Descemet Stripping Automated Endothelial Keratoplasty Using Cultured Corneal Endothelial Cells in a Rabbit Model

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Objective: To investigate the feasibility of Descemet stripping automated endothelial keratoplasty (DSAEK) using cultured human corneal endothelial cells (HCECs) in an animal model.

Methods: Descemet stripping automated endothelial keratoplasty grafts were produced by seeding cultured HCEC suspensions onto human corneal stromal discs. Three insertion techniques were assessed in an ex vivo model. The feasibility of DSAEK grafts with cultured HCECs was examined in a rabbit model. Rabbits received stromal disc transplants with cultured HCECs (c-DSAEK) or without HCECs (controls).

Results: The HCECs on the DSAEK grafts had a consistent size and polygonal shape. Mean (SD) percentage of cell loss in the taco-folding group (38.7% [5.2%]) was significantly greater than that in the Busin glide (11.6% [3.1%]; P = .001) and lens glide (18.0% [5.4%]; P = .007) groups. Corneal transparency gradually recovered in the c-DSAEK group, whereas edema persisted for up to 28 days in controls. Histologic examination after surgery revealed donor HCECs covering the posterior surface of the graft in the c-DSAEK group.

Conclusions: Further enhancements of the efficacy and safety of DSAEK using cultured HCECs will make this a clinically feasible alternative therapy for corneal endothelial dysfunction.

Clinical Relevance: Descemet stripping automated endothelial keratoplasty using cultured HCECs may be a novel therapeutic approach to treat corneal endothelial dysfunction.


Descemet stripping automated endothelial keratoplasty (DSAEK) has gained popularity as a tissue-selective corneal transplant method for the treatment of corneal endothelial dysfunction. This procedure improves postoperative visual function and reduces the risks associated with penetrating keratoplasty, such as large astigmatism and expulsive hemorrhage. Descemet stripping automated endothelial keratoplasty requires a donor cornea, however, and therefore, the worldwide shortage of donor corneas limits the applicability of this procedure. If cultured human corneal endothelial cells (HCECs) can be used in corneal transplant, many patients with corneal endothelial dysfunction could be treated using only 1 donor cornea. Therefore, several groups, including ours, have investigated the use of HCECs in the treatment of corneal endothelial dysfunction. Because DSAEK has become a clinically feasible method of corneal endothelial transplant, the development of DSAEK using cultured HCECs will help to meet the increased demand for corneal transplant. Thus, we investigated the feasibility of DSAEK using cultured HCECs in an animal model.

Methods

HCEC Culture

Primary culture of HCECs was performed as described previously. Briefly, cultures were established from the remainders of donor corneas that were used for full-thickness corneal transplant. The explants were placed endothelial cell side down onto a 35-mm tissue culture dish coated with bovine extracellular matrix and the dishes were placed in a carbon dioxide incubator. Cultured cells from the fourth or fifth passages were used in this study.

Preparing Corneal Stromal Discs

After setting a human donor sclerocorneal button onto the artificial anterior chamber system (Katena, Denville, New Jersey), an inci-
sion was made near the limbus to a depth of 120 to 150 μm and the cornea was dissected along the layer of the incision depth with tissue-dissecting knives (Katena). Then, a corneal stromal disc was punched out with an 8.0-mm trephine. Two to 3 discs could be obtained from 1 donor.

SEEDING CULTURED HCECs ON STROMAL DISCS

The corneal stromal discs were placed in a 24-well, non-treated (non–tissue culture) plate (BD Biosciences, Franklin Lakes, New Jersey). To observe HCEC localization after in vivo transplant, cultured HCECs were labeled with the fluorescent tracer PKH26 (Sigma-Aldrich, St Louis, Missouri) before making the DSAEK grafts. An HCEC suspension (4.0 × 10⁵ cells in 0.5 mL of culture medium) was added to each stromal disc, and the plates were centrifuged at 1000 rpm (176g) for 10 minutes to enhance the attachment of the HCECs to the discs. Incubation at 37°C and 5% carbon dioxo was performed for 4 weeks. The medium also contained 6% dextran to adjust the osmotic pressure, thereby preventing the stromal discs from swelling.

