Fluorescent In Situ Hybridization (FISH) on Corneal Impression Cytology Specimens (CICS): Study of Epithelial Cell Survival after Keratoplasty

Muriel Catanese,1,2 Cornel Popovici,3 Hélène Proust,2 Louis Hoffart,2 Frédéric Matonti,3 Isabelle Cochereau,1 John Conrath,2 and Eric E. Gabison1

PURPOSE. To assess corneal epithelial cell survival after keratoplasty.

METHODS Corneal impression cytology (CIC) was performed on sex-mismatched corneal transplants. Fluorescent in situ hybridization (FISH) with sex chromosome-specific probes was performed to identify epithelial cell mosaicism and therefore allocate the donor or recipient origin of the cells. Twenty-four samples of corneal epithelial cells derived from 21 transplanted patients were analyzed. All patients received post-operative treatment using dexamethasone eye drops, with progressive tapering over 18 months, and nine patients also received 2% cyclosporin eye drops.

RESULTS. Out of the 24 samples reaching quality criteria, sex mosaicism was found in 13, demonstrating the presence of donor-derived cells at the center of the graft for at least 211 days post keratoplasty. Kaplan-Meier analysis established a median survival of donor corneal epithelial cells of 385 days. Although not statistically significant, the disappearance of donor cells seemed to be delayed and the average number of persistent cells appeared to be greater when 2% cyclosporine was used topically as an additional immunosuppressive therapy.

CONCLUSIONS. The combination of corneal impressions and FISH analysis is a valuable tool with negligible side effects to investigate the presence of epithelial cell mosaicism in sex-mismatched donor transplants. Epithelial cells survived at the center of the graft with a median survival of more than one year, suggesting slower epithelial turnover than previously described. (Invest Ophthalmol Vis Sci. 2011;52:1009–1013) DOI:10.1167/iovs.10-5394

Numerous animal models and human studies have tried to assess corneal epithelial cell turnover, particularly after transplantation. These studies presented conflicting results, some demonstrating that the grafted epithelium was quickly replaced by the recipient epithelium within few weeks,1–5 while others suggested long-term survival of the donor epithelium over several months or years.6–7 The advance of techniques assessing human corneal epithelial cell renewal allowing better accuracy led to the consensus that the grafted epithelium is gradually replaced after transplant. These techniques include analysis of sex chromatin in histology samples,8–9 sex chromosomes determination by fluorescent in situ hybridization (FISH) in sex-mismatched grafts,10–13 and DNA fingerprinting to determine the presence of epithelial cells from multiple origin (recipient or donor) in any transplanted corneas.14–16 These studies agree that the grafted epithelium is gradually replaced after transplant.

Several theories have been proposed to explain central epithelial cell renewal. Thoft theory was based on the XYZ hypothesis, wherein corneal epithelial cells renewal result from conjunctival cells centripetal migration.17–19 The current model localizes corneal epithelial stem cells in the limbus. Homeostasis of the corneal epithelium is believed to be maintained through a balance between the proliferation of transient amplifying cells derived from stem cells strictly localized in the basal layer of corneal limbus and the desquamation of the cells at its surface.20,21 However, using different animal models, Majo et al.21 demonstrated that there are epithelial stem cells in the central cornea of mammals that renew the corneal epithelium in physiology. However, in humans, the model of corneal stem cells only located in the limbus is still used and the physiological renewal of human central corneal epithelium remains a puzzling question and would require additional investigation to understand the complete corneal epithelium stem cell story.22

The purpose of this study was to assess central corneal epithelial cell survival after sex-mismatched keratoplasty. Fluorescent in situ hybridization (FISH) was performed on human corneal impression cytology specimens (CICS) taken at different time points after keratoplasty and statistical analysis was performed to extrapolate central corneal epithelial cell survival in living patients.

MATERIALS AND METHODS

Patients

Our research adhered to the tenets of the Declaration of Helsinki and this study has been conducted with the approval from the Marseille University ethics committee and after obtaining patient informed consent. Among the 114 patients who received corneal transplants between January 2007 and June 2008 in the department of ophthalmology, Timone Hospital in Marseille, France, 52 patients received a sex-mismatched graft (donors whose sex was different from that of the recipient). Of these 52 patients, 35 (16 women, 19 men) were enrolled in this study. (The others refused to participate or could not be
Three patients had multiple CICs to assess donor cells survival within the same individual.

