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総合研究報告書表紙

厚生労働科学研究費補助金

再生医療実用化研究事業

培養細胞または幹細胞を用いた再生ヒト角膜内皮移植の実用化に関する研究

平成２０年度～２１年度　総合研究報告書

研究代表者　三村　達哉

平成２２（２０１０）年　５月

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Ｉ．総括研究報告

厚生労働科学研究費補助金（再生医療実用化研究事業）
（総合）研究報告書

培養細胞または幹細胞を用いた再生ヒト角膜内皮移植の実用化に関する研究

研究代表者　三村達哉　虎の門病院眼科　医師・研究員

研究要旨
角膜ドナー不足を解消するために、再生した角膜内皮により角膜混濁を治療することを目的としている。培養ヒト角膜内皮細胞あるいは、ヒト角膜組織幹細胞を用いて、角膜内皮を再生した。再生内皮は生体内同様の機能を有し、動物眼に移植することにより水疱性角膜症の治療に有効であった。

Ａ．研究目的：本邦の角膜移植手術におけるドナー角膜不足は深刻であり、ドナー角膜を必要としない、人工角膜の開発に期待がかかっている。ヒト角膜は角膜上皮、実質、内皮にわかれ、その中で角膜内皮細胞は角膜透明性維持に最も重要であるが、生体内では増殖能を失っているために、加齢に伴った角膜内皮細胞の減少が角膜透明性減少の原因となっている。我々は角膜再生を目的として、細胞外基質、培養細胞あるいは生体幹細胞を用いた角膜上皮、実質、内皮の再生について精力的に研究を行っている。角膜上皮混濁に対しては、自己の健常部分より採取して培養した角膜上皮細胞、結膜上皮細胞、あるいは口腔粘膜上皮細胞のシートによる眼表面再生医療を既に行っている。しかし、角膜混濁を来たす症例は全層が障害されていることが大半で、その多くは角膜内皮細胞の障害による不可逆的な水疱性角膜症である。本研究では、角膜内皮に焦点をあて、培養ヒト角膜内皮細胞を用いた角膜内皮シートあるいは幹細胞を移植することにより混濁した角膜を透明にする治療の実用化を目的としている。

Ｂ．研究方法：

研究計画および方法

培養角膜内皮細胞を用いた角膜再生と内皮体性幹細胞を用いた角膜再生の二大項目に分けて、研究を行う予定である。培養角膜内皮細胞を用いた内皮シートはこれまでの我々の動物実験にて術後角膜透明性の維持に有効であることが既に証明されており、この２年間で臨床応用をめざして、内皮シートの更なる改良を目指して実用化を目指す。

幹細胞研究では研究用輸入ヒト角膜より選択的に角膜幹細胞を採取し、生体外で培養し、角膜内皮細胞に分化誘導することにより、角膜再生医療に応用することを目的とする。

Ｉ 分化培養角膜細胞を用いた再構築角膜

分化した培養細胞を用いる利点としては、通常の培養法で容易に分化した細胞が得られることと、既に分化した細胞は内皮以外に分化することなく移植した後も内皮の機能を果たしうることが期待される。年度別計画は以下の通りである。

平成 20 年度
① ヒト角膜内皮細胞のバンク化
② 動物種由来製剤完全フリーの培養液の開発
③ 角膜内皮シートの生体慣用性キャリアーの開
④ 基質を使用しない角膜内皮単層シートの作成

平成 21 年度
① 内皮シートの移植法ならびに器具の開発
② 動物眼への内皮シートの移植ならびに長期観察
③ 臨床応用

II 角膜幹細胞を用いた再構築角膜

幹細胞を用いた研究に関しては、角膜内皮より選択的に幹細胞を採取し、生体外で培養し、幹細胞自身を移植する方法あるいは幹細胞を内皮細胞に分化誘導することにより内皮シートを作成して移植する方法がある。角膜幹細胞の採取法については、メチルセルロースを含んだ培養液を用いたスフェアー法により、角膜の各層より組織幹細を採取することに我々は既に成功している。一般的に、術後 allo 移植よりは auto 移植の方が、拒絶反応を起こしにくいため、自己細胞の移植が理想となる。また分化した培養細胞を移植しても、術後生体内での増殖能は期待できない。そこで、患者の健常眼の片眼から自己の幹細胞を選択的に採取して、罹患眼に移植する方法は、移植後も細胞は増殖する可能性があり、細胞供給源となる可能性がある。拒絶反応抑制と移植後の細胞供給源の利点を兼ね備えた自己幹細胞移植についても、検討した。年度別に以下のテーマについて研究を行った。