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HISTOLOGIC EXAMINATIONS OF THE CULTURED DSAEK GRAFTS

After incubation for 28 days, cultured DSAEK grafts were examined under a light microscope (model BX-50; Olympus, Tokyo, Japan) and images were saved to a personal computer. Some grafts were fixed in 10% formalin (Wako Pure Chemicals, Osaka, Japan) and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Naperville, Illinois) at −20°C. Frozen OCT compound–embedded sections were cut at 8-μm thickness, placed on silane-coated microscope slides (Muto, Tokyo), stained with hematoxylin–eosin, and observed with light microscopy. In grafts of 2 rabbits, the cell number in a 0.1-× 1.0-mm square was counted at 4 different sites after staining with 0.2% alizarin red S for 1.5 minutes. Two other grafts were used to observe the fluorescence of the whole mount sample under a fluorescence microscope (models BH2-RFL-T3 and BX50; Olympus) with an excitation wavelength of 420 nm and an emission wavelength of 480 nm. For electron microscopy observation, 2 samples were immersed in a fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde (Wako Pure Chemicals) in 0.1M phosphate-buffered saline at pH 7.4 and were observed with scanning and transmission electron microscopy.

COMPARING GRAFT INSERTION TECHNIQUES IN AN EX VIVO MODEL

Because corneal endothelial cell loss is one of the most significant problems of the DSAEK procedure, damage to the grafts induced by the 3 DSAEK graft insertion techniques was compared. A porcine sclerocorneal button was placed into an artificial anterior chamber (Katena), and the anterior chamber was replaced with a balanced salt solution until normal ocular tension was achieved. Two side ports and a 4-mm corneal incision were made, and then the anterior chamber maintainer was attached. Three incision techniques were assessed: taco-folding technique using forceps (DSAEK forceps; Moria, Doylestown, Pennsylvania), pull-through technique using a Busin glide (Moria), and pull-through technique using a lens glide (Alcon, Fort Worth, Texas). After inserting and attaching the graft with air injection, the sclerocorneal button was dyed with 0.25% trypan blue for 1 minute and 0.2% alizarin red S for 1 minute. Stained corneas were fixed in 2.5% glutaraldehyde solution for 10 minutes and digital photographs were taken. The ratio of purple stained area representing the HCEC damaged area to the whole disc area was examined. Four discs for each group were prepared. Statistical analysis was performed using 1-way analysis of variance and post hoc Tukey test. A P value of less than .05 was considered to be significant.

TRANSPLANT OF DSAEK GRAFT IN A RABBIT MODEL

New Zealand white rabbits were used to assess the feasibility of DSAEK grafts with cultured HCECs in an in vivo model. The animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were purchased from Saitama Experimental Animals Inc (Saitama, Japan). Fourteen New Zealand white male rabbits weighing approximately 2.5 kg were prepared for the study. Before surgery and examinations, the rabbits were anesthetized intramuscularly with ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo) and xylazine hydrochloride (10 mg/kg; Bayer, Munich, Germany) and also topically with an oxybuprocaine hydrochloride eyedrop solution, 0.4% (Santen, Osaka, Japan). The rabbits were divided into 2 groups: a cultured-graft DSAEK group (c-DSAEK) in which a stromal disc with cultured HCECs was transplanted and a control group (controls) in which a stromal disc without HCECs was transplanted. Both groups comprised 7 eyes of 7 rabbits.

The surgical procedure was similar to DSAEK performed in a clinical setting. An anterior chamber maintainer (Alcon) was positioned and a core vitrectomy was performed using a vitreous cutter (Accuris or Inniti; Alcon). The Descemet membrane in the central 8-mm area was peeled off using a Price hook (Moria). A 4-mm corneal incision was made with a slit knife (Mani, Tochigi, Japan). The graft was pulled through the incision into the anterior chamber with a Busin glide (Moria) and capsulorrhexis forceps (Asico, Westmont, Illinois). After the graft insertion, air was injected into the anterior chamber to attach the graft onto the posterior surface of the cornea. After 10 minutes, the air was reduced to prevent pupillary block. Topical levofloxacin (ophthalmic solution, 0.3% (Santen), and betamethasone sodium phosphate ophthalmic solution, 0.1% (Shionogi, Osaka) were instilled once a day for 1 week.

