culture medium CorneaMax; Eurobio, Les Ulis, France) containing 2% fetal calf serum with incubation at 31°C in sealed T25 culture flasks for a period of 2 weeks and counted (both supernatant and adherent fractions) at various time points (day 3, 6, 8, 10, 13, and 15) for 15 days. Briefly, for each cell line, the supernatant fraction was removed, and shed cells were counted separately in a hemocytometer (Thoma, Bad Blankenburg, Germany). The remaining adherent cells were incubated with 0.05% trypsin (Eurobio) for 2 minutes and counted by the same method.

For the Alamar blue assay, cells were harvested from ongoing cultures and 10³ to 3.10³ cells/well were plated on 96-well microplates (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) and a minimum of 48 hours of incubation time in the corresponding media was allowed for cell attachment before any additions to the wells. Alamar blue reagent (10% vol/vol; Serotec, Cergy Saint-Christophe, France) was added to quadruplicate wells, to result in a final volume of 200 µL. Blank wells containing the medium with reagent but no cells served as the control. Plates were incubated in respective conditions for 3 hours and fluorescence analyzed (excitation 530 nm, emission 590 nm) after a 15-minute equilibration to room temperature with a microplate fluorescence reader (Fluoroskan Ascent, Labsystems, Cheshire, UK). Tests were performed as endpoint assays at various time points over 2 weeks (days 3, 5, 7, 10, 12, and 14) and the data expressed as fluorescence emission intensity units as a function of time of incubation.

Evaluation of Adherence to Human Donor Cornea: Cell Tracking Ex Vivo

Membrane Labeling. Fluorescent labeling of cancer cells was performed with the fluorescent probe PKH67 (Sigma-Aldrich). Two million cells were incubated with 4 µL of the dye in 1 mL diluent C. After 3 minutes in the dark, 2 mL fetal calf serum was added, to neutralize free dye in the solution and, after multiple rinses, to remove excess dye, a small sample was checked for viability with 0.5% trypan blue and stain adequacy, with a 530-nm FITC filter. Labeled cells were used for further cell-seeding procedures with the appropriate controls.

Cell Seeding on Endothelium. Human corneas used in this study were eye bank donor corneas (St. Etienne Eye Bank) considered unsuitable for therapeutic keratoplasty (endothelial cell count be-

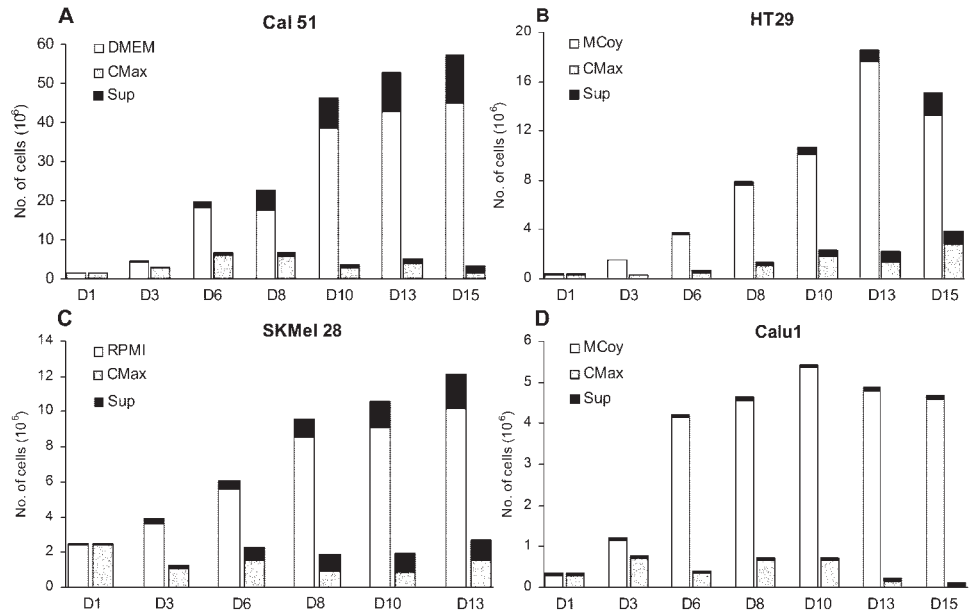
tween 1800–2000 cells/mm² or medical contraindication of the donor regardless of endothelial quality) and organ cultured (CorneaMax; Eurobio) for at least 7 days. They were seeded with the fluorescent-labeled cell suspension at various concentrations (10⁶, 5 × 10⁴, 10⁴, and 10³ cells per cornea) and incubated at 37°C in the presence of 5% CO₂ for 6 hours. Next, the corneas were rinsed gently with physiologic saline (Balanced Salt Solution [BSS]; Alcon, Rueil Malmaison, France), exactly as is usual during a routine corneal retrieval and suspended in 100 mL of the medium (CorneaMax; Eurobio) using a 6-0 black silk suture and stored at 31°C. Storage medium was renewed after 48 hours (in accordance with routine practice) and corneas were stored for 2 weeks. Corneas seeded with nonlabeled cells were similarly stored for immunohistochemical procedures, which also served as the control.

Counterstaining. At the end of 2 weeks, corneas were removed from OC, observed under bright-field and fluorescence microscope (FITC filter) and sampled for further tests. Staining of the endothelial cell borders was performed with application of 0.2% Alizarin red (Sigma-Aldrich), as previously described.¹⁸ Cell nuclei were stained with Hoechst 33342 10 µg/mL (Cambrex Bioscience, Emerainville, France) by keeping in the dark for 10 minutes at room temperature. The whole corneas were flatmounted in antifade medium (DakoCytomation, Trappes, France) with four radial nicks at the periphery and observed under a fluorescence microscope with the appropriate filters (450 nm for Hoechst and 530 nm for PKH67). Autofluorescence was eliminated by appropriate filter adjustments and comparison with the control.