### Statistical Analysis

Survival (persistence of donor cells) was estimated by Kaplan-Meier analysis. The effects of different factors, including use of 2% cyclop- sone, fluorometholone drops, post-operative Day 1 epithelial defect, and age of the donor, on survival were first studied by univariate analysis: log-rank test (for qualitative cofactors) and Cox regression (for quantitative cofactors). A multivariate analysis was also performed by the Cox model (Cox proportional hazard model, forward stepwise analysis based on likelihood ratio). Finally, the link between the average number of residual cells and the different cofactors was tested by the Wilcoxon nonparametric test. The P value considered to be significant was P < 0.05.

### Surgical Indications

The indications of corneal transplants are described in Table 1. The main indication was the nonimmune failure of previous transplants, followed by endothelial dystrophies, keratoconus, and infectious keratitis (mainly herpes).

### Surgical Procedure

All grafts were obtained from the French Grafting Institute (Marseille). Before grafting, banking time, at 31°C in organ culture, did not exceed 10 days before postoperative cell count and graft descemet. Eight of the patients underwent penetrating keratoplasty (PK), of which twelve were carried out in a conventional way, one was combined with an anniotic membrane transplantation (PK + AMT), and five were made using a femtosecond laser (Femtec 20/10 Perfectvision, Nidek; Heidelberg, Germany) for the trephination of both donor and recipient (PK FEMTEC). Three patients had a deep anterior lamellar keratoplasty (DALK), of which two were carried out manually and one using the same femtosecond laser (FEMTEC DALK). The trephinations were central in all cases. The diameter of the donor cornea ranged from 7.0 to 8.5 mm. All the grafts were performed using a combination of 16-bites edge-to-edge running sutures and eight interrupted single sutures.

Post-operative Day 1, five patients had a central or paracentral corneal epithelial defect (total in patient 2), which healed within 1 to 3 days.

All patients received post-operative treatment using anti-inflammato- tory steroid eyedrops (dexamethasone, 1 mg/mL) three times a day for 6 months, plus artificial tears (carbomer) three times a day for 6 months. Nine patients also received 2% topical cyclosporin (qid), due to previous graft failures (eight patients), and to a past history of Goujrot-Sjogren syndrome (one patient, case No. 8).

### Fluorescent In Situ Hybridization

After oxybuprocain topical anesthesia and saline eye wash, corneal impression cytology were performed by affixing a 16-mm glass slide on the central cornea during slit-lamp examination. The cover slips were then glued on glass slides and stored at 20°C until use. Prior staining, the cells were fixed for 5 minutes at room temperature in a 3:1 ethanol/acetic acid solution. To facilitate probe penetration, the samples were treated with 0.01N hydrochloric acid–pepsin solution for 10 minutes at 37°C.

The slides were then washed twice for 5 minutes in a PBS solution at room temperature followed by a rinse of 5 minutes in a PBS-MgCl2 solution. Post-fixation was made in 1% formaldehyde in PBS-MgCl2, for 10 minutes at room temperature. The slides were then washed in PBS for 5 minutes and air dried, after which they were immersed in three successive ethanol baths (70, 85, and 100%) for three minutes.

To identify the sex chromosomes, two commercial probes (CEP-X/Y; Abbott Molecular, Abbott Laboratories, Des Plaines, Illinois) were used: the centromeric probe specific to X chromosome (DXZ1) and the CEPI (DYZ1, satellite III DNA), which recognizes the heterochromatic region of the long arm of the Y chromosome (DYZ1). The probe solution was then deposited on the glass coverslip and sealed. The glass slides were heated to 73°C on a hot plate for 3 minutes to denature the DNA and then incubated overnight at 37°C in a humid atmosphere. After hybridization the covers were removed and the slides washed in a solution 0.4X SSC, 0.01% Tween for 2 minutes at 73°C and a solution 1X SSC for one minute at room temperature. The samples were counterstained with DAPI (Abbott Molecular, Abbott Laboratories) and examined by fluorescence microscopy (Zeiss Axios- phot microscope; Zeiss, Germany).

The centromeric probe of the X chromosome (DXZ1) emitted a red signal and satellite probe III, which recognizes the heterochromatic region of the long arm of the Y chromosome (DYZ1) emitted a green signal. Only the nuclei of cells containing two fluorescent spots were counted: two red signals in female cells, a green and a red signal in male cells (Fig. 1). An average of 67 nuclei per patient was counted.

### RESULTS

Patient's characteristics, clinical details including donor and recipient ages, surgical indication, past-history of post-operative epithelial defect after transplant, post-operative treatments, and FISH on CICs results are included in Table 1.

Grafts had no past-history of corneal rejection and were clear at the time of sampling, with no apparent inflammation. The discomfort experienced by the patient was minimal (2 on an analogic scale ranging from 1 to 10) and lasted for no more than 24 hours.

