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綜 説

ぶどう膜炎の診断：微量検体検査による網羅的検索
— Diagnosis for uveitis : comprehensive examination system using ocular samples —

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はじめに

　眼内炎症の原因として，ウイルス，細菌，真菌，寄生虫などの各種病原微生物があり，視力予後に直結する。これらの迅速でかつ正確な診断は適正な治療に不可欠である。また，その原因となる外来性抗原は多彩で，包括的な診断が求められる。そのうえ，眼局所から得られる検体は常に微量で，網羅的な診断検査を行うには工夫が必要となる。我々は，少量検体にて短時間に多項目の眼感染症検査を行う診断法を開発した。PCR（polymerase chain reaction）にてスクリーニングする各種病原微生物として，HSV-1，HSV-2，VZV，EBV，CMV，HHV-6，-7，-8 のヘルペスウイルス DNA，細菌 16S rDNA（細菌全般），真菌 28S rDNA（真菌全般），カンジダ・アスペルギルス 18S rDNA，トキソプラズマ・トキソカラの寄生虫 DNA の 14 種類の外来性抗原 DNA がある。ウイルスおよび寄生虫関連 DNA の同定は，マルチプレックス定性 PCR およびリアルタイム定量 PCR の組み合わせを用いて検査，また，細菌・真菌はブロードレンジリアルタイム定量 PCR で眼感染症症例を検査し，その有効性を報告した。本稿では，眼局所の微量検体検査による網羅的検索に基づく最新の眼科感染症診断法について述べたい。

I. 眼局所検体の採取方法

　眼科的な検体として，前房水，硝子体，涙液，網膜下液などの眼内液，あるいは虹彩，結膜，角膜擦過物などの眼組織がある（表）。いずれも微量であるが，PCR の検体としては十分に足りる。また，検体を凍結しておけば，後日利用することもできるので，疑わしい検体は凍結保存しておくのが望ましい。表にその一般的な採取方法を記載した。前房水は前部ぶどう膜炎（虹彩炎），角膜内皮炎などの前眼部の眼内炎症性疾患に有効で，0.1 ml の量で十分に網羅的な検査が行える。硝子体は硝子体手術（PPV）の際に採取されるものを検査に使用する。サイトカインや DNA 量を定量する場合もあるので還流前の生の検体を採取するのが望ましい。眼表面炎症性疾患（角膜炎，結膜炎など）では，涙液や角膜擦過物を採取する。涙液は量が取れないので eye wash 法（生食を用いて眼表面を洗うようにしてその後の涙液とともに生食を回収）で得られた希釈涙液を PCR 検査に使用する。そ

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表　眼の検体の種類および採取方法

検体の種類	手技(手法)	採取量
前房水	房水ピペット＊または30G針シリンジ	0.1 ml
硝子体	硝子体切除(PPV)	0.5 ml
涙液	希釈涙液(eye wash 法)	0.5 ml 生理食塩水
角膜組織	角膜擦過物	可能な限り
結膜組織	結膜切除	可能な限り
眼内組織(虹彩・網膜など)	眼内手術	可能な限り

＊房水ピペット：30G針が付いたピペットでキャピラリー現象を利用して前房水を採取(ニプロ株式会社)
PPV：pars plana vitrectomy

の他，網膜，虹彩，結膜，角膜擦過物などの眼組織はその小さな組織からDNA抽出処理を行う。一般的な眼検体の処理は，検体を遠心分離し，沈査の細胞成分は核酸DNAを抽出，また検体の上清はPCR以外の検査(培養，スメア，蛋白測定など)に使用する。

　以前から診断目的にはこれらの眼局所検体が用いられていたが，得られる検体量は微量であることから可能な検査は限られていた。数年前まではこのような微量検体を用いて検査可能な核酸の検出や特異抗体(Q値測定)の同時測定を行っていた。その当時はPCR法を用いても核酸は1〜2項目が限度であった。しかし現在はこのような微量検体でも迅速かつ網羅的に10種類以上と多くの項目の核酸DNAを検査できるシステムが作成され，実際臨床応用されてきている。

