Evidence of Long-term Survival of Donor-Derived Cells after Limbal Allograft Transplantation

Jun Shimazaki,1 Minako Kaido,1 Naosbi Shinozaki,1 Sbigeto Shimmura,1 Batmunkh Munkbat,2 Masao Hagihara,2 Kimiyosbi Tsuji,2 and Kazuo Tsubota1

PURPOSE. Severe destruction of the corneal limbus causes conjunctival invasion and subsequent visual loss. Limbal allograft transplantation (LAT) was recently proposed for the treatment of these disorders. However, whether the method functions as a stem cell transplantation of the corneal epithelium remains unclear. This study provided evidence that donor-derived corneal epithelial cells survive long after LAT.

METHODS. Epithelial cells on the paracentral cornea in patients who have undergone LAT were subjected to fluorescence in situ hybridization (FISH) and polymerase chain reaction restriction fragment length polymorphism (RFLP) analysis. X and Y chromosomes were detected using sex chromosome–specific probes in the FISH analysis, and HLA-DPB1 antigens were examined in the RFLP analysis. Eyes receiving conventional penetrating keratoplasty (PKP) served as controls.

RESULTS. Donor-derived epithelial cells were detected in three of five eyes (60.0%) in the FISH analysis and in seven of nine eyes (77.8%) in the RFLP analysis. Among these eyes, one and three eyes in the FISH and RFLP analysis, respectively, had both donor- and recipient-derived cells. In control PKP eyes, none of the eyes in the FISH analysis and one of eight eyes (12.5%) in the RFLP analysis had donor-derived cells.

CONCLUSIONS. These results suggest that donor-derived cells survive much longer after LAT than those after PKP, and that LAT may function as stem cell transplantation of the corneal epithelium.


From the 1Department of Ophthalmology, Tokyo Dental College, Chiba, Japan; and the 2Department of Transplantation Immunology, Tokai University School of Medicine, Kanagawa, Japan.

Submitted for publication July 7, 1998; revised January 22, 1999; accepted March 3, 1999.

Proprietary interest category: N.

Reprint requests: Jun Shimazaki, Department of Ophthalmology, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba, 272-3518 Japan.
Immune, Sandoz, Basel, Switzerland) was administered intravenously with a starting dose of 3 mg/kg for 1 week, with trough levels of 100 to 150 ng/ml maintained for at least 6 months. Topical antibiotics (0.3% ofloxacin, Tarivid; Santen Pharmaceutical, Osaka, Japan), corticosteroid (0.1% dexamethasone, Santen; Betason; Santen Pharmaceutical), autoserum dissolved in physiological saline, and 0.05% cyclosporin A dissolved in α-cyclodextrin were used five times a day. In patients who underwent PKP without LAT, systemic cyclosporin A was not used. Systemic administration of corticosteroid and/or topical cyclosporin A was used in some cases.

### Surgical Procedure for LAT

All surgical procedures were performed with patients under retrobulbar anesthesia. After excising inflamed conjunctiva and subconjunctival fibrosis, PKP was performed when the central cornea was opaque. The grafts were preserved in preservation media (Optisol GS, Chiron, Irvine, CA) for several days. Simultaneous cataract extraction and/or intraocular lens implantation; PEA, phacoemulsification and aspiration of the lens; IOL, posterior chamber intraocular lens extraction; AMT, amniotic membrane transplantation; PEA, phacoemulsification and aspiration of the lens; IOL, posterior chamber intraocular lens implantation; ASR, anterior segment reconstruction; AV, anterior vitrectomy; ACIOL, anterior chamber intraocular lens implantation; PED, persistent epithelial defect of the cornea; Conj, conjunctivalization.

#### Clinical Sampling

Epithelial cells in the paracentral cornea (approximately 0.5 mm in diameter) in the inferotemporal region were obtained using fine forceps. For the FISH analysis, cells were placed on a glass slide with saline, then dried, and fixed with absolute alcohol. For the RFLP analysis, the samples were placed in microtubes with physiological saline and kept frozen until analysis.