Out of the 40 samples taken by CIC, 16 could not be analyzed, as five samples were cell free and 11 samples were met with technical problems during the various stages of the FISH procedure. The average age of the 21 patients was 56 years (12 to 84 years) and the mean follow-up time was 167 days (from 2 to 497 days). Of the 24 CIC samples that had been analyzed, cell nuclei of the opposite sex were found on thirteen cases, demonstrating the presence of cells derived from the donor (Fig. 1). The degree of sex mosaicism ranged from 1.4 to 53.8% (Table 1). Donor cells were detectable for at least 211 days after keratoplasty. The eleven other corneal impression cytology specimens showed only chromosomes from the host. Kaplan-Meier survival analysis was established (curve 1) and a median survival of 385 days (SD = 87.60) was estimated. In univariate analysis of the different cofactors effects, the disappearance of donor cells seemed to occur at a later stage in patients who received 2% cyclosporine (curve 2). Furthermore, the average number of residual donor cells seems to be more important when using 2% cyclosporine (5% vs. 12%) (Table 2), however the difference in survival did not reach statistical significance (P = 0.711 and 0.481, respectively), probably due to low statistical power of our study (small size).

The presence of post-operative ulcer in D1 and age of the donor were not significantly related to the persistence of donor cells.

### DISCUSSION

In this study, we show that corneal impression cytology using glass cover slips was technically appropriate, fast, easy and reproducible. This technique allows the collection of epithelial cells in a very minimally invasive way (comparable to Goldman applanation), with no detectable side effects noted for over 1 year after the study.