Histopathology and Immunohistochemistry. Nonlabeled cell-seeded corneas were fixed for 24 hours in 10% formalin at the end of a 2-week preservation in OC. For histopathological studies, 5-µm-thick paraffin-embedded sections were dewaxed and stained with hematoxylin-eosin. Immunostaining was performed on dewaxed and rehydrated tissue sections with an automated immunostainer (TechMate 500 Plus; DakoCytomation) by the avidin-biotin peroxidase complex technique and heat-induced epitope retrieval (TRS; DakoCytomation). The following primary antibodies were used: Melan-A (1:25), epithelial membrane antigen (EMA; 1:200), cytokeratin (CK) 903 (1:75), AE1/AE3 (1:50), CK7 (1:50), CK20 (1:50), CK18 (1:100), TTF-1 (1:50), and p53 (1:100; all from DakoCytomation). Slides were processed according to the manufacturer's protocols for immune complex detection using a detection system (EnVision; DakoCytomation) with diaminobenzidine (DAB) chromogen (K 5007) or aminoethylcarbazole (AEC), (K3464; DakoCytomation). They were counterstained with Harris hematoxylin, dehydrated in alcohol, and mounted. Positive controls included tissue sections known to be positive for the antibody used. Negative controls included sections of cornea processed in parallel with omission of the primary antibody. Slides were examined by light microscopy (DMR microscope; Leica Microsystems, Rueil-Malmaison, France).

HLA Typing of the Dissected Endothelium. The endothelium of seeded OC-preserved corneas was removed from the underlying stroma by careful dissection of Descemet's membrane (DM) serving as a carrier. The DM was subjected to HLA typing for class I alleles (A and B). Genomic DNA was isolated after tissue digestion with proteinase K (10 mg/mL; Eurobio), then extraction with 1 vol each of buffered phenol, buffered 1:1 mixture of chloroform-isoamyl alcohol (phenol:Sevag; 24:1), and isoamyl alcohol followed by precipitation with an equal volume of cold absolute ethanol (all from Sigma-Aldrich). Molecular typing of HLA A and HLA B was achieved with a polymerase chain reaction-sequence-specific oligonucleotide (PCR-SSO) reverse hybridization line probe assay (DynaL RELI SSO HLA A and B Typing kit; Dynal Biotech, Warral, UK) according to the manufacturer's instructions. The test was based on three major steps: PCR target amplification, hybridization of the amplified products to an array of immobilized SSO probes and detection of the probe-bound amplified product by colorimetric reaction. The HLA A and B type for each cell line was

FIGURE 1. Comparative proliferation with time (days) of breast adenocarcinoma Cal51 (A), colon adenocarcinoma (B), cutaneous melanoma SKMel28 (C), and pulmonary epidermoid carcinoma Calu1 (D), in recommended media versus that in OC conditions (CorneaMax [CMax] Eurobio). Proliferation in all cell lines was considerably reduced in OC. Cell desquamation (supernatant [Sup] fraction) increased progressively with increasing duration of storage in all cell lines except Calu1. Despite reduced proliferation in OC, persistent cell population was noted in all cell lines at the end of 2 weeks.



obtained by comparison of hybridization data with the expected outcomes for known alleles. The interpretation of the HLA type was based on a characteristic pattern of positive probes for each cell line in a software program (PMP; Dynal Biotech).

Primary Cell Culture. The DM of seeded and OC-preserved corneas were carefully dissected in small strips with sterile precautions and incubated in sterile six-well tissue culture plates (BD Biosciences Labware) in respective recommended media supplemented with 10% fetal calf serum, antibiotics, and glutamine at 37°C in a 5% CO₂ humidified atmosphere. Medium was renewed every 3 days. Cellular proliferation was noted, either in the form of clusters or migrating sheets of cells from the edge of the DM in approximately 1 to 2 weeks time, bearing morphologic resemblance to respective cancer cell lines. After primary cultures reached confluence, cells were subcultured and maintained in medium containing 10% fetal calf serum for another week to ensure formation of a confluent monolayer. Confluent cultures were then trypsinized, genomic DNA extracted, and HLA matching

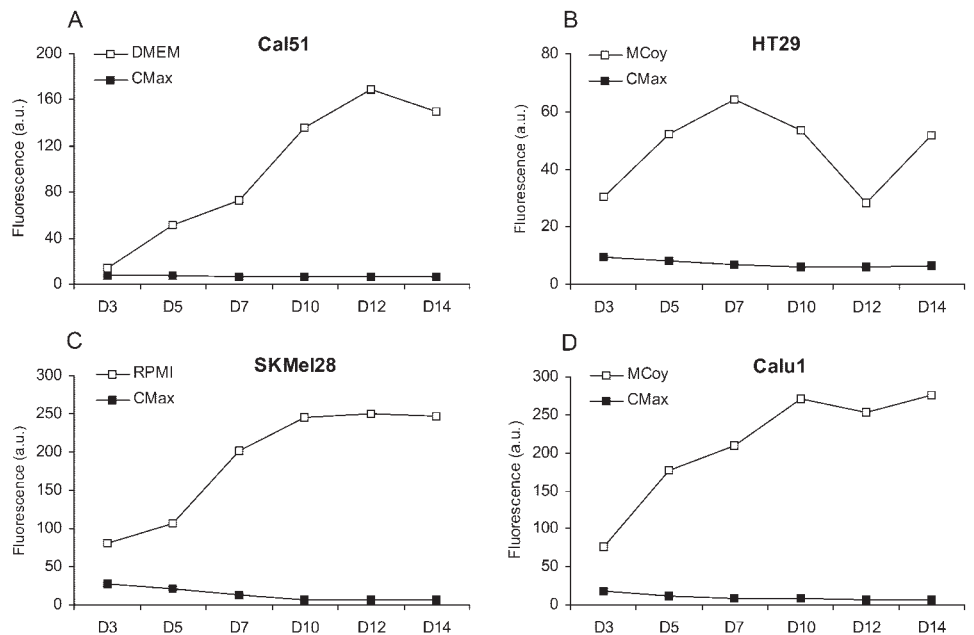
performed for cell line identification by the PCR-SSO technique, as described previously.

