Sex chromatin of donor corneal epithelium in rabbits

Shigeru Kinoshita, Judith Friend, and Richard A. Thoft

The survival of donor corneal epithelium was investigated in rabbits after they received unilateral 8 mm diameter lamellar keratoplasties with living donor tissue of a different sex from the host. Three weeks, 6 weeks, and 12 weeks postoperatively, cultured corneal epithelia, grown from 5 mm diameter central buttons, the adjacent 0.75 mm wide donor rings, the peripheral 10 to 13 mm diameter rings, and 5 mm diameter central host buttons were used for sex-chromatin analysis. The results indicated that some of the donor corneal epithelium survived up to 12 weeks postoperatively. Even in the absence of overt epithelial rejection, however, a time-dependent decrease of donor corneal epithelium and simultaneous invasion of host corneal epithelium were demonstrated.

Key words: lamellar keratoplasty, epithelial retention, sex chromatin, ocular surface, donor corneal epithelium, tissue culture, rabbit, centripetal movement of epithelium

In the past four decades the fate of the donor corneal epithelium has been explored by many investigators through a variety of methods. In general, the early work showed a rapid loss of the donor epithelium after grafting. For example, Dohlman, by means of P32 label, showed that the donor epithelium disappeared within 6 to 10 days. In 1969, however, Khodadoust and Silverstein, with use of very fresh donor tissue, demonstrated the survival of donor epithelium for up to 6 months in rabbits by means of immunological methods. Since then, other studies have also suggested long-term survival of donor epithelium in humans and animals for months and even years. Such studies have concentrated on determining retention or loss of the entire corneal epithelium, and the results are still not conclusive. There are no studies aimed at determining whether there is a gradual dilution of the epithelium postoperatively in cases where an overt epithelial rejection has not occurred.

One problem in the study of epithelial retention is that of finding an appropriate marker. Since the epithelium has a very short turnover time (7 to 11 days in rabbits), it is necessary to use a marker such as sex chromatin, Y chromatin, or HL-A antigen that is passed on in cell division and to develop an accurate, reproducible method to demonstrate that marker. Epithelial sex chromatin in cornea has been demonstrated by many authors, but unlike sex chromatin in oral mucosa, it is difficult to show corneal epithelial sex chromatin routinely and accu-
rately with standard histological methods. As a result there have been relatively few accurate applications of sex chromatin staining to epithelial retention studies. On the other hand, Basu et al. and others have successfully applied sex chromatin typing to studies of corneal stromal and endothelial retention.

Because of the increased interest in epithelial survival, both after grafting and in procedures such as conjunctival transplantation that are used in the treatment of ocular surface disease, there is a renewed interest in resolving the conflicting evidence of the past and in determining the extent of donor epithelial survival. This article presents the results of studies on the composition of the donor corneal epithelium for up to 12 weeks after lamellar keratoplasty in rabbits. The method we developed to show sex chromatin in cultured corneal epithelial cells is similar to the one that has been applied to studies of stromal and endothelial cell retention.

Materials and methods

Animal preparation and follow-up

Surgical procedure. Albino rabbits weighing 2.5 to 3.0 kg were used in all experiments. The anesthesia used for operation, removal of sutures, and taking of sample tissues was intramuscular ketamine hydrochloride with chlorpromazine hydrochloride maintained with ether inhalation. No topical anesthetics were used. For each set of operations, a pair of rabbits of different sex were used, and the corneal lamellar button taken from one rabbit was used as the donor button in the other rabbit.

After anesthesia, the eyeball was proptosed and secured. An 8 mm diameter trephine was used to cut the cornea to about half its depth. A razor blade was then used to make a sharp incision at the 10 o'clock position. This was followed by splitting the corneal stroma at one-half to two-thirds depth with a synechiae spatula. After a pocket was made in the cornea, the 8 mm circle was completely reincised with corneal scissors. The 8 mm diameter corneal buttons were immediately exchanged between the two rabbits and anchored on the host cornea with 12 interrupted sutures of 9-0 nylon. During operation, special attention was paid to avoiding damaging donor corneal epithelium. Antibiotic ointment was instilled into the operated eye immediately postoperatively. Antibiotic eye drops were sometimes employed on the first and second postoperative days but never thereafter. All sutures were removed with microscissors on the seventh day postoperatively. Steroids were never applied to any operated eye.

Observation. After staining with Richardson stain, the eyes were examined with the slit-lamp microscope immediately after surgery, every day for the first postoperative week, immediately after removal of sutures, twice a week for the subsequent 3 weeks, and once a week for the subsequent 2 months. Special attention was paid to signs of epithelial rejection, epithelial defect formation, and corneal neovascularization.

Sampling of corneal tissue. After 3, 6, or 12 weeks, corneal samples were taken. As shown in Fig. 1, the samples were a 5 mm diameter donor central button (D1), a doughnut-shaped ring of donor tissue between 5 and 6.5 mm in diameter (D2), a doughnut-shaped host corneal ring between 10 and 13 mm in diameter (H1), and a 5 mm diameter central corneal button from the host contralateral eye (H2). These tissues were immediately transferred to a vial containing 5 ml Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum at 4° C.