平成 20 年度
① 内皮幹細胞の局在:未分化マーカーを用いた免疫染色、各エリアにおけるスフェアー形成率
② 内皮幹細胞の分化誘導:分化誘導培地にて幹細胞を培養し、分化マーカーの発現を確認する
③ 採取した内皮幹細胞による内皮シートの再構築。
④ 再構築内皮シートの機能解析:再構築した内皮シートに実質の水分をハイドレーションする機能が備わっているか Na^+ -K^+ ATPase 依存性のポンプ機能の解析を行う。

平成 21 年度

⑤ 再構築内皮シートの移植実験：臨床応用に向けて動物眼への移植実験を行う。

⑥ 移植法：内皮幹細胞の移植法ならびに、移植用器具を開発する。

⑦ 臨床応用の準備：倫理委員会での承認ならびに移植コーディネートの準備

（倫理面への配慮）

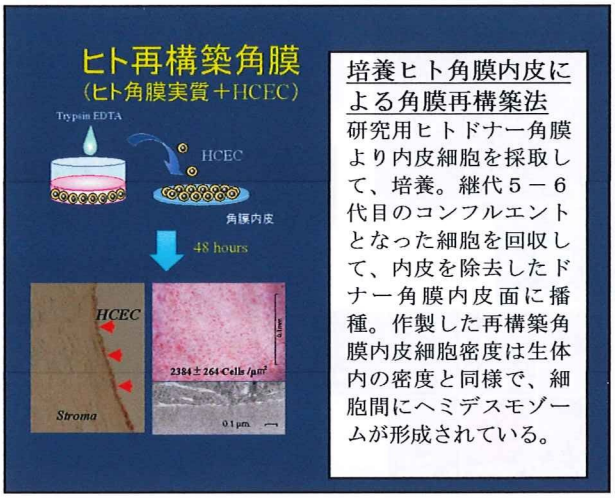
すべての研究は東京大学倫理委員会の承認を得て行う。角膜幹細胞を用いた人工角膜の人への移植を前提とした研究であるため、倫理委員会の指針、動物実験の対する指針、および研究に関与するあらゆる倫理指針を遵守する。動物の取り扱いは、苦痛を伴うものは必ず全身麻酔下に行い、両眼が失われる可能性のある場合は片眼のみに処置を行う。全ての実験において動物は the Association for Research in Vision and Ophthalmology の規約および、実験動物の飼養及び保管等に関する基準（総理府）に従って扱う。人を扱う研究では，ヘルシンキ宣言（世界医師会総会 World Medical Assembly）の勧告に従って行う。また遺伝子解析はヒトゲノム・遺伝子解析研究に関する倫理指針（文部科学省、厚生労働省、経済産業省）に従い、幹細胞の取り扱いはヒト幹細胞を用いた臨床研究に関する指針（厚生労働省）を遵守する。患者を対象とする臨床試験においては十分な説明をした後、文書による同意を得てから行う（インフォームド・コンセント）。

C．研究結果：

培養内皮細胞あるいは、組織幹細胞は、生体内と同様にポンプ機能を有し、そして ZO-1 蛋白に代表される細胞間の tight junction を形成しうることを証明した。また我々は水疱性角膜症に対し、培養角膜内皮細胞とコラーゲンで再構築した内皮細胞シートが in vivo で有効であるを証明した。

内皮層より選択的に採取した組織幹細胞は神経未分化マーカーNestin を発現した。また分化誘導培地で培養して得られた細胞は角膜内皮様の正六角形細胞に分化し、RT-PCR 法にて内皮細胞と同様の遺伝子発現パターンを示した。織幹細胞を用いて再構築した内皮シートは十分な強度を持ち、移植可能であった。