II. 微量検体を用いた眼感染症の網羅的診断法

　図1に我々の施設で現在行われている眼感染症に対する網羅的診断システムを示した。対象疾患は，すべての眼炎症性疾患で，感染性ぶどう膜炎，感染性眼内炎，眼内リンパ腫，網膜血管炎などの活動性眼内炎症を有する患者からインフォームド・コンセントを得て眼内液や眼内組織も採取する。眼表面炎症性疾患では角膜擦過物や涙液などを採取する。また非感染性ぶどう膜炎でも感染を否定する目的で検体を採取する場合がある。まず最初は2つのステップでスクリーニングPCR検査を行う(図1)。ステップ1では，ウイルスおよびぶどう膜炎の原因となる外来性抗原のマルチプレックス定性PCRおよびリアルタイム定量PCRを行う。またステップ2では，細菌全般(細菌16S)および真菌全般定量PCR(真菌18S/28S)をブロードレンジ定量PCRで行う(図1)。上記の2つのステップのスクリーニング検査に加えて，症例によってはオプション検査として図1に示すような各種外来性抗原DNA検出のための定性・定量PCR検査も行う。HTLV-1(proviral DNA：HTLV-1関連ぶどう膜炎)，風疹ウイルス(Fuchs虹彩異色性虹彩毛様体炎)，アデノウイルス(流行性角結膜炎)，エンテロウイルス(流行性角結膜炎)，アクネ菌(*Propionibacterium acnes*：術後遅発性眼内炎)，クリプトコッカス(真菌性眼内炎)，アカントアメーバ(角膜炎)，フザリウム(角膜炎)，結核(結核性ぶどう膜炎)，梅毒(梅毒性ぶどう膜炎)，クラミジア(クラミジア結膜炎)，バルトネラ(猫ひっかき病)などは疑う場合のみ検査を行う。上記感染症以外に悪性のリンパ腫細胞が眼内に浸潤する眼内リンパ腫があり，生命予後に関与するので疑う場合は積極的に検査を行う。眼内リンパ腫の診断には一般的な病理検査に加えて，IgH遺伝子再構
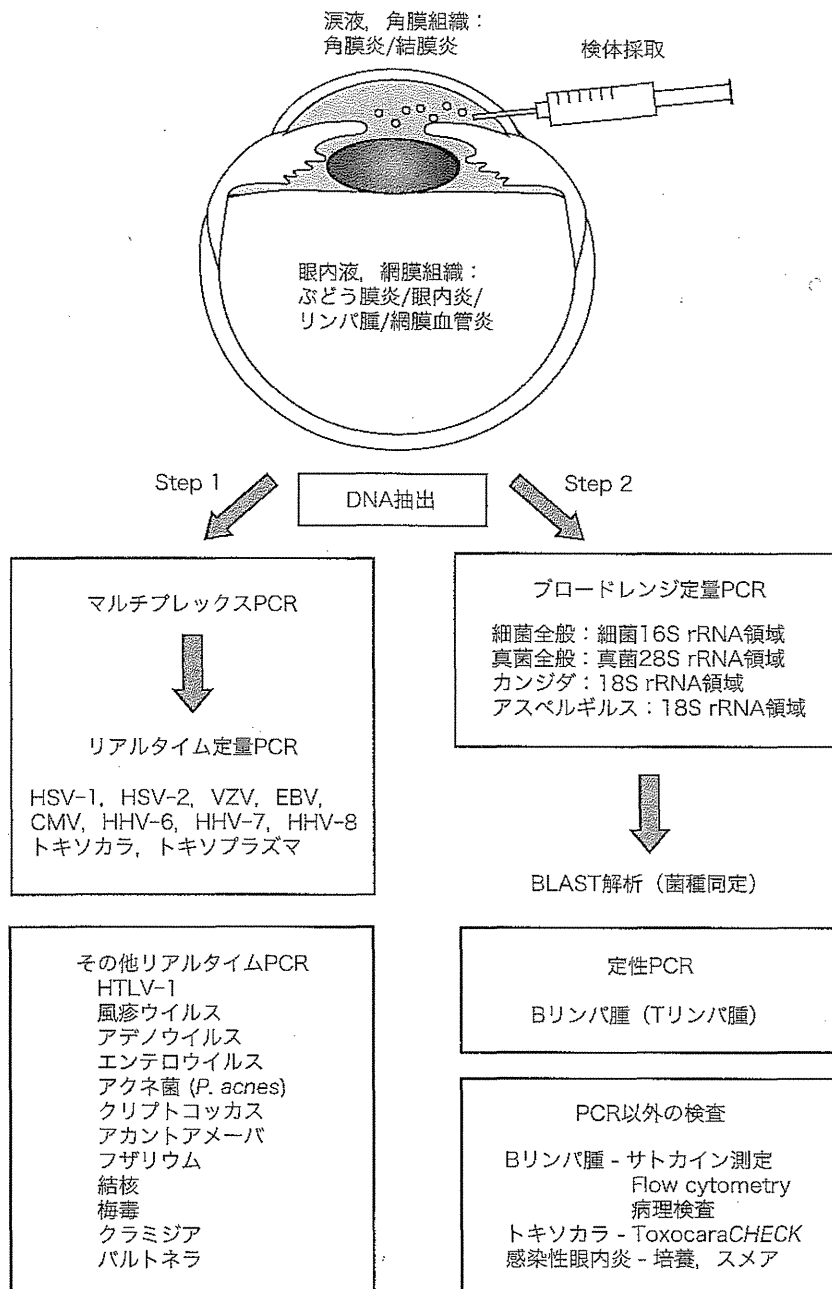
涙液，角膜組織：
角膜炎/結膜炎