FISH analysis was conducted successfully on CICs in sex-mismatched transplants. It is a highly sensitive technique.
## Table 1. Characteristics of Patients and Results of FISH Analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)/Sex</th>
<th>Type of Graft</th>
<th>Indication</th>
<th>Ulcer at D1</th>
<th>2% Cyclosporine</th>
<th>Follow-up Days</th>
<th>Cells Counted</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>43/M 70/F</td>
<td>PK</td>
<td>Keratoconus, second graft</td>
<td>N</td>
<td>Y</td>
<td>2</td>
<td>80</td>
<td>70 10 12.5% D</td>
</tr>
<tr>
<td>1b</td>
<td>43/M 70/F</td>
<td>PK</td>
<td>Keratoconus, second graft</td>
<td>N</td>
<td>Y</td>
<td>2</td>
<td>25</td>
<td>18 7 28% D</td>
</tr>
<tr>
<td>2</td>
<td>30/M 63/F</td>
<td>DALK</td>
<td>Keratoconus</td>
<td>Y</td>
<td>N</td>
<td>2</td>
<td>100</td>
<td>100 0 R</td>
</tr>
<tr>
<td>3</td>
<td>76/F 82/M</td>
<td>PK</td>
<td>Infectious keratitis</td>
<td>N</td>
<td>N</td>
<td>9</td>
<td>18</td>
<td>18 0 R</td>
</tr>
<tr>
<td>4</td>
<td>80/F 50/M</td>
<td>KT FEMTEC</td>
<td>Endothelial graft failure</td>
<td>N</td>
<td>N</td>
<td>22</td>
<td>115</td>
<td>85 30 26% D</td>
</tr>
<tr>
<td>5a</td>
<td>50/M 70/F</td>
<td>PK</td>
<td>Endothelial dystrophy</td>
<td>N</td>
<td>N</td>
<td>29</td>
<td>123</td>
<td>86 37 30% D</td>
</tr>
<tr>
<td>5b</td>
<td>50/M 70/F</td>
<td>PK</td>
<td>Endothelial dystrophy</td>
<td>N</td>
<td>N</td>
<td>87</td>
<td>143</td>
<td>138 5 3.5% D</td>
</tr>
<tr>
<td>6</td>
<td>24/M 35/F</td>
<td>PK</td>
<td>Keratoconus</td>
<td>N</td>
<td>N</td>
<td>29</td>
<td>36</td>
<td>35 1 2.7% D</td>
</tr>
<tr>
<td>7</td>
<td>51/M 85/M</td>
<td>PK FEMTEC</td>
<td>Endothelial dystrophy</td>
<td>N</td>
<td>N</td>
<td>29</td>
<td>16</td>
<td>11 5 31.2% D</td>
</tr>
<tr>
<td>8</td>
<td>77/F 80/M</td>
<td>PK</td>
<td>Cornea guttata</td>
<td>Y</td>
<td>Y</td>
<td>50</td>
<td>75</td>
<td>50 25 33.3% D</td>
</tr>
<tr>
<td>9a</td>
<td>47/F 18/M</td>
<td>PK</td>
<td>Infectious keratitis, second graft</td>
<td>N</td>
<td>Y</td>
<td>73</td>
<td>39</td>
<td>18 21 53.8% D</td>
</tr>
<tr>
<td>9b</td>
<td>47/F 18/M</td>
<td>PK</td>
<td>Infectious keratitis, second graft</td>
<td>N</td>
<td>Y</td>
<td>157</td>
<td>65</td>
<td>45 20 30.8% D</td>
</tr>
<tr>
<td>10</td>
<td>77/M 70/M</td>
<td>PK FEMTEC</td>
<td>Cornea guttata</td>
<td>N</td>
<td>N</td>
<td>85</td>
<td>70</td>
<td>70 0 R</td>
</tr>
<tr>
<td>11</td>
<td>16/M 18/F</td>
<td>PK</td>
<td>DALK failure</td>
<td>N</td>
<td>N</td>
<td>116</td>
<td>13</td>
<td>13 0 R</td>
</tr>
<tr>
<td>12</td>
<td>81/F 75/M</td>
<td>PK</td>
<td>Endothelial graft failure</td>
<td>Y</td>
<td>N</td>
<td>169</td>
<td>71</td>
<td>70 1 1.4% D</td>
</tr>
<tr>
<td>13</td>
<td>73/M 72/F</td>
<td>PK FEMTEC</td>
<td>Endothelial Dystrophy, second graft</td>
<td>Y</td>
<td>Y</td>
<td>192</td>
<td>66</td>
<td>63 3 4.5% D</td>
</tr>
<tr>
<td>14</td>
<td>37/M 74/F</td>
<td>DALK</td>
<td>Keratoconus</td>
<td>N</td>
<td>N</td>
<td>211</td>
<td>20</td>
<td>19 1 5% D</td>
</tr>
<tr>
<td>15</td>
<td>37/M 37/M</td>
<td>PK + AMT</td>
<td>Endothelial dystrophy, second graft</td>
<td>N</td>
<td>Y</td>
<td>307</td>
<td>11</td>
<td>11 0 R</td>
</tr>
<tr>
<td>16</td>
<td>57/M 58/M</td>
<td>PK FEMTEC</td>
<td>Viral keratitis</td>
<td>N</td>
<td>N</td>
<td>323</td>
<td>41</td>
<td>41 0 R</td>
</tr>
<tr>
<td>17</td>
<td>76/M 69/M</td>
<td>PK</td>
<td>Endothelial dystrophy, second graft</td>
<td>Y</td>
<td>Y</td>
<td>385</td>
<td>124</td>
<td>124 0 R</td>
</tr>
<tr>
<td>18</td>
<td>84/F 82/M</td>
<td>PK</td>
<td>Endothelial Dystrophy, second graft</td>
<td>N</td>
<td>Y</td>
<td>399</td>
<td>&gt;200</td>
<td>&gt;200 0 R</td>
</tr>
<tr>
<td>19</td>
<td>78/F 74/M</td>
<td>PK</td>
<td>Endothelial dystrophy</td>
<td>Y</td>
<td>N</td>
<td>400</td>
<td>38</td>
<td>38 0 R</td>
</tr>
<tr>
<td>20</td>
<td>73/F 78/M</td>
<td>PK</td>
<td>Endothelial Dystrophy, second graft</td>
<td>N</td>
<td>Y</td>
<td>400</td>
<td>25</td>
<td>25 0 R</td>
</tr>
<tr>
<td>21</td>
<td>12/F 34/M</td>
<td>DALK</td>
<td>Viral keratitis</td>
<td>N</td>
<td>N</td>
<td>497</td>
<td>&gt;100</td>
<td>&gt;100 0 R</td>
</tr>
</tbody>
</table>

R, recipient; D, donor; M, male; F, female; PK, penetrating keratoplasty; DALK, deep anterior lamellar keratoplasty; AMT, amniotic membrane transplant; Y, yes; N, no.
which allows distinguishing accurately the donor or the recipient origin of the collected cells.

FISH has already been used to evaluate cell survival using sampling techniques other than CIC. Shimazaki et al.\textsuperscript{26} sampled 0.5 mm of donor epithelium with fine forceps during slit lamp examination. Although the method allowed the collection of 20 to 50 cells, it is likely to be more traumatic for the transplant than CIC and the samples only concerned a small area of the graft. Egarth et al.\textsuperscript{10} obtained the corneal epithelial cells by gently smearing the nylon sutures onto a glass slide at the time of suture removal. While the number of cells harvested was greater, this technique can only analyze the periphery of the graft and does not differentiate epithelial cells from keratocytes. The corneal impression cytology technique that we used in this study permits the collection of epithelial cells detached from the surface of the graft in the central cornea, reflecting a more homogeneous population of grafted epithelial cells.