Tissue culture of corneal epithelium. The epithelial samples were established in tissue culture by the following techniques. The tissues were transferred from the vial to Lab-Tek culture slides (two-chamber slide; Lab-Tek Products, Naperville, Ill.) and were cultured in 0.5 ml of Dulbecco's modified Eagle's medium with 20% fetal calf serum in 5% carbon dioxide–balance air incubator at 37° C. Sixty-eight to 70 hr later, when an outgrowth of
Fig. 2. Cultured rabbit corneal epithelial cells stained with thionin after hydrolysis. A, Cells in the normal female sample show clear dense spots, which are condensed X chromatin (sex chromatin), just inside the nuclear membranes (long arrows). Larger cells such as the one in the left lower corner were eliminated for sex-chromatin counting, since they sometimes had more than two sex-chromatin bodies. B, Cells in the normal male sample show no such nuclear densities. Small arrow indicates an artifact, which proper focusing showed to be outside of the cell. (×2400.)
Sex-chromatin of donor corneal epithelium

Corneal epithelium had been obtained, the tissue explant was removed from the slide. The epithelial cells were continued in culture in 2 ml of Dulbecco's modified Eagle's medium with 2% fetal calf serum for 24 hr longer and were then fixed with 95% ethanol for 24 hr prior to staining. Sex-chromatin staining and counting. Air-dried slides with fixed cultured corneal epithelium were immersed in 1N HCl at 58° to 60° C for 7.5 min, followed by staining with 0.45% thionin solution (pH 5.7) for 60 min. Then the stained slides were washed with tap water, dehydrated with 70%, 95%, and 100% ethanol, immersed in xylene, and mounted.

Sex-chromatin frequency in epithelial samples were counted by two persons by a single masked method. Each person checked at least 200 nuclei under X400 magnification with a Zeiss microscope. The mean value of the percentage of sex chromatin containing nuclei counted by the two persons was used as the sex-chromatin frequency of the sample counted. Only cells in the periphery of the outgrowth of D1 and H2 were counted. In the outgrowth of D2, cells in the peripheral outside areas were counted, and in the outgrowth of H1 the peripheral inside areas were counted (Fig. 1).

[D-H] value. The [D-H] value is the absolute difference between the percentage of sex chromatin-containing cells in the donor button (D) and the percentage in the host contralateral eye (H2). This value was adopted to make it possible to correlate the data of a male donor graft on a female host with that of a female donor graft on a male host.

Results

Demonstration of sex chromatin. Fig. 2 shows cultured rabbit corneal epithelium stained with thionin after hydrolysis in non-operated female and males. In cells from female epithelium, sex chromatin was seen just inside the nuclear membranes (Fig. 2, A); male nuclei showed no such nuclear densities (Fig. 2, B).

Table I. Sex-chromatin frequency in cultured corneal epithelium in rabbits

<table>
<thead>
<tr>
<th>Sex</th>
<th>No.</th>
<th>% Sex chromatin–containing cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>10</td>
<td>39.3 ± 2.8</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>1.4 ± 0.5</td>
</tr>
</tbody>
</table>

*Mean value ± S.D.

Table II. Sex-chromatin frequency in cultured donor epithelial samples 3 weeks after lamellar keratoplasty

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Donor sex</th>
<th>5 mm diameter donor central circle (D1)</th>
<th>6.5 mm diameter donor central ring (D2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>1.5</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>44.3</td>
<td>40.9</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>47.4</td>
<td>43.1</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>38.7</td>
<td>40.5</td>
</tr>
</tbody>
</table>

Retention of donor epithelium

Experimental lamellar keratoplasty. Sixteen rabbits that had received unilateral 8 mm diameter lamellar keratoplasties with tissue from a rabbit of the opposite sex were used for sex-chromatin analysis of retention of donor epithelium. In those eyes, no epithelial defects were observed in the donor button except at the sutures immediately after surgery. Those defects were healed on the first or second postoperative day. Corneal vascularization was observed in the superior region on the third day and extended gradually; it rapidly disappeared after removal of sutures, however, and did not reach the donor-host border. Overt epithelial rejection, as characterized by an epithelial rejection line indicating epithelial loss and leukocyte infiltration, was not detected in our routine observations. These operated eyes were completely quiet at 12 weeks postoperatively.
Sex-chromatin frequency in the cultured donor corneal epithelium. Table II shows the sex-chromatin frequency in epithelial samples 3 weeks after lamellar keratoplasty. In D1, all five samples had sex-chromatin frequencies characteristic of the donor tissues. In D2, one (No. 2) of the five indicated some mixed characteristics of donor and host, and the other four had donor sex-chromatin frequency.