RESULTS

Viability Tests

For all cell lines, proliferation was reduced in the OC media compared with that in respective recommended media (Fig. 1). Proliferation of the Cal51 cell line was the least affected in OC, whereas that of the Calu1 line was the most affected. Progressive desquamation with increasing storage duration was noted for all cell lines except Calu1. The percentage viability was also diminished for all four cell lines in OC as shown by the lesser reduction of Alamar blue dye (Fig. 2). Though proliferation and viability were markedly reduced, complete extinction was not observed for any cell line in OC conditions.

FIGURE 2. Comparative viability with time (days) of breast adenocarcinoma Cal51 (A), colon adenocarcinoma (B), cutaneous melanoma SKMel28 (C), and pulmonary epidermoid carcinoma Calu1 (D), in recommended media versus that in OC (CorneaMax [CMax]; Eurobio). The viability was considerably less in OC-conditioned medium as shown by reduction in of Alamar blue staining that produced lower fluorescence values on cytofluorimetry. Despite reduced viability in OC, complete extinction was not observed in any cell line at the end of 2 weeks.



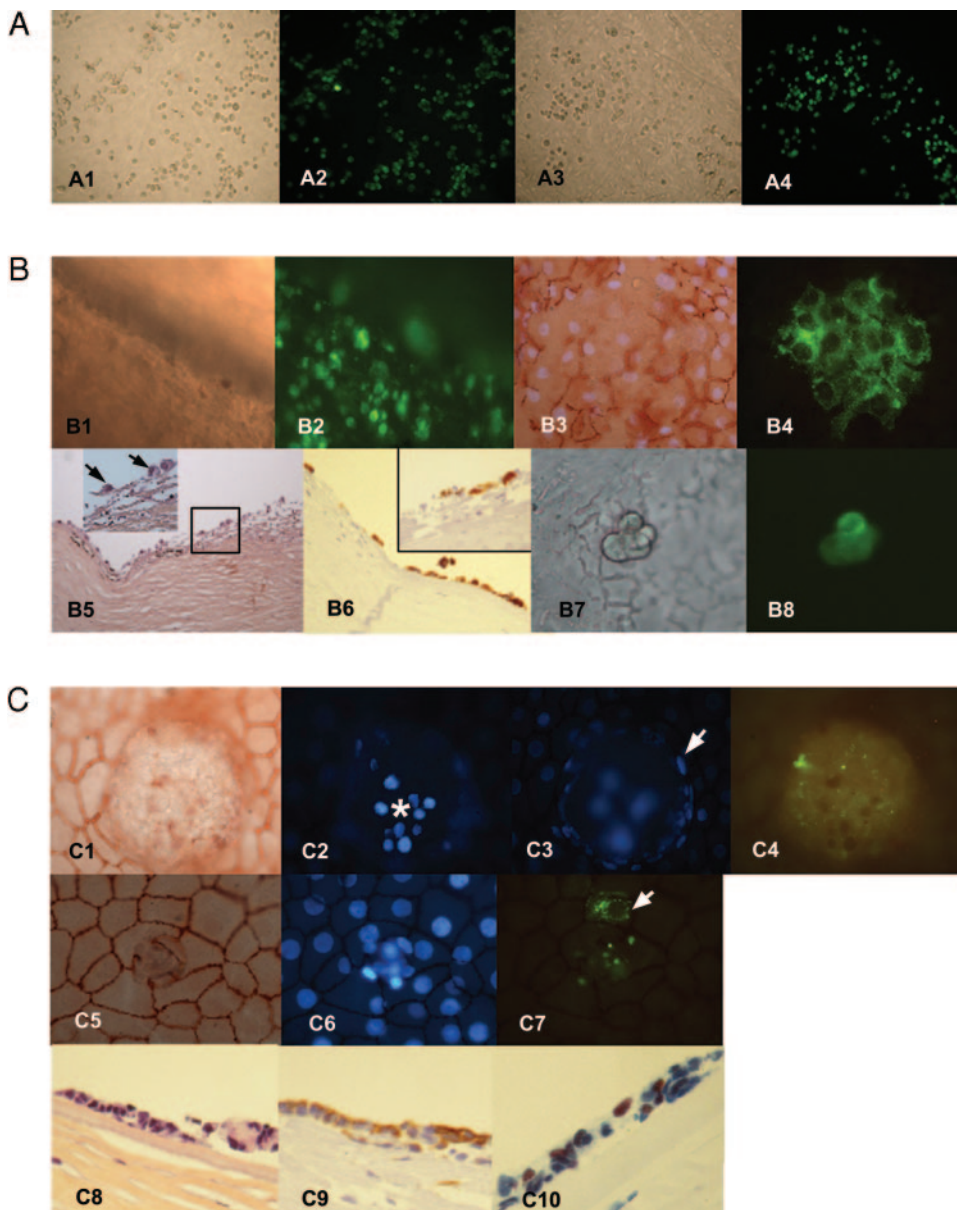


FIGURE 3. (A) Light micrographs of a cornea seeded with 5×10^4 PKH67-labeled Calu1 cells at 6 hours after inoculation and incubation at 37°C . Cellular aggregates were scattered throughout the endothelial surface before rinsing (A1, bright-field; A2, FITC) whereas after gentle rinse with physiologic saline solution, cells were fewer and were predominantly arranged along the corneal folds (A3, bright-field; A4, FITC). Original magnification, $\times 10$. (B) Light micrographs of a whole cornea seeded with 10^6 PKH67-labeled SKMel28 cells on day 15 of OC and counterstained with Hoechst 33342 and Alizarin red, showing cellular adherence in sheets on the juxtatrabecular region (B1, bright-field; B2, FITC) and in clusters on the midperipheral endothelium (B3, bright-field; B4, FITC). Histopathological examination of a section (H-E stain) confirmed the presence of abnormal cells with giant hyperchromatic nuclei in the trabecular region (B5, arrows in inset), which show positive