#### FISH

Samples slides were treated with 100 mg/ml RNase A in 2× SSC at 37°C for 1 hour, incubated with 0.01 N HCl containing 50 mg/ml pepsin for 10 minutes at 37°C, and fixed with 1% formaldehyde in phosphate-buffered saline (PBS) containing 50 mM MgCl₂. After washing with PBS, cells were dehydrated in an ethanol series and then denatured by incubating in 70% formaldehyde and 2× SSC at 71°C for 2 minutes.

FISH was performed with sex chromosome–specific dual color probe (CEP X SpectrumOrange/CEP Y SpectrumGreen; Vysis, Downers Grove, IL). Hybridization using each probe was carried out according to the manufacturer’s recommendation. The samples were counterstained with 4’6-diamino-2-phenylindole (DAPI). Signals from fluorescein isothiocyanate (FITC), rhodamine, the two color probes, and DAPI were visualized by fluorescence microscope (Nikon, Tokyo, Japan) with triple-band-pass filter. X chromosomes were visualized as

---

### Table 1. Patient Profile and Results of FISH Analysis

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/ Sex</th>
<th>Donor Gender</th>
<th>Original Disease</th>
<th>LAT Shape</th>
<th>Other Surgical Methods</th>
<th>Follow-up (Days)</th>
<th>Postoperative Complication</th>
<th>FISH Result</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73F</td>
<td>M</td>
<td>P-OCP</td>
<td>R</td>
<td>PKP, ICCE, AMT</td>
<td>348</td>
<td>XY:14, XX:1</td>
<td>Mix</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77F</td>
<td>M</td>
<td>OCP</td>
<td>R</td>
<td>PKP, ECCE, IOL, AMT</td>
<td>349</td>
<td>PED, Conj</td>
<td>XX</td>
<td>Recipient</td>
</tr>
<tr>
<td>3</td>
<td>69M</td>
<td>F</td>
<td>OCP</td>
<td>R</td>
<td>PKP, AMT</td>
<td>691</td>
<td>PED</td>
<td>XX</td>
<td>Donor</td>
</tr>
<tr>
<td>4</td>
<td>70F</td>
<td>M</td>
<td>FSA</td>
<td>C</td>
<td>PKP</td>
<td>504</td>
<td>PED</td>
<td>XY</td>
<td>Donor</td>
</tr>
<tr>
<td>5</td>
<td>69M</td>
<td>F</td>
<td>SJS</td>
<td>R</td>
<td>PEA, IOL, AMT</td>
<td>355</td>
<td>Conj</td>
<td>YY</td>
<td>Recipient</td>
</tr>
<tr>
<td>6</td>
<td>58M</td>
<td>F</td>
<td>BK</td>
<td></td>
<td>PKP</td>
<td>460</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30F</td>
<td>M</td>
<td>Scar</td>
<td></td>
<td>PKP</td>
<td>375</td>
<td>XY</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>24M</td>
<td>F</td>
<td>Scar</td>
<td></td>
<td>PKP</td>
<td>362</td>
<td>YY</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>84F</td>
<td>M</td>
<td>BK</td>
<td></td>
<td>PKP, IOL, ASR, AV</td>
<td>365</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>58F</td>
<td>F</td>
<td>Scar</td>
<td></td>
<td>PKP</td>
<td>429</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>30M</td>
<td>F</td>
<td>BK</td>
<td></td>
<td>PKP, IOL</td>
<td>277</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>81F</td>
<td>M</td>
<td>BK</td>
<td></td>
<td>PKP, ACIOL, ASR, AV</td>
<td>294</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>13*</td>
<td>15M</td>
<td>F</td>
<td>KC</td>
<td></td>
<td>PKP</td>
<td>399</td>
<td>XX</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>14*</td>
<td>15M</td>
<td>F</td>
<td>KC</td>
<td></td>
<td>PKP</td>
<td>630</td>
<td>YY</td>
<td>Recipient</td>
<td></td>
</tr>
</tbody>
</table>