On the other hand, other methods of analysis on CIC on filters have also been described by other authors including the analysis by DNA polymorphism and polymerase chain reaction (PCR)\textsuperscript{27} or by genetic fingerprint of DNA with microsatellites.\textsuperscript{14,15} These molecular biology based procedures endow a risk of contamination and misdiagnosis.

The corneal epithelium renewal rate has been estimated by various experimental approaches with conflicting results.\textsuperscript{22} While some suggest this rate to be about two weeks,\textsuperscript{1,2,28–30} the epithelial replacement after corneal transplants in animal models was reported by others to last from 12 weeks to 6 months. Using autoradiography after intravitreal injection of 3H-thymidine in the rabbit, Haddad et al.\textsuperscript{31} demonstrated that corneal epithelial cells are still tagged after 90 days of experiment. Khinoshita et al.\textsuperscript{8} have studied in rabbit the survival of donor corneal epithelium by analyzing sex chromatin after lamellar keratoplasty. They noted that the donor cells survived up to 12 weeks after surgery.\textsuperscript{3} This long-term survival of donor epithelial cells had been predicted by Khodadoust\textsuperscript{6} and Silverstien\textsuperscript{7} based on clinical observation of epithelial rejection. Indeed, epithelial rejection frequently starts at the edge of the graft and usually occurs 6 months (or later) after corneal transplants. Other investigations using FISH analysis assessed the long term survival of donor cells in human corneas. While Kobayashi et al.\textsuperscript{13} evaluated the maximum survival of the donor’s epithelium at 2 months, Wollensak et al.\textsuperscript{11} performed FISH analysis on explanted corneal transplants and reported the complete replacement of the epithelium within the first 11 months after keratoplasty. In a more recent study, Lagali et al.\textsuperscript{32} showed that as early as 3 months after transplantation, donor epithelial cells were completely replaced by the recipient epithelial cells.

Analysis of samples collected at different times after the transplant suggests a slow and gradual decrease of donor epithelial cells over time. Kaplan-Meier analysis of our results revealed that the median of donor epithelial cell survival was 385 days. This may still represent an underestimation of CEC survival as the technique only takes into account the most superficial cells, donor epithelial cells could be present deeper in the epithelial compartment. Additionally, corticosteroids were progressively tapered 6 months post-operatively, therefore asymptomatic epithelial cell rejection may take place after this, favoring the progressive replacement of donor cells by recipients epithelial cells. Although cyclosporine topical treatment was only used in high risk keratoplasties, the survival rate of corneal epithelial cells did not differ from the group of patients at lower risk of rejection. Future studies investigating a greater number of pa-
TABLE 2. Percentage of Persistent Cells Depending on the Use of 2% Cyclosporine

<table>
<thead>
<tr>
<th>Percentage of Cells</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>Least</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.31</td>
<td>0.33</td>
</tr>
<tr>
<td>Valid, n</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Variance</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>

patients treated by cyclosporine and topical steroids are needed to confirm the protective role of this combination on donor epithelial cell survival.

Majo et al. (Majo F, et al. J OVS 2005;46:ARVO E-Abstract 2087; Majo F, et al. J OVS 2006;47:ARVO E-Abstract 3018) recently demonstrated in several animal models, that the central corneal epithelium had a great capacity for regeneration over a period of several months. They subsequently demonstrated that the central corneal epithelium contained stem cells which permitted self-renewal. A more recent study equally brought to light that the central corneal epithelium had a capacity to proliferate and migrate at least as actively as the paralimbic region even after ablation of the limbus. We therefore hypothesized that in human, donor epithelial stem cells or long surviving transient amplifying cells could persist for months or years on the recipient ocular surface, their disappearance may be due to chronic asymptomatic rejection. In conclusion, we demonstrate that epithelial cells survived at the center of the graft for at least 7 months with a median reaching more than one year. Asymptomatic epithelial rejection may be responsible for the progressive replacement of donor epithelial cells by the cells of the recipient. We also show that FISH on CIC is minimally invasive, easy and reliable test to apply after penetrating keratoplasty or lamellar limbal graft. It can also be used to investigate epithelial cell fate and the role of immunosuppressive drugs in grafted patients.

References