Table III shows sex-chromatin frequency 6 weeks after lamellar keratoplasty. One out of the four samples of the 5 mm diameter region (D1) had mixed characteristics (No. 7), and the other eyes had donor corneal sex-chromatin frequency. In the 6.5 mm diameter region (D2) of the same animal, the female and male cells showed approximately the same degree of mixing as in the 5 mm diameter sample.

Table IV shows sex-chromatin frequency 12 weeks after lamellar keratoplasty. In contrast to the data at 3 and 6 weeks (Tables II and III), four out of the seven 5 mm diameter samples (D1) had mixed donor and host characteristics (Nos. 12, 13, 15, and 16). Those four and one additional animal (No. 11) showed mixed sex characteristics in the more peripheral region (D2). However, none of the samples showed a complete replacement of donor epithelium by the host.

**Discussion**

Our data on epithelial retention show a gradual dilution of the donor corneal epithelium by host cells without any clinical signs of epithelial rejection, confirming the survival of at least some of the donor corneal epithelium for at least 12 weeks postoperatively. Previous studies by Khodadoust and Silverstein predicted complete retention of the donor epithelium based on the observation that induced epithelial rejection always started precisely at the margin of the graft. Since the area very close to the edge of the graft retained a significant proportion of...
donor cells even 12 weeks postoperatively, it is not surprising that epithelial rejection would start at the donor-host border. However, in the study by Silverstein et al., epithelial rejection of corneal xenograft was appreciably slower and milder at 3 months postoperatively than that in the first postoperative weeks, which might also imply a dilution of the donor corneal epithelium with time after surgery and not a complete epithelial retention.

Of considerable interest is the question of why this dilution occurs. We can postulate at least four explanations as follows: (1) The donor corneal epithelium is not viable, necessitating replacement by host cells. (2) There is subclinical donor epithelial rejection. (3) There is continuous random mixing of corneal epithelial cells, even in the normal eye, which results in a gradual decrease of donor cells on a graft in an operated eye. (4) There is a continuous centripetal motion of epithelial cells.

With regard to the viability of donor tissue, the tissue used in these experiments was very fresh. Less than 10 min elapsed between removal of the graft from one eye and placement of the first four anchoring sutures in the host bed. The sex-chromatin frequencies of the central corneal epithelial samples, 3 weeks postoperatively, were what would have been expected had no host infiltration occurred. This retention of donor epithelium for 3 weeks suggests that the grafted tissue was viable at the time of transplant. In addition, it has been shown in similar experiments that the basal cells of corneal epithelium in the donor button after lamellar keratoplasty can incorporate tritiated thymidine as early as the first postoperative day, and the rate of uptake is higher than normal for the first 2 months after grafting, returning to normal thereafter. These data all indicate that the donor epithelium is viable postoperatively. Although the possibility of mechanical injury to the donor epithelium, especially the peripheral cells, cannot be neglected, clinical observation with staining showed no punctate staining or defect in donor epithelium immediately after opera-

Fig. 3. Relationship between |D-H| value and postoperative time. |D-H| is expressed as the absolute mean value ± standard error, with the number of measurements in parentheses. The correlation coefficient (r) is calculated for each of three groups. The |D1-H2| values (○) show r = -0.521 (p < 0.05). For |D2-H2| values (●), r = -0.558 (p < 0.05), and for |H1-H2| values (●), r = 0.129 (p > 0.1). The |D-H| data from 6.5 mm diameter donor ring is statistically different from those of the 5 mm diameter donor central circle at 12 weeks postoperatively (p < 0.05).
the host. On the other hand, the Y-chromatin frequency in the peripheral host corneal epithelium in the operated eye was normal, suggesting no centrifugal movement of donor cells even though very rare X chromatin-containing cells were found. It is possible, of course, that such mixing might occur in cases such as the one reported where the host ocular surface epithelium is abnormal. If the host has normal epithelium, as in the animal experiments reported here, there is no evidence of dilution of host with graft cells, indicating no random mixing and no centrifugal epithelial cell movement after grafting.

A centripetal movement of cells would, on the other hand, result only in a dilution of the graft by host cells, not vice versa, which is what we have observed. Furthermore, at 12 weeks postoperatively, the 6.5 mm diameter region had significantly more of the sex-chromatin characteristics of the host epithelium than did the 5 mm diameter region, again indicating a centripetal motion of epithelial cells in these operated eyes. Whether this motion is characteristic only of operated eyes or whether it also occurs in normal eyes is not clear. In an investigation of human keratoplasty, Kaye also suggested centripetal movement of donor corneal epithelium. He measured the change in dot density, which occurs between sutures about 6 weeks after keratoplasty, and found that these dots were seen to move into the central portion of the cornea after removal of sutures. In addition, Buck suggested that centripetal cell migration, but not centrifugal movement, played a significant role in epithelial covering of denuded central cornea in mice. These findings imply that corneal epithelium has centripetal cell movement.

The results of this study suggest that donor epithelium after lamellar keratoplasty may be replaced by host tissues if the surrounding host cells are healthy. Whether that is also the case if the host epithelium is abnormal or if the grafted tissue is peripherally placed as in conjunctival transplantation is unknown.

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