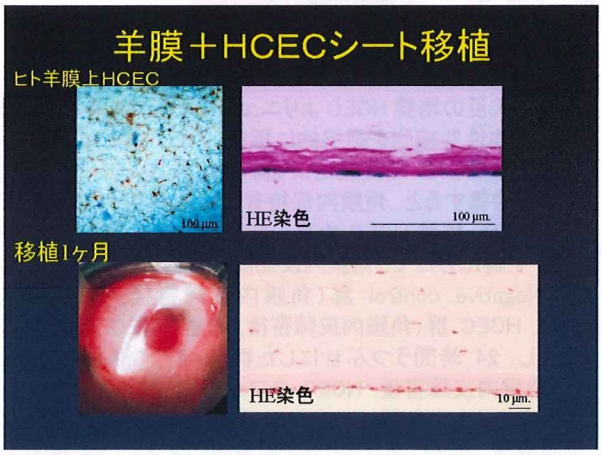
内皮細胞を除去することにより角膜が混濁した家兎水疱性角膜症モデルに角膜内皮組織幹細胞を移植したところ、透明性および角膜厚は正常に回復した。経過観察中、術後拒絶反応は認められず、角膜透明性を維持した。



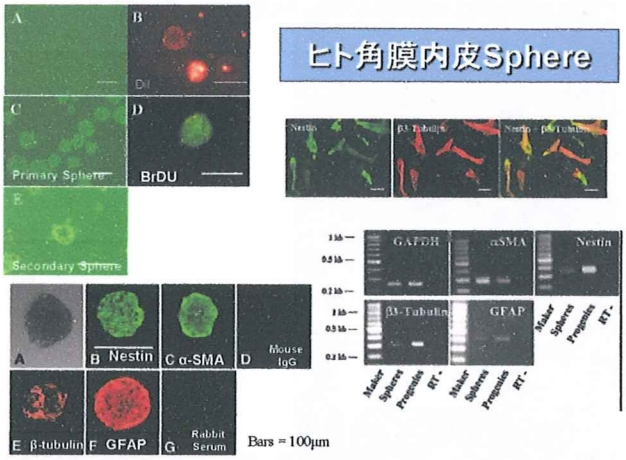
培養ヒト角膜内皮による角膜再構築法

研究用ヒトドナー角膜より内皮細胞を採取して、培養。継代５－６代目のコンフルエントとなった細胞を回収して、内皮を除去したドナー角膜内皮面に播種。作製した再構築角膜内皮細胞密度は生体内の密度と同様で、細胞間にヘミデスモゾームが形成されている。

再構築角膜を家兎角膜に移植後１ヶ月目
角膜透明性が維持され、内皮面に蛍光標識した内皮細胞が観察される。

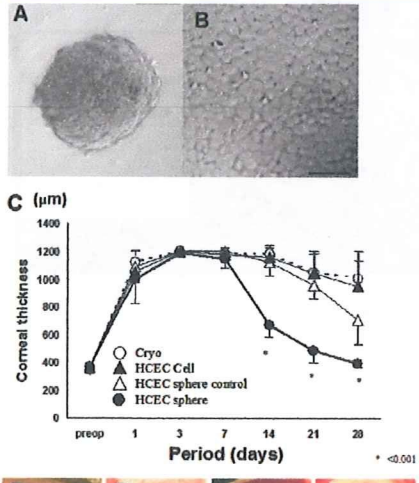


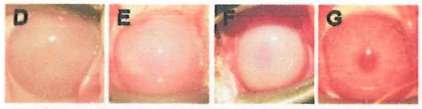
羊膜上に培養ヒト角膜内皮細胞を播種して再構築角膜内皮シートを作製。内皮細胞を除去して、角膜浮腫を起した家兎角膜内皮面に移植。移植後は角膜浮腫が改善し、角膜厚が薄くなり、角膜透明性が維持された。



浮遊培養法（スフェアー法）によるヒト角膜内皮前駆細胞の採取。ドナーからのヒト角膜内皮細胞より 1 次および二次 sphere が形成される。sphere は BRDU の取り込みが高く、また免疫染色およびＲＴ-PCR では sphere に神経幹細胞マーカーNestin が強く発現する。