immunostaining (brown reaction products indicating positive immunolocalization) with anti-Melan A antibody (B6). Cellular adherence was also noted with a reduced inoculum of 10^3 cells (B7, bright-field; B8, FITC). Original magnification: (B1, B2, B5) $\times 10$; (B3, B4, inset, B5) $\times 40$; (B6, B7, B8) $\times 20$. (C) Light micrographs (C1–C7) of a cornea seeded with 10^3 PKH67-labeled Cal51 cells on day 15 of OC and counterstained with Hoechst 33342 and Alizarin red, showing malignant cell adherence in clusters (focus on surface, * (C2) and base, arrow (C3) of the cluster) on the endothelium [(C1, C5) bright-field; (C2, C3, C6), UV; (C4, C7) FITC]. A morphologically normal endothelial cell just above the cluster in C7 (arrow) showed positive fluorescence, indicating either the phagocytosis of cancer cell debris by the endothelial cell

or an endothelial “integration” of the cancer cell. Histopathological examination of a section (H-E stain) of a cornea seeded with 10^6 cells showed cellular adherence in the trabecular region (C8), which showed positive immunohistochemical staining with anti-AE1-AE3 antibody (brown cytoplasmic staining, C9) and anti-p53 antibody (brown nuclear staining, C10). Original magnification, $\times 40$.

Light and Fluorescence Microscopy of Whole Cornea

Corneas seeded with PKH67 labeled cells after 6 hours of incubation on light microscopy (bright field) showed the presence of abnormal cells on the endothelial surface including the extreme periphery. On fluorescence microscopy, cancer cells were clearly delineated by their bright spotty membrane staining due to the presence of the green fluorescent marker. Cell clumps were scattered throughout the endothelial surface, with dense aggregates on the central cornea (settled due to gravity) before rinsing. During gentle rinsing with physiologic saline, loosely attached or cells in suspension were washed off. The remaining cell aggregates were smaller and fewer and were predominantly arranged along the endothelial folds (Fig. 3A).

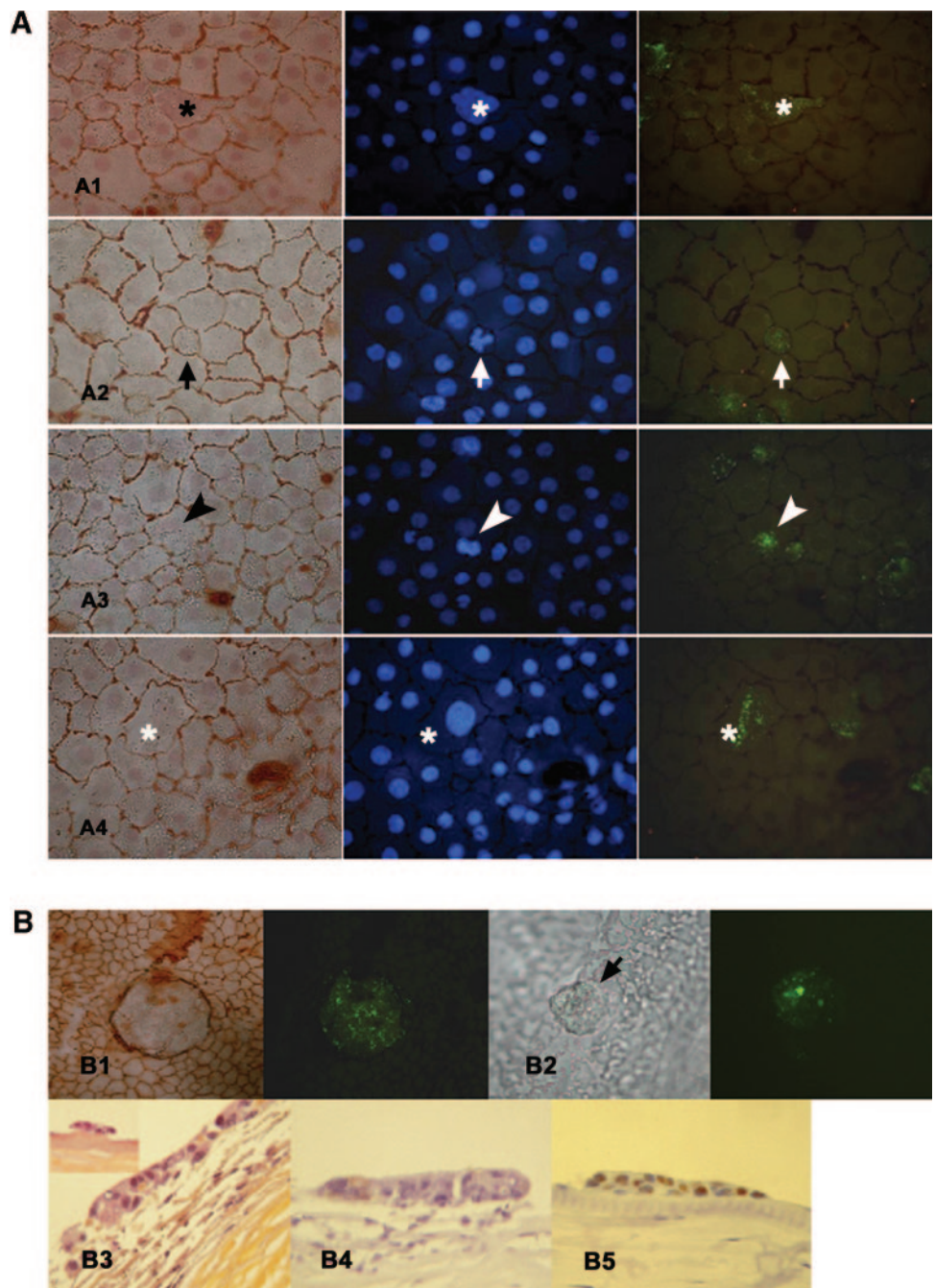
At the end of 2-week storage in OC, on light microscopy after counterstaining, similar abnormal cells were observed on