The treated eyes were observed with a slitlamp microscope (Olympus) and photographed until day 28 after surgery. Central corneal thickness was measured with an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan). The average corneal thickness was compared between the c-DSAEK and control groups. The P value for statistical significance in this evaluation was set to P = .007 after Bonferroni correction for multiple comparisons because the corneal thickness was compared at 7 different postoperative points. Intraocular pressure was measured using a pneumotonometer (Alcon).

HISTOLOGIC EXAMINATION

At 28 days after transplant, the rabbits were killed with an overdose of pentobarbital sodium (Dainippon Pharmaceutical, Osaka) injected under deep anesthesia and the corneas were excised. Some corneas were fixed in 10% formalin and embedded in OCT compound at −20°C. Frozen OCT compound–embedded sections were cut at an 8-μm thickness, placed on silane-coated microscope slides, stained with hematoxylin–eosin, and observed with light microscopy. In 2 corneas of the c-DSAEK group, the number of cells in a 0.1× 0.1-mm square was counted at 4 different sites after staining with 0.2% alizarin red S for 1 minute. To examine the fluorescence of PKH26-


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labeled HCECs in a whole mount sample, 2 other corneas in the c-DSAEEK group were observed under a fluorescence microscope with an excitation wavelength of 420 nm and an emission wavelength of 480 nm. Immunostaining for zonula occludens 1, a tight junction–associated protein, was performed with these samples after fixation with methanol.

RESULTS

DSAEEK GRAFTS WITH CULTURED HCECs

Although slight stromal edema was observed in the grafts after 4 weeks of culture, the transparency of the grafts recovered after briefly soaking the grafts in a high-osmolarity liquid of 10% glycerol. Light microscopic observation showed slight stromal edema, but the layer structure of the stroma was not affected. The HCECs on the DSAEEK grafts formed a monolayer (Figure 1A) and had a consistent size and a polygonal shape (Figure 1B). Mean (SD) cell density was 1656 (156.8) cells/mm² (range, 1400-1850 cells/mm²). Electron microscopic observation demonstrated attaching cells on the stroma (Figure 1C). These microscopic findings were similar to those in normal corneal endothelial cells in vivo.

COMPARING GRAFT INSERTION TECHNIQUES IN AN EX VIVO MODEL

Figure 2 shows representative photographs of vital staining after graft insertion using 1 of the 3 insertion techniques. The grafts inserted with the taco-folding method showed 2 parallel bands of dense staining corresponding to the regions contacted by the forceps. The grafts inserted with either a Busin glide or lens glide showed several stained lines corresponding to wrinkles that were probably formed while passing through the incision. Mean (SD) percentage of cell loss in the taco-folding, Busin glide, and lens glide groups was 38.7% (5.2%), 11.6% (1.5%), and 18.0% (5.4%), respectively. Mean (SD) percentage of cell loss area in the taco-folding group was significantly greater than that in the Busin glide (P = .001) and lens glide (P = .007) groups.

IN VIVO DSAEK MODEL

Slitlamp examination showed that all grafts attached to the posterior surface of the recipient rabbit cornea. Representative anterior segment photographs at day 1 and day 21 are shown in Figure 3. Corneal edema developed after surgery in both groups. The edema decreased and transparency recovered gradually in the c-DSAEEK group, whereas the edema persisted for 28 days in the control group. Figure 4 shows the time course of central corneal thickness. At 21 and 28 days after surgery, central corneal thickness was significantly smaller in the c-DSAEEK group than in the control group. Vessel invasion into the corneal stroma was observed in 2 eyes of the control group and in 1 eye of the c-DSAEEK group. In those eyes, intraocular pressure was elevated to 30 to 40 mm Hg. The other eyes showed no apparent complications, including intraocular pressure elevation.

HISTOLOGIC EXAMINATION

Figure 5 shows the result of histologic examination with light microscopy of the cornea at 28 days after the surgery. The edge of the recipient’s Descemet membrane was observed in both groups. Stromal edema was observed in both groups and the degree of edema varied among samples. The rear surface of the graft in the c-DSAEEK group was covered with a cell monolayer and fluorescence microscopy showed that the cells were of donor origin (Figures 5B and...
C. Immunostaining for zonula occludens 1 was observed at the intercellular lesion, suggesting that a junction formed between the cells (Figure 5D).