ヒト角膜内皮細胞由来体性幹細胞の移植





継代 5 代目の培養 HCEC よりニューロスフェアー法により角膜内皮体性幹細胞を選択的に採取し(A)，内皮障害のある家兎に移植した．培養細胞より得られたスフェアーを培養皿上で再培養すると，角膜内皮特有の正六角形の形態を示している(B)．培養 HCEC 由来の幹細胞または培養 HCEC を次の 4 群に分けて，角膜内皮細胞を障害した家兎に移植した．Negative control 群（角膜内皮障害後，無治療群，n=6)，HCEC 群(角膜内皮障害後，培養 HCEC を前房内に注入し，24 時間うつぶせにした群，n=6)，Sphere-face-up 群(角膜内皮障害後，HCEC スフェアーを前房内に注入し，体位を自由にした群，n=6)，Sphere-face-down 群(角膜内皮障害後，HCEC スフェアーを前房内に注入し，24 時間うつぶせにした群，n=6)．C：術後角膜厚（縦軸）．術後経過（横軸）．Sphere-face-down 群の角膜厚は 3 日，7 日，14 日，21 日，28 日目において他のコントロールと比較して有意に低下している(p<0.001)．術後前眼部を観察すると Negative control 群(D)，HCEC 群(E)，Sphere-face-up 群(F)では角膜混濁が強いが，Sphere-face-down 群では角膜は透明で実質浮腫は認められない(G)．

D．考察：培養角膜内皮細胞および角膜内皮組織幹細胞による再構築角膜はの移植後長期成績を動物眼で今後検討する必要があるが、今後の臨床応用に対して大いに期待できる方法であると考えられた。

E．結論　再生内皮は生体内同様の機能を有し、動物眼に移植することにより水疱性角膜症の治療に有効であった。

F．健康危険情報　本研究の結果により、健康に及ぼす危険事項は確認できなかった。

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三村達哉シンポジウム・角膜内皮の幹細胞と老化
② 第 112 回日本眼科学会総会　4 演題発表
③ 第 14 回日本糖尿病眼学会総会　1 演題発表
④ 第 62 回日本臨床眼科学会 7 演題発表
⑤ 第 33 回角膜カンファランス 6 演題発表
⑥ ARVO2008 (USA フロリダ)　1 演題発表
⑦ 第 113 回日本眼科学会総会　7 演題発表
⑧ 第 62 回日本臨床眼科学会 4 演題発表
⑨ 第 34 回角膜カンファランス 2 演題発表
⑩ TERMIS, 2009 国際再生医療学会(Seoul)1 演題発表
⑪ 第 114 回日本眼科学会総会　2 演題発表
⑫ ARVO2010 (USA フロリダ)　1 演題発表

H．知的財産権の出願・登録状況

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2．実用新案登録　なし
3．その他・賞罰　本年度 4 回受賞
①三村達哉　東京大学医師会医学賞受賞　平成 20 年
②三村達哉　第 112 回日本眼科学会総会 座長賞 平成 20 年
③三村達哉　第 112 回日本眼科学会総会　学術展示優秀賞　平成 20 年
④三村達哉　第 33 回角膜カンファレンス・第 25 回日本角膜移植学会　内田賞　平成 21 年

別紙４

II. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

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代表論文の1報のみ添付する。

LABORATORY SCIENCES

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

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

Norihiko Honda, MD; Tatsuya Mimura, MD, PhD; Tomohiko Usui, MD, PhD; Shiro Amano, MD, PhD

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% [1.5%]; $P=.001$) 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.

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DESCEMET STRIPPING AUTOmated endothelial keratoplasty (DSAEK) has gained popularity as a tissue-selective corneal transplant method for the treatment of corneal endothelial dysfunction.[1-4] 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.[5-10] 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.

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METHODS

HCEC CULTURE

Primary culture of HCECs was performed as described previously.[10] 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, nontreated (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 graft. An HCEC suspension (4.0×10^5 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 dioxide was continued for 4 weeks. The medium also contained 6% dextran to adjust the osmotic pressure, thereby preventing the stromal discs from swelling.

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 2 grafts, the cell number in a 0.1×0.1-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 insertion techniques were assessed: taco-folding technique using a forceps (DSAEK forceps; Moria, Doylestown, Pennsylvania), pull-through technique using a Busin glide[11] (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 (Accurus or Infiniti; 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, 0.5% (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 pneumatonometer (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-

labeled HCECs in a whole mount sample, 2 other corneas in the c-DSAEK 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

DSAEK 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 DSAEK grafts formed a monolayer (**Figure 1** A) and had a consistent size and a polygonal shape (Figure 1B). Mean (SD) cell density was 1656 (156.8) cells/mm^2 (range, 1400-1850 cells/mm^2). 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-DSAEK 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-DSAEK 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-DSAEK 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.