検体採取

眼内液，網膜組織：
ぶどう膜炎/眼内炎/
リンパ腫/網膜血管炎

Step 1　　DNA抽出　　Step 2

マルチプレックスPCR

リアルタイム定量PCR

HSV-1，HSV-2，VZV，EBV，
CMV，HHV-6，HHV-7，HHV-8
トキソカラ，トキソプラズマ

ブロードレンジ定量PCR

細菌全般：細菌16S rRNA領域
真菌全般：真菌28S rRNA領域
カンジダ：18S rRNA領域
アスペルギルス：18S rRNA領域

BLAST解析（菌種同定）

その他リアルタイムPCR
HTLV-1
風疹ウイルス
アデノウイルス
エンテロウイルス
アクネ菌（P. acnes）
クリプトコッカス
アカントアメーバ
フザリウム
結核
梅毒
クラミジア
バルトネラ

定性PCR

Bリンパ腫（Tリンパ腫）

PCR以外の検査

Bリンパ腫 - サトカイン測定
Flow cytometry
病理検査
トキソカラ - ToxocaraCHECK
感染性眼内炎 - 培養，スメア

図1　微量検体を用いた網羅的PCR診断検査

ぶどう膜炎や眼内炎などの眼内炎症性疾患，あるいは結膜炎，角膜炎などの眼表面性疾患から検体を採取して，細胞成分は核酸DNAを抽出，上清はPCR以外の検査（培養，スメアなど）に使用する。PCRは2つのステップでスクリーニング検査を行う（赤枠）。(1)ウイルスおよびぶどう膜炎マルチプレックスおよびリアルタイム定量PCR，(2)細菌全般（細菌16S）および真菌全般定量PCR（真菌18S/28S）をブロードレンジ定量PCR。DNA抽出に40分，マルチプレックス定性PCRは90分，リアルタイム定量PCRはわずか60分で検査可能と迅速に多くの結果を得ることができる。
ToxocaraCHECK：トキソカラ抗体検査，PCR：polymerase chain reaction

成(モノクローナリティの証明)の同定を定性PCR検査で,またIL(インターロイキン)-10およびIL-6の眼内液サイトカイン測定を行う。いずれの場合も検体量が少ないので効率的に使用する必要がある。