the endothelial surface in all cell lines. For the SKMel28 cell line, adherence in the form of a monolayer was noted predominantly near the corneal periphery and the trabecula. Adhesion was minimal in the central or midperipheral cornea and was mainly in the form of cells aggregated loosely in clusters (Fig. 3B).

Adherence in the form of tightly packed clusters with whorled appearance and faint spotty fluorescence was noted in the Cal51 cell line, scattered throughout the endothelium, both centrally and peripherally (Fig. 3C). Of particular interest was the observation of certain morphologically normal endothelial cells adjacent to the clusters with positive fluorescence, indicating either the phagocytosis of cancer cell debris by the endothelial cell or some kind of “integration” of the cancer cell into the endothelium.

The Calu1-seeded corneas had a characteristically different appearance (Fig. 4A). The endothelial margins were intact,

FIGURE 4. (A) Light micrographs (bright-field, UV, and FITC) of a cornea seeded with 5×10^4 Calu1 cells labeled with PKH67 and organ cultured for 2 weeks. Counterstaining with Alizarin red (*left column*; bright-field) highlighted the intercellular junctions and irregularly shaped endothelial mosaic. The counterstaining of nuclei with Hoechst 33342 (*middle column*, UV) demonstrated the presence of chromatin abnormalities (giant nucleus, hyperchromatism, *) and postmitotic changes (bi-lobed nuclei, *arrows*). The green-fluorescence-labeled cells (*right column*; FITC) represent adherent cancer cells. Two distinct types of cell adhesion were noted: cancer cells superimposed on endothelium without any underlying endothelial interconnections (A3) and more commonly, abnormal cells in the form of a pseudo-hexagon embedded within the endothelium with borders well delineated by Alizarin red (A1, A2, A4). (B) Light micrographs (bright-field and FITC, respectively) of a cornea seeded with 10^6 HT29 cells labeled with PKH67 on day 15 of OC and counterstained with Hoechst 33342 and Alizarin red showing presence of large clusters on the endothelium (B1). Similar clusters are also noted for an inoculum of 10^3 cells, sometimes barely distinguishable from the surrounding endothelium on bright-field microscopy (B2). Histopathological section (H-E stain) confirms presence of abnormal cells with large hyperchromatic nuclei on the trabecular region (B3). These cells show weakly positive immunohistochemical staining of the vacuolated cytoplasm (*arrow*) with anti-CEA antibody (B4) but show prominent nuclear staining with the anti-p53 antibody (B5). Original magnification: (A1–A4, B3–B5) $\times 40$; (B1, B2).



well delineated by the Alizarin red, and shiny refractile intracytoplasmic inclusions were observed, irrespective of their proximity to cancer cells. The adherent cancer cells were well delineated by the presence of chromatin abnormalities in the form of giant, hyperchromatic nuclei, with nonuniform chromatin distribution revealed by nuclear staining with Hoechst 33342 in combination with the green fluorescent marker. Postmitotic changes (bi-lobed nuclei) were present, indicating cell multiplication. Two distinct types of PKH67-positive cells were noted: First, cancer cells superimposed on endothelium without any underlying interconnections. The two nuclei of endothelial and cancer cells were clearly visible separate from each other, the latter displaying chromatin abnormalities and more brilliantly stained with Hoechst 33342. Second, certain PKH67-positive cells with abnormal nuclei seemed to integrate within the endothelial cells forming a pseudo-hexagon. The endothe-

lial cell nucleus could not be distinguished separately, though a distinct Alizarin red-stained hexagonal margin was delineated.

For the HT29 cell line, clusters of abnormal cells were visible both on the central cornea and over the trabecula (Fig. 4B). Cells were more likely to adhere to small patches of bare DM denuded of endothelium. For low inoculums, clusters were often smaller and sometimes indistinguishable from the surrounding endothelium but could be clearly delineated on fluorescence microscopy.