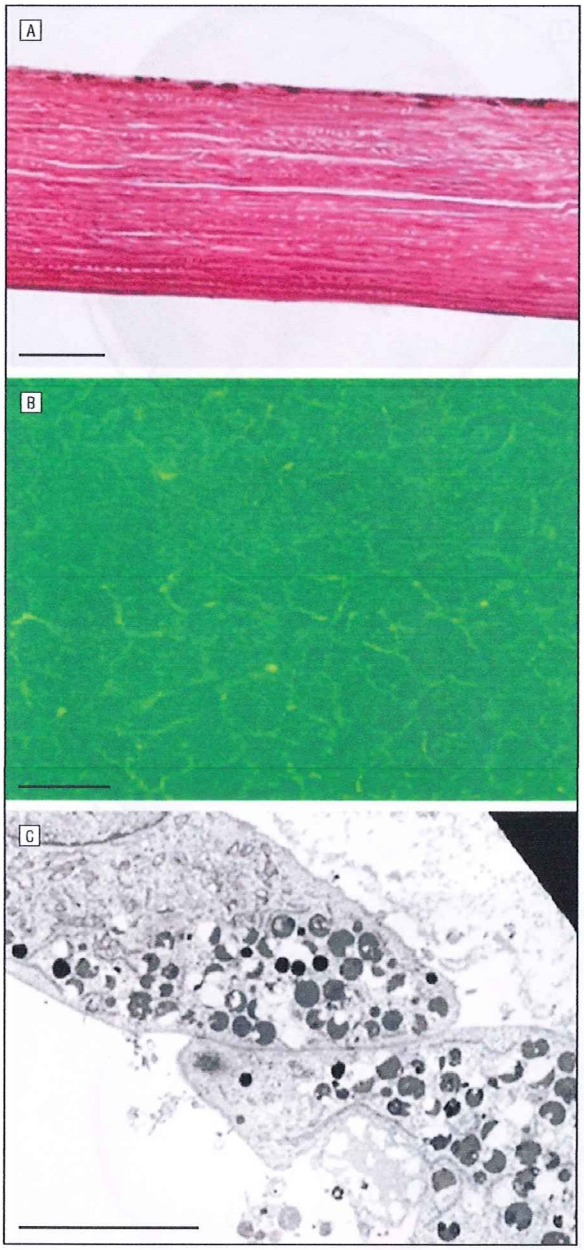

Figure 1. Histologic observation of the graft for Descemet stripping automated endothelial keratoplasty (DSAEK). A, Light microscopy with hematoxylin-eosin staining of the graft showed that human corneal endothelial cells (HCECs) seeded on the DSAEK graft formed a monolayer. Bar=50 µm. B, Phase contrast microscopy showed that HCECs on the DSAEK graft had a consistent size and a polygonal shape. Bar=50 µm. C, Transmission electron microscopy demonstrated an attached cell monolayer. Bar=5 µm.

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-DSAEK group was covered with a cell monolayer and fluorescence microscopy showed that the cells were of donor origin (Figures 5B and