III. マルチプレックスPCR法:ウイルスや寄生虫の診断

微量検体でも迅速に簡易的に10種類以上の多くの核酸DNAを検査できるシステムが作成され,実際臨床応用されるようになってきている(マルチプレックスPCR,別名,多項目迅速PCR:図2)[1)2)]。このマルチプレックスPCRの最大の特徴は,数種類のウイルスなどの外来性抗原を同時に迅速に検出できることである。その手順を図2に示した。以前の一般的なPCRのようにゲル内のバンド検出で陽性か陰性かを判定するのではなく,融解曲線で判定する(図2)。曲線が大きい場合,DNA量が多いことがわかり半定量できる利点がある。このマルチプレックスPCRに加えて,その核酸の量を定量化する検査,リアルタイム定量PCRが最近使用されるようになった。このリアルタイム定量PCRは経過中に何度か検体を採取できればその経過中の病原体DNAコピー数が把握できる利点がある。また,治療前に眼局所内のDNAコピー数を把握できるために,実際,治療薬の量の決定の参考にもなる。我々は,ウイルス性ぶどう膜炎の前房水から高コピー数のVZV-DNAやCMV-DNAが検出され其の眼局所のウイルス量と眼内組織破壊が相関し,早期診断・早期治療が重要であることを報告している[3)4)]。その他の利点として,眼表面炎症性疾患(角膜炎,結膜炎など)の涙液検体は複数の外来性抗原が検出される可能性があり,このPCR検査は有用である。また,免疫不全患者の検体からも複数の抗原DNAが検出されるこ