C, C-shaped; R, ring-shaped; FSA, familial subepithelial amyloidosis of the cornea; OCP, ocular cicatricial pemphigoid; P-OCP, pseudo-OCP; SJS, Stevens-Johnson syndrome; BK, bullous keratopathy; LK, lipid keratopathy; KC, keratoconus; ICCE, intracapsular cataract extraction; AMT, amniotic membrane transplantation; PEA, phacoemulsification and aspiration of the lens; IOL, posterior chamber intraocular lens implantation; ASR, anterior segment reconstruction; AV, anterior vitrectomy; ACIOL, anterior chamber intraocular lens implantation; PED, persistent epithelial defect of the cornea; Conj, conjunctivalization.

* Right and left eyes of the same patient.
In brief, the reaction mixture containing 0.5 mg genomic DNA, 10 μl 10× PCR buffer, 10 μl solution containing dNTPs, mixed primers, 2.5 U Taq polymerase (TakaraTaq; Takara Shuzo, Shiga, Japan) and MilliQ water for a total volume of 100 μl, was incubated for 30 cycles of 96°C denaturation (60 seconds), 56°C annealing (60 seconds), and 72°C extension (120 seconds), followed by an additional extension (72°C, 5 minutes), in an automated oil-bath PCR thermal sequencer (model TSR-300; Iwaki Glass, Chiba, Japan). After PCR, 5-μl aliquots of the PCR product with 1.5 μl of gel loading buffer were checked for amplification by 10% acrylamide gel electrophoresis, and the allelic types were determined from the RFLP patterns.

**RFLP Analysis**

Genomic DNAs from the sample cells were extracted by the conventional phenol-chloroform method. HLA-DPB1 alleles were genotyped by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. A test typing kit (Sumitomo Metal Industries, Tokyo, Japan) was used. In brief, the reaction mixture containing 0.5 mg genomic DNA, 10 μl 10× PCR buffer, 10 μl solution containing dNTPs, mixed primers, 2.5 U Taq polymerase (TakaraTaq; Takara Shuzo, Shiga, Japan) and MilliQ water for a total volume of 100 μl, was covered with 1 drop of mineral oil in a 0.5-ml microcentrifuge tube. PCR amplifications were carried out by 30 cycles of 96°C denaturation (60 seconds), 56°C annealing (60 seconds), and 72°C extension (120 seconds), followed by an additional extension (72°C, 5 minutes), in an automated oil-bath PCR thermal sequencer (model TSR-300; Iwaki Glass, Chiba, Japan). After PCR, 5-μl aliquots of the PCR product with 1.5 μl of gel loading buffer were checked for amplification by 10% acrylamide gel electrophoresis in 0.5× TBE buffer in MUPID (Cosmo Bio, Tokyo, Japan). When the DNA sample was amplified for the target DPB1 loci, 10-μl aliquots of the PCR product were digested with enzyme solution, which contained 2 U restriction endonucleases and reaction buffer solution, for 3 hours to overnight at 37°C. Seven restriction enzymes (Bsp1280I, BssHII, Cfr13I, Ddel, EcoNi, FokI and Rsal) were used. Three milliliters gel loading buffer with proteinase K (Boehringer Mannheim, Mannheim, Germany) was added and incubated for 30 minutes at 37°C. The digested fragments were detected by 10% acrylamide gel electrophoresis, and the allelic types were determined from the RFLP patterns.

**RESULTS**

**Clinical Outcomes and Complications**

Although clarity of the central cornea improved in all eyes receiving LAT, various postoperative complications such as persistent epithelial defects or partial conjunctival invasion were noted. Eyes with conventional PKP were free from these complications in all but one eye, which had a persistent epithelial defect (Table 2).