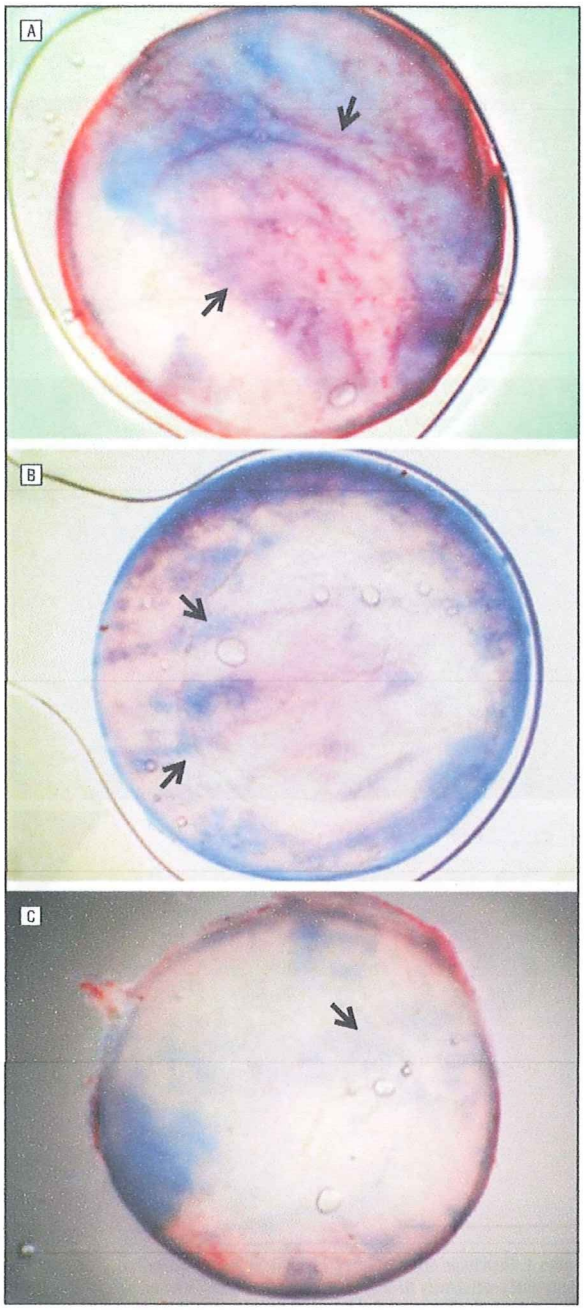

Figure 2. Representative photographs of vital staining after graft insertion with 1 of 3 insertion techniques (taco-folding [A], Busin glide [B], and lens glide [C]). A, Two parallel bands (arrows) of dense staining, probably corresponding to regions contacted by the forceps, were observed. B and C, Several stained lines (arrows) corresponding to wrinkles that were probably formed while passing through the incision were also observed.

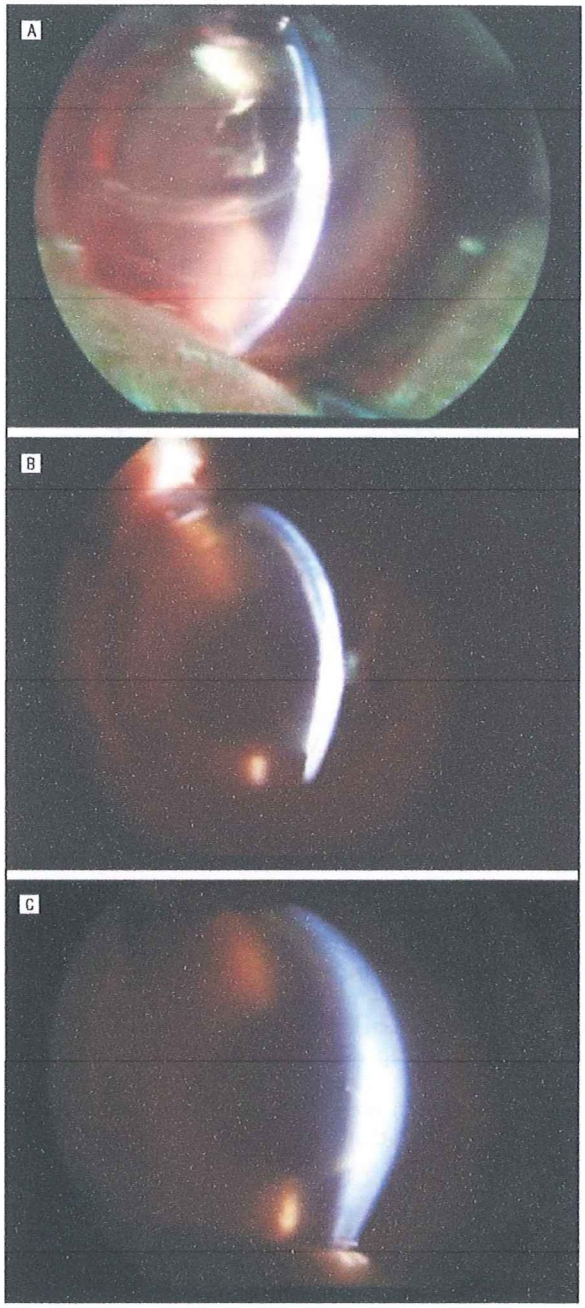

Figure 3. Representative anterior segment photographs after surgery. A, In the cultured-graft Descemet stripping automated endothelial keratoplasty (DSAEK) group, the DSAEK graft attached to the posterior surface of the cornea and corneal edema persisted at 1 day after surgery. B and C, At 21 days after surgery, corneal edema was greater in the control group (C) than in the cultured-graft DSAEK group (B).