とがある。1検体のみの場合,検体処理からPCR結果の判定までわずか3〜4時間で,10種類以上の病原性抗原DNA同定のためのスクリーニング検査は終了する。

IV. ブロードレンジPCR法:細菌や真菌の診断

ブロードレンジPCRとは,リボソーマルRNA(rRNA)遺伝子を標的にしたPCR法で,原核生物では16S rRNA,真核生物では18S/28S rRNAといったそれぞれの種で保存された遺伝子を標的にしたものである。ほとんどの細菌が保有する遺伝子であるハウスキーピング遺伝子,16S rRNA(リボソームの蛋白合成に関与,真菌の場合は18S/28S rRNA)はよく用いられるハウスキーピング遺伝子のひとつであり,これを検出することで菌の存在が証明できる。実際には,世の中に存在する3万種以上の細菌の多くを網羅できると考えられ,臨床の場でも非常に重要な検査になっている。我々は,この細菌16S PCRとリアルタイム定量PCRを組み合わせた遺伝子検査システムを確立し,細菌性眼内炎や真菌性眼内炎の検査に応用し(図3),その有効性についての報告を行っている[5)6)]。眼感染症の中でもこれらの菌種は急速な経過をたどることがあるので,迅速かつ包括的な診断検査は必要不可欠である。また,ぶどう膜炎などの眼炎症性疾患では免疫抑制剤,特にステロイドが治療の中心となる。しかし,感染性ぶどう膜炎や感染性眼内炎に対してステロイドを単独で投与すると逆に細菌や真菌を眼局所内で増やす結果となる。このブロードレンジPCR検査は感染性ぶどう膜炎や眼内炎を否定するうえでも重要な検査となる。実際,多くの非感染性ぶどう膜炎症例で"感染"をこの検査で否定でき,安心してステロイド投与できるようになっている。このように,原因不明の眼炎症疾患の検体
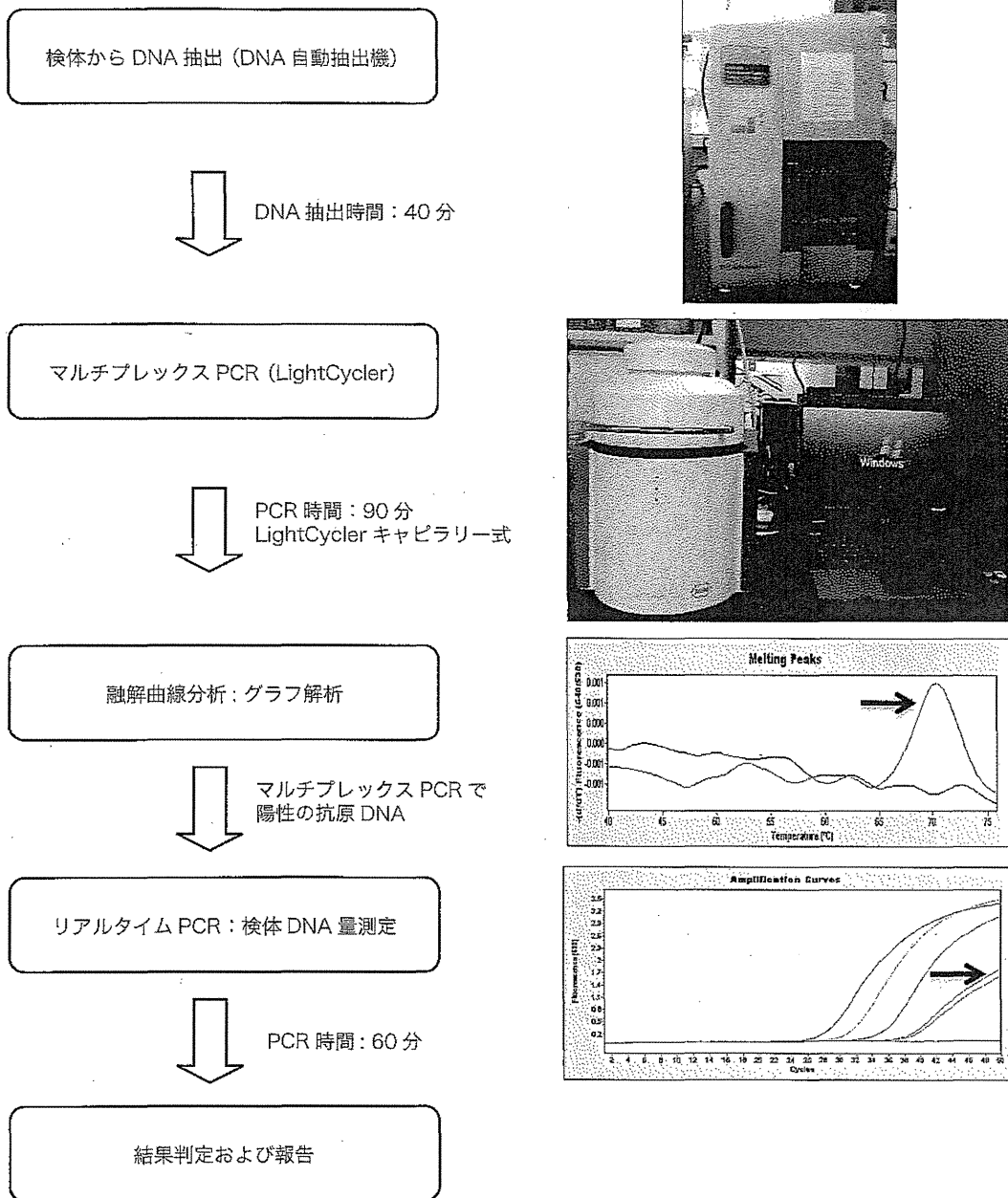
図2　マルチプレックス PCR ＆ リアルタイム PCR 検査の流れ