Histopathology and Immunohistochemistry

Histologic sections stained with H-E stain confirmed presence of cancer cells with prominent hyperchromatic nuclei on the endothelial surface close to the trabecula for the SKMel28, Cal

TABLE 1. HLA Analysis Results for the Cutaneous Melanoma (SKMel 28) Cell Line

SKMel 28	Reactive Probes for Loci A	HLA A	Concordance	Reactive Probes for Loci B	HLA B	Concordance
Reference alleles	2, 5, 12, 14, 19, 22, 25, 31, 34, 38, 40	A*11	—	4, 10, 14, 18, 28, 29, 31, 40, 48, 54, 56, 60	B*40	—
Descemet's membrane seeded with 1000 cells	3, 5 , 6, 11, 13, 15, 19 , 22 , 23, 25 , 26, 30, 31 †, 34, 36, 38 , 39, 40	A*01, *02	72.7%	5, 6, 7, 8, 13, 15, 16, 28 , 29 , 30, 32, 40 , 43, 48 , 49, 52, 53, 56 , 59	B*08, *35	41.7%
Primary culture	2, 5, 12, 14, 19, 22, 25, 31, 34, 38, 40	A*11	100%	4, 10, 14, 18, 28, 29, 31, 40, 48, 54, 56, 60	B*40	100%

Numbers in bold represent probes positive for both the donor cornea and cancer cell line.

* International HLA nomenclature style.

† Probe unique for cancer cell line.

51, and HT29 cell lines at higher cell inoculums (5×10^4 – 10^6 ; Figs 3, 4). No cancer cells were visible in cross sections in the Calu1-seeded corneas, probably due to technical reasons, though evidence of adherence was seen on cell tracking. Immunostaining with the anti-Melan A antibody confirmed the presence of positively stained SKMel28 cells on the peripheral cornea and trabecula (Fig. 3B). Positive immunolocalization of p53 (nuclear) and AE1/AE3 (cytoplasmic) was noted for the Calu1-seeded corneas (Fig. 3C). Corneal sections seeded with HT29 cells stained positive for p53 (nuclear) and carcinoembryonic antigen (cytoplasmic; Fig. 4B). No immunochemical staining was detected in the Calu1-seeded corneas, though they should have been positive for AE1/AE3, EMA, and TTF-1 and negative for CK 7.

HLA Expression Analysis

Tables 1, 2, 3, and 4 show the results of the HLA typing tests for the different cell lines. The first row (Reference alleles) indicates the typing (HLA A and B) obtained with the DNA derived from pure cancer cell lines, which served as reference for further HLA matching. The second row (Descemet's membrane seeded with 1000 cells) indicates the typing obtained with the DNA derived from the dissected DM of corneas seeded with 1000 cancer cells of each cell line and stored in OC. Because the inoculate volume was very feeble compared with that of the DM of donor cornea, typing corresponded

principally to the HLA type of the donor cornea. Of interest, presence of probes common to the cancer cell line and donor cornea was observed (shown in bold), which indicates the possibility that cancer cells also contributed to the typing pattern (concordance varying between 61% and 83% for HLA A and between 42% and 100% for HLA B, depending on the cell line). This is further supported by the fact that probe 31 (Table 1), which is positive uniquely for allele A*11 (SKMel28 cell line), was detected in the DM sample, indicating that the source was most likely the cancer cells. The third row indicates the typing obtained with DNA derived from primary culture of the DM. A complete matching with the reference alleles was observed indicating a cell population identical with the pure cell line.

DISCUSSION

Transmission of malignancy by corneal transplantation is very rare, and the case reports are extremely limited or even anecdotal. This study addresses two aspects of the issue: Can tumor cells survive in OC conditions, and do cells adhere to corneas. The conditions required for transmission of viable cancer cells through the corneal graft to the recipient are multiple. First, the development of an intraocular metastasis from the primary site is imperative. The incidence of ocular metastases is approximately 300,000 cases a year, most often from breast (60%)

TABLE 2. HLA Analysis Results for Lung Epidermoid Carcinoma (Calu1) Cell Line

Calu1	Reactive Probes for Loci A	HLA A	Concordance	Reactive Probes for Loci B	HLA B	Concordance
Reference alleles	2, 8, 9, 10, 11, 12, 15, 19, 20, 24, 27, 28, 30, 32, 34, 36, 38, 40	A*26, *29	—	6, 9, 10, 14, 18, 25, 28, 29, 30, 31, 43, 46, 48, 51, 53, 56, 57	B*15, *44	—
Descemet's membrane seeded with 1000 cells	3, 5, 6, 8 , 9 , 11 , 12 , 13, 14, 15 , 19 , 22, 23, 25, 26, 27 , 30, 32 , 34 , 36 , 37, 38 , 40	A*02, *03	66.6%	1, 6, 7, 9, 10 , 13, 14 , 15, 18 , 20, 28 , 29 , 30 , 31 , 32, 39, 43, 46 , 48, 50, 51, 53, 54, 56 , 57 , 60	B*07, *15†	93.8%
Primary culture	2, 8, 9, 10, 11, 12, 15, 19, 20, 24, 27, 28, 30, 32, 34, 36, 38, 40	A*26, *29	100%	6, 9, 10, 14, 18, 25, 28, 29, 30, 31, 43, 46, 48, 51, 53, 56, 57	B*15, *44	100%

Numbers in bold represent probes positive for both the donor cornea and cancer cell line.

* International HLA nomenclature style.

† HLA allele common to cornea and cancer cell line.