**FISH Analysis**

Three of five samples contained epithelial cells with donor-derived chromosomes (Table 1). Figure 1A shows the fluorescence micrograph of a 69-year-old man with ocular cicatricial pemphigoid who had LAT from a female donor (case 5). All cells in the sample, which were collected at 99 weeks after surgery, showed two red fluorescence signals in each cell, indicating that these cells were of donor origin. Two other samples (cases 1 and 4) also showed the presence of donor-derived cells. In case 1, 14 of 15 cells collected showed both X and Y chromosomes (donor origin, Fig. 1B), and only one cell had two X chromosome signals (recipient origin). The remaining two cases had only host-derived cells, which corresponded to the two cases that had either post-
operative conjunctival invasion or corneal neovascularization. Cells from the nine PKP cases were all of host origin (Table 3; Fig. 1C).
plasty, and keratoepithelioplasty, are transplantations of transient amplifying cells and terminally differentiated cells.\textsuperscript{14} Therefore, it is understandable that donor epithelium is depleted within several months after surgery. The results of the present study in which donor epithelial cells were detected up to 30 months after LAT strongly suggest that LAT functions as a stem cell transplantation.

Although the clinical success of LAT is remarkable, previous studies failed to show the long-term survival of donor-derived cells after LAT. Swift et al.\textsuperscript{10} studied donor cell survival in rabbit limbal dysfunction models using sex-chromatin analysis. They showed that the number of epithelial cells positive for the Barr body (female origin) was not significantly different in female rabbits receiving LAT from male donors than in control animals. The most probable reason that we found more donor-derived cells than Swift et al. is the difference in postoperative treatment. Relatively strong postoperative immunosuppression was used in our study, whereas topical steroid was used only in a subgroup of animals.\textsuperscript{10}

Interestingly, in Swift et al., more donor-derived epithelial cells were observed in eyes receiving topical steroid than in those that did not. We believe that intense care against immunologic rejection is a key to longer survival of donor-derived epithelial cells. In human studies, Williams et al.\textsuperscript{9} investigated the survival of donor-derived epithelial cells in an LAT patient using short tandem-repetitive DNA polymorphism. They reported that donor-derived cells were detected at the 12th postoperative week but disappeared by the 20th week, despite strong systemic immunosuppression. One possible explanation is that they placed limbal grafts segmentally (from 4 to 8 o'clock and 10 to 2 o'clock). Host cells may therefore have invaded the central cornea through nasal and temporal openings of the limbal grafts. In our experience, a ring-shaped graft blocks conjunctival invasion to the cornea more efficiently than do segmentally placed grafts.

Interestingly, some of the samples in our series had both donor and recipient cells. It is not clear whether this coexistence of donor and recipient cells is a stable condition or represents slow but continuous replacement of donor-to-recipient cells. Recent studies show that persistence of donor-derived cells after organ transplantation is a relatively common phenomenon when molecular analysis is used (microchimerism).\textsuperscript{12,13,15} Whether the chimeric distribution on the cornea contributes to the graft acceptance in LAT, as is postulated in liver transplantation,\textsuperscript{15} is unclear. The limbal area of eyes undergoing LAT was totally covered by fibrous tissues before surgery that was completely excised during surgery. Therefore, recipient limbal stem cells are unlikely to survive, and recipient-derived cells found in the samples were presumably of host conjunctival epithelium origin. Phenotypic change of the conjunctival to corneal epithelium has been observed when limbal cells are totally destroyed.\textsuperscript{16} We did not find any differences in clinical outcomes, including the development of postoperative complications between eyes with or without donor-derived epithelial cells. More studies are needed to elucidate the clinical significance in donor cell survival.

In conclusion, the present study indicates that donor-derived corneal epithelium survived for up to 30 months after LAT, which is significantly longer than conventional keratoplasty. LAT is likely to function as a stem cell transplantation of the corneal epithelium.

Acknowledgments

The authors thank Mitsubishi Kagaku Bio-Clinical Laboratories for technical and advisory support.

References


Downloaded from iovs.arvojournals.org on 10/11/2019