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-

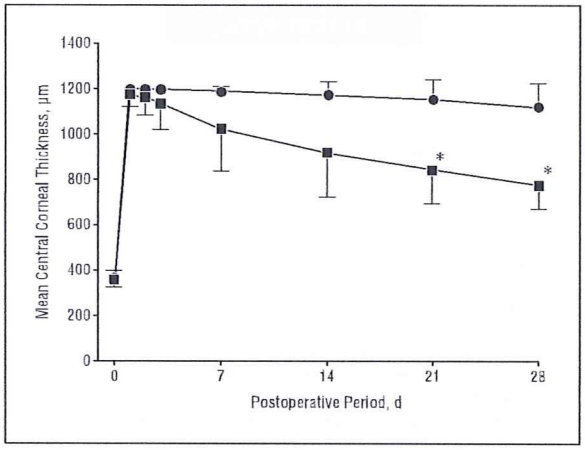

Figure 4. Time course of central corneal thickness in the cultured-graft Descemet stripping automated endothelial keratoplasty group and the control group. Values are expressed as mean (SD). *Central corneal thickness in the cultured-graft Descemet stripping automated endothelial keratoplasty group was significantly smaller than that in the control group at 21 and 28 days after surgery.

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.[14] 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[15,16] and gelatin hydrogels,[17] 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.[18] 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,[19] 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

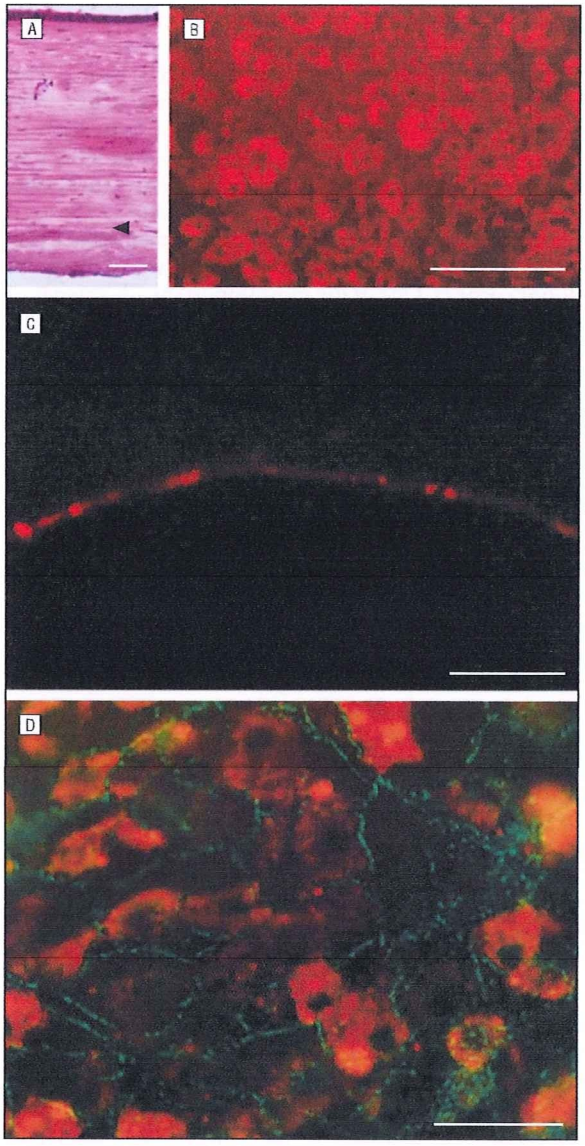

Figure 5. Histologic observation of the corneas in the cultured-graft Descemet stripping automated endothelial keratoplasty group at 28 days after the surgery. A, A cell monolayer was observed on the posterior surface of the graft. The interface of the graft and the host is indicated by an arrowhead. B and C, The posterior surface of the cornea was covered with fluorescent cells in the whole-mount preparation (B) and in the section (C). D, Immunostaining for zonula occludens 1 was observed at the intercellular lesion. Bars=100 µm.

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[20] 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[21] 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/mm^2 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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