TABLE 3. HLA Analysis Results for Adenocarcinoma Colon (HT29) Cell Line

HT29	Reactive Probes for Loci A	HLA A	Concordance	Reactive Probes for Loci B	HLA B	Concordance
Reference alleles	1, 3, 5, 7, 11, 15, 16, 19, 22, 25, 26, 31, 34, 36, 38, 39, 40	A*01, 24	—	6, 8, 10, 14, 15, 16, 18, 25, 28, 29, 30, 43, 46, 48, 53, 56, 57	B*35, *44	—
Descemet's membrane seeded with 1000 cells	3, 6, 7, 11, 13, 19, 23, 25, 26, 30, 31, 34, 36, 38, 39, 40	A*02	61.1%	3, 6, 8, 10, 13, 14, 15, 16, 18, 25, 28, 29, 30, 31, 43, 46, 48, 49, 52, 53, 56, 57, 58	B*18, *44†	100%
Primary culture	1, 3, 5, 7, 11, 15, 16, 19, 22, 25, 26, 31, 34, 36, 38, 39, 40	A*01, *24	100%	6, 8, 10, 14, 15, 16, 18, 25, 28, 29, 30, 43, 46, 48, 53, 56, 57	B*35, *44	100%

Figures in bold represent probes positive for both the donor cornea and cancer cell line.

* International HLA nomenclature style.

† HLA allele common to cornea and cancer cell line.

or lung primaries (19%).^{19,20} The overall incidence of ocular metastases among deaths due to solid cancers was 4% in a study on 716 eyes obtained from patients who had malignant neoplasms at the time of death.²¹ In another comparative study performed on the frequency of intraocular metastases in patients who had died of cancer, the authors found microscopic metastases in 12.6% of cases and gross metastasis in 1%.⁹ Ocular involvement in metastatic disease primarily occurs in the uvea, mainly in the choroid, with spreading to the ciliary body and iris being relatively rare.^{22,23} However, iris and anterior chamber involvement has been described in metastatic solid cancers, such as malignant melanoma,^{24,25} renal cell carcinoma,^{26,27} and gastric carcinoma,²⁸ as well as in circulating hematologic malignancies such as leukemia.^{13,29,30} Occurrence of benign disorders such as secondary amyloidosis in the corneal graft after transplantation has been reported.³¹ Sarcoid nodules mimicking iris tumors have been reported in a patient where the graft tissue was derived from a donor with metastatic renal cell carcinoma.³² It is to be noted that donors with hematologic malignancies (leukemia and lymphoma), primary anterior segment tumors and retinoblastoma are excluded for graft purposes by eye bank associations worldwide.

The next important requirement is the adherence of malignant cells on the endothelium within the 7- to 9-mm trephination zone. Corneal avascularity makes the transmission of blood-borne viable tumor cells in metastatic disease very unlikely. In our study, cellular adherence was observed preferentially close to the trabecula, a zone devoid of endothelial cells, for most cell lines. This suggests that cancer cells are more likely to adhere to the trabecular collagen matrix and less likely to adhere to the intact endothelium. This finding could partly explain the extreme rarity of cancer transmission, since only the central 7 to 9 mm of donor cornea is trephined for surgical use. We also observed that cancer cells were more likely to adhere to areas of exposed DM. The DM adjacent to the endothelium is composed of collagen IV and VIII and fibronectin, which are known to favor cell adhesion.³³ The scientific corneas used in the study had already been stored for more than a week before implantation of cancer cells and therefore inevitably had endothelial cell loss, especially in the folds induced by hypotony and stromal swelling during OC.³⁴

The next vital point required for transmission is adequate cell viability during corneal storage. Two of the long-term retrospective studies of 73 and 47 recipients of grafts from

TABLE 4. HLA Analysis Results for Adenocarcinoma Breast (Cal51) Cell Line

Cal51	Reactive Probes for Loci A	HLA A	Concordance	Reactive Probes for Loci B	HLA B	Concordance
Reference alleles	1, 7, 8, 11, 12, 15, 16, 19, 24, 25, 26, 28, 30, 31, 36, 38, 39, 40	A*24, *29	—	6, 10, 14, 18, 25, 29, 30, 46, 48, 49, 53, 57	B*44	—
Descemet's membrane seeded with 1000 cells	1, 2, 3, 5, 7, 8, 11, 12, 14, 15, 16, 19, 22, 25, 26, 31, 34, 36, 38, 39, 40	A*11, *24†	83.3%	3, 6, 7, 8, 10, 13, 14, 15, 16, 18, 19, 23, 25, 26, 28, 29, 30, 35, 43, 44, 46, 48, 49, 53, 54, 56, 57	B*27, *35	100%
Primary culture	1, 7, 8, 11, 12, 15, 16, 19, 24, 25, 26, 28, 30, 31, 36, 38, 39, 40	A*24, *29	100%	6, 10, 14, 18, 25, 29, 30, 46, 48, 49, 53, 57	B*44	100%

Figures in bold represent probes positive for both the donor cornea and cancer cell line.

* International HLA nomenclature style.

† HLA allele common to cornea and cancer cell line.

donors dying of systemic malignancy were from the United States where donor tissue is stored hypothermically (4°C).^{3,5} In two other series reported from Europe, donor tissue was preserved either by OC ($n = 40$)² or in a moist chamber ($n = 19$).⁴ None of these studies reported any case of transmission, even on long-term follow-up ranging up to 29 years. Both the commercially available media used for corneal OC (CorneaPrep/Max; Eurobio, and Inosol; Chauvin-Opzia, Toulouse, France) contain only 2% fetal bovine serum instead of the 10% required during standard cell culture. Corneal preservation is maintained at 31°C for a maximum quarantine period of 1 month in sealed containers with no access to CO₂. Tissue deswelling in medium containing 4% to 8% dextran, mechanical factors like fluid turbulence during transport, vertical suspension with a suture during conservation and repeated preoperative rinse contribute to detachment of loosely adherent cells, if any, on the endothelial surface. All these factors in combination should explain the poor cell viability during OC. However, since OC was used in at least two of the reported cases of transmission, we were interested in experimentally addressing the questions of cell adherence and viability during OC.