手順としては DNA 自動抽出機（右上写真）を用いて検体から DNA を抽出する。マルチプレックス PCR（別名，多項目迅速 PCR）検査は，多い場合は 10 種類以上のウイルスなどの外来性抗原を同時に迅速に検出できる新しい PCR 検査法。検体から DNA を抽出後，Accuprime Taq を用いてそれぞれの抗原 DNA 特異的プライマーを混合して，マルチプレックス PCR を行う。数種類の抗原を数本のキャピラリーを用いて同時に検査する（LightCycler キャピラリー式：右中央写真）。PCR 反応後，ハイブリダイゼーションプローブの混合液と PCR 産物を混合し，融解曲線分析を行い，抗原 DNA の同定を行う。右中央のグラフは代表症例で，前房水からマルチプレックス PCR にて HSV2-DNA が検出されたもの（→）。同時に HSV-2 以外の他のヘルペスウイルス DNA（HSV-1，VZV，EBV，CMV，HHV-6，HHV-7，HHV-8）はすべて陰性であることが判明する。マルチプレックス PCR で陽性の場合は定量リアルタイム PCR 検査へ移行する（右下グラフ，→は症例の結果）。
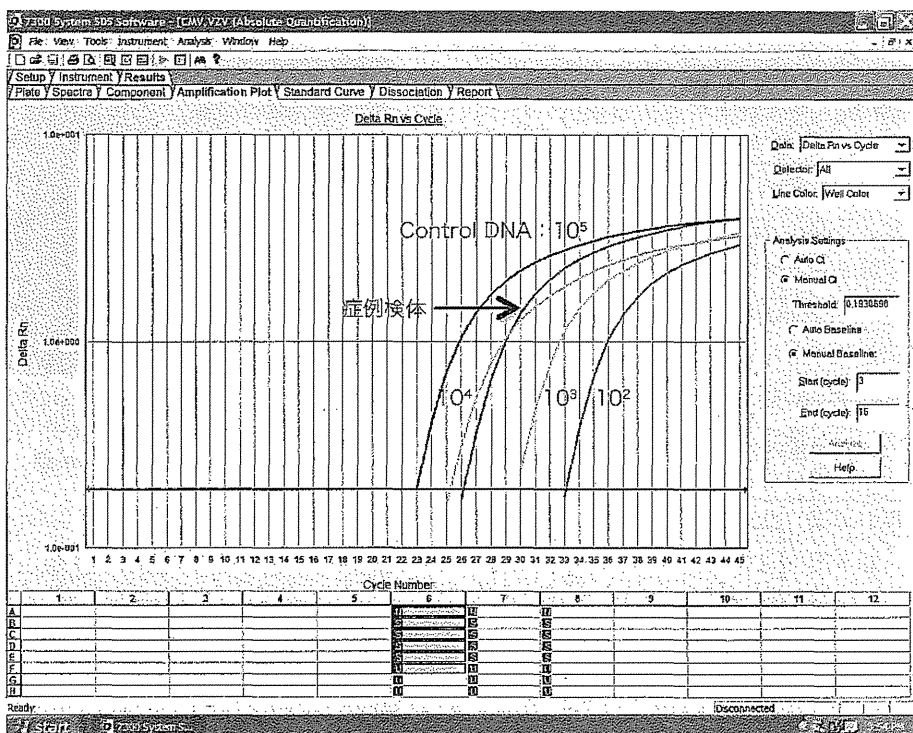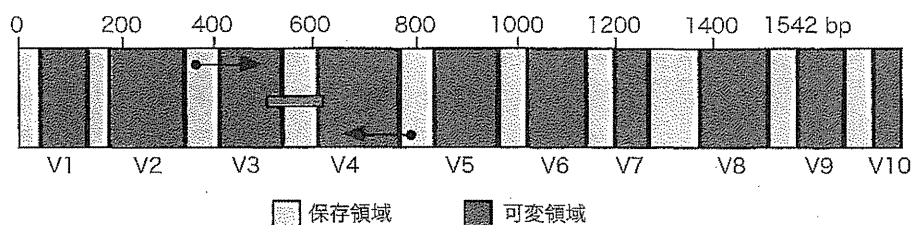
検査項目：細菌16S rDNA, 真菌28S rDNA, カンジダ・アスペルギルス18S rDNAなど

図3 ブロードレンジPCR法

細菌 16S rRNA 領域 (rDNA) の特異的なプライマーと TaqMan プローブを設計し，定量 PCR 検査を構築。同じ原理で，真菌 18S や真菌 28S rRNA 領域の定量リアルタイム PCR もデザインした。細菌の配列には保存領域（非可変領域）と可変領域が交互に存在し，保存領域をプライマーとプローブを用いて PCR で増幅させる。理論的にはヒトに感染する 60〜70％の細菌種をカバーできると考えられている。下グラフはこの定量 PCR で診断された細菌性眼内炎の PCR の結果のグラフ。コピー数の算出方法は，未知 DNA 濃度のテストサンプル（症例検体）と検量線作成のためのスタンダードサンプル（control DNA：10^2, 10^3, 10^4, 10^5）を同じ条件下で PCR をかけて解析し，サンプルの Ct 値を算出して検量線に当てはめることでそのサンプル内の DNA 濃度を知ることができる。