COMMENT

In this study, HCECs seeded on DSAEK grafts had a morphology similar to HCECs in vivo and contributed to reduce corneal edema in an animal model. The transplanted cells were very likely to be HCECs because our previous studies using the same culture technique confirmed the properties of the cultured cells as HCEC, including the pump function and the expressions of sodium potassium adenosine triphosphatase and zonula occludens 1.12,13 These findings suggest that DSAEK grafts made using cultured HCECs are clinically feasible. Sev-
eral modifications must be made, however, before DSAEK using cultured HCECs can be performed in a clinical setting. First, the HCEC density on the DSAEK grafts must be increased to an in vivo level. The cell density of the HCEC solution and the methods of cell seeding and attachment must also be improved. Second, the quality of the cultured HCECs must be suitable for transplant. Because older donors provide more senescent HCECs with karyotype abnormalities, HCECs from younger donors are more suitable for transplant. Moreover, HCECs from early culture passages are likely to have better quality. Finally, because the corneal stroma for DSAEK grafts must be of a smooth surface, it should be made using a microkeratome or femtosecond laser.

There are several possible methods of delivering the HCECs, including the use of carriers, such as collagen sheets and gelatin hydrogels, but these methods increase the risk of inserting foreign bodies into the human eye, which may induce excessive inflammation and result in a higher risk of rejection and graft failure. Because our method used human corneal stroma, the risk of inducing inflammation is thought to be low. Although 1 eye in the c-DSAEK group showed inflammation with corneal angiogenesis and intraocular pressure elevation, most of the eyes did not exhibit excessive inflammation. The stress of the surgical procedure may have induced the inflammation, because in preliminary studies, peeling off the Descemet membrane without graft transplant frequently caused fibrin formation and massive angiogenesis (data not shown).

The use of an HCEC sheet without a carrier has also been reported. Although it is an attractive concept, establishing a method for delivering the HCEC sheet without a carrier is difficult because the HCEC sheet is so fragile. Similarly, using an HCEC sheet supported only by the Descemet membrane was reported by Melles et al, but the surgical procedure is difficult for most surgeons to perform reliably.

The damage to the grafts by the 3 graft insertion techniques was compared and the mean percentage of cell loss in the taco-folding group (38.7%) was significantly greater than that in Busin glide (11.6%) and lens glide (18.0%) groups. Bahar et al reported in a clinical study that endothelial cell loss in the Busin guide-assisted DSAEK group (25%) was significantly lower than that in the forceps-assisted DSAEK group (34.3%). Mehta et al reported mean cell damage of 9% to 9.2% following lens glide insertion and 32% to 38% following the taco-folded insertion in a wet laboratory DSAEK model. These previous studies reporting larger cell loss with the taco-folding method of insertion are in good agreement with the findings of the present study. Moreover, the cell loss percentages in these studies were similar to those in our
study, suggesting that the attachment of cultured HCECs on DSAEK graft is as good as HCECs in vivo.

Using our method, more patients could be treated with the material obtained from 1 donor. Enough HCECs from a single donor can be proliferated to make more than 10 DSAEK grafts. Moreover, because it is not necessary to use fresh corneal stroma, preserved sclerocorneal buttons not suitable for penetrating keratoplasty can be used to make DSAEK grafts. Thus, the current method of using cultured HCECs might compensate for the shortage of donor corneas. One of the most important advantages of our method is that it is based on a common clinical procedure, and therefore, performing it in a human eye is not difficult for DSAEK surgeons.

In the in vivo investigation, the postoperative recovery was slow. At 1 month after surgery, the average central corneal thickness in the c-DSAEK group was 776 µm, which was greater than the sum of the original graft thickness (350 µm) and the DSAEK graft (150 µm). Because the average cell density of 1656 cells/mm² is usually more than enough to clear up most of the cornea at 1 month after surgery, this delayed recovery might be due to the relatively low quality of the HCECs. Refinements in the methods used to enhance the density and quality of the seeded HCECs for DSAEK grafts are necessary to improve the efficacy of DSAEK with cultured HCECs. Given the fact that this study describes an animal experiment in 7 eyes (and 7 control eyes) with 1 month follow-up, it would be fair to state that the results are preliminary in vivo animal data. A longer follow-up with more subjects is necessary to assess how the endothelial density tends to decrease over time. In conclusion, with further enhancements in efficacy and safety, DSAEK using cultured HCECs can be an alternative therapy for corneal endothelial dysfunction in the near future.

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