Our *in vitro* and *ex vivo* experiments demonstrate that under laboratory conditions, metastatic cancer cells adhere to the inner face of donor corneal tissue and retain the capacity to survive and proliferate during storage in OC. Cell lines differ in their viability potential and the pattern of adherence to corneal endothelium. Adherence was predominant in the corneal periphery but also noted on the central cornea depending on the cell type. Signs of proliferation like postmitotic changes in the nuclei and formation of tight clusters consisting of rapidly multiplying daughter cells were observed in various cell lines. Cancer cells, despite their enormous metabolic demand were surprisingly able to survive after 2 weeks in stringent conditions of OC. A switching mechanism is known to exist that triggers profound readjustment of tumor cell metabolism when proliferative activity begins to stagnate, and that is likely to initiate other, yet unidentified, energy-consuming processes.³⁵ They also probably benefited from the microenvironment offered by the corneal tissue itself as is evident from the fact that cell survival *ex vivo* was more remarkable than that *in vitro*. Although histopathology and immunohistochemistry were positive only with large inoculates (studies of cross sections cannot screen the whole cornea), cell tracking (observation of the whole surface in a flatmounted cornea) demonstrated cancer cell adherence, even for a weak inoculum of 1000 cells. It is to be noted that of 1000 cells initially inoculated, only a small fraction adhered to donor endothelium after 6 hours of incubation under standard conditions. Positive HLA matching studies on DM demonstrated adherence of such a tiny cell load on the endothelium. The successful isolation of cancer cells on primary culture provided a conclusive evidence of the viability of these adherent cells.

The membrane marker PKH67 is reported not to have any effects on cell adhesion or proliferation.³⁶ Dividing cells show progressive decrease of fluorescence intensity, since daughter cells share the stained surface of the parent cell. This explains the faint staining noted for rapidly multiplying clusters seen in various cell lines.³⁷ No contiguous migration of the dye to neighboring cells is known to occur.³⁸ In our experiment with Calu1 and Cal51 cells, the rare staining of hexagonal shaped cells with normal nucleus, looking similar to normal endothelial cells raises two possibilities: the phagocytosis of cancer cells debris by endothelial cells and/or some kind of "integration" between cancer and endothelial cells. The corneal endothelial cell, in human and animal models is known to display phagocytic function and is one of the proposed mechanisms for retrocorneal pigmentation in pigment-dispersion syndromes.³⁹⁻⁴¹ The presumed integration of cancer cells bearing

chromatin anomalies like giant or dividing nuclei into the corneal endothelial cell with preservation of the hexagonal cell margins for the lung carcinoma cells is extremely interesting. Human corneal endothelium is known to express adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and nerve cell adhesion molecule (NCAM), but whether this could explain this phenomenon is not entirely clear.^{42,43} We also do not know if such integration was possible because of adherence in a zone of denuded endothelium or due to their individual invasive potential linked to function of collagenases and matrix metalloproteinases.^{44,45}

Whether metastatic cells adhere to corneal endothelium *in vivo* is not yet known. The three reported cases of transmission could indicate that they probably did. However, the PCR tests performed to establish transmission in two recent cases had obvious pitfalls. In a case reported by McGeorge et al.⁷ regarding development of a presumed metastasis 19 months after the corneal graft, evidence was based on the presence of a single matching allele (3.0) between the donor and the tumor whereas concomitant absence of another allele 1.2 (expressed by the donor but lacking in the tumor) was not accounted for. In the case reported by Florén et al.,⁸ although the tumor contained foreign HLA DR alleles, PCR studies could not be performed on the donor because of the lack of available tissue samples. Thus, absolute proof of transmission remains to be established.

Although our results may appear to lead to conflict with the clinical experience, we have to interpret them judiciously in view of several factors not yet addressed. First, all tests were *ex vivo*, hence excluding a very vital parameter, the host immune mechanism. Next, the cell concentrations used in our experiments were arbitrary because no clinical data exist on the likely aqueous cell load in metastatic disease. Preserved corneas with possible endothelial cell depletion could have artifactually facilitated cell adhesion. The anterior chamber constitutes a dynamic environment due to the presence of aqueous currents, temperature variations, and continuous aqueous circulation, in contrast to the static conditions of incubation at 37°C during cell-seeding procedures that facilitate adherence. To investigate the possibility of adherence to and survival of cancer cells on human corneal endothelium *in vivo*, animal experimentation is needed to simulate the conditions inside the anterior chamber. Cell tracking procedures in the form of corneal grafts seeded with PKH-labeled or eGFP-transfected tumor cells in animal models could indicate the fate of these cells in the face of host immune mechanisms. Extending the study to other cancer cell lines as well as further reduction of the inoculate volume would be necessary, to study the individual survival and proliferation pattern in OC conditions in a more clinically significant context. The absence of reports of corneal endothelium-mediated metastasis in humans to date is an argument in favor of such studies.

In conclusion, although in laboratory conditions cancer cells adhere to donor corneal tissue stored in OC and subsequently survive and retain proliferative capacity, the course of events *in vivo* remains yet to be determined. In the absence of any absolutely confirmed case of transmission, our results should not bring into question the current clinical practice of harvest of donor tissue from deaths due to solid organ malignancies. However, in view of our results, we recommend thorough examination of the iris and anterior chamber structures during tissue harvest as well as careful examination of the endothelial surface before surgery. The use of good-quality donor corneas with intact endothelium would further lower the risk of tumor cell adhesion. Further research should be designed to investigate possible cell adherence *in vivo* and subsequent survival in the recipient—the two major obstacles that must be overcome for the transmission of cancer.

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