から多種類の外来性抗原感染の有無をスクリーニングできるので，結果的に感染症が除外でき，症例によってはステロイド中心の抗炎症療法を行えるようになる。

V．ブラスト解析を用いて菌種の迅速同定へ

細菌 16S ブロードレンジ PCR での陽性検体は，菌の同定目的でブラスト解析（BLAST：
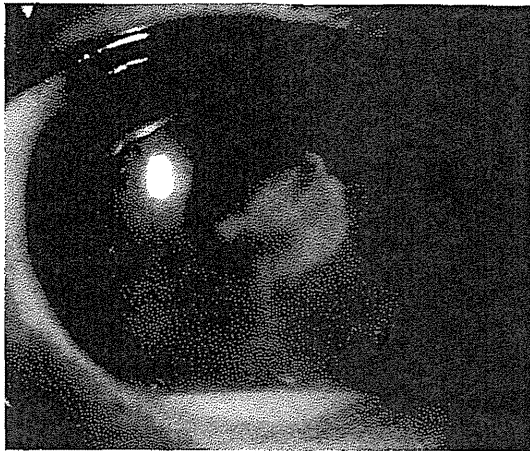
白内障術後眼内炎の症例
PCR 定量：1.4×10^8 copies/m*l*
細菌培養：*Streptococcus oralis*
BLAST 解析：*Streptococcus* sp.

```
>□gb|AY005040.1|  Streptococcus sp. oral strain 7A 16S ribosomal RNA gene, partial
sequence
Length=1474

 Score = 1131 bits (612),  Expect = 0.0
 Identities = 613/614 (99%), Gaps = 0/614 (0%)
 Strand=Plus/Plus

Query  1    TGCAAGTAGAACGCTGAAGCTTGGTGCTTGCACCGAGCGGATCAGTTGCGAACGGGTGAG  60
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Query  601  ACTTGAGTGCAAGA  614
            ||||||||||||||
Sbjct  631  ACTTGAGTGCAAGA  644
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図4 ブラスト解析

細菌16S ブロードレンジ PCR での陽性検体は，菌の同定目的でブラスト解析を行う。プライマーを用いて増幅した PCR 産物を GeneBank データベースでダイレクトシークエンスする。本症例は，白内障術後の感染性眼内炎疑いの症例で，写真のように前房蓄膿がみられる激しい眼内炎症があり，硝子体検体から高コピー数の細菌 DNA が検出された。その後，菌の同定目的にブラスト解析を行った。その結果，細菌培養結果と同じ *Streptococcus* 属が検出されていた（identities＝99％）。

Basic Local Alignment Search Tool) を行う。プライマーを用いて増幅したPCR産物をGeneBankデータベースでダイレクトシークエンスする。100%一致 (あるいは98%以上) する菌を同定菌とする。この検査を用いれば一般的な細菌培養検査より早期に菌種同定まで行える。理論的には細菌や真菌陽性検体のすべてにおいて菌種同定が可能である。**図4**に我々が行ったBLAST解析がその診断に有用であった細菌性眼内炎の症例を提示した。しかし実際にはコストの問題 (人材費, シークエンサーが高額, など) や技術の問題 (検体内のDNA量や質の問題がある場合うまく解析できない, など) があり多くの施設で検査できるようになるにはまだ時間がかかるであろう。

おわりに

ぶどう膜炎・眼内炎の診断はここ数年で急速に進歩した。この新しい検体検査システムの期待される効果として, 難治性眼炎症疾患の原因となる外来性抗原を迅速に短時間で同定することができ, 早期診断・早期治療へとつながる。また, 原因不明であった患者から眼科関連性が不明であった感染抗原が検出されるという新しい知見が得られる可能性もある。さらには原因特定以外に感染性疾患を除外することができることから臨床の場で重要な検査となることが期待される。しかしながら, 偽陽性, 偽陰性の症例が少なからず存在し, PCR検査の結果だけで

診断するのは危険である。実際の診断には, その特徴的な眼所見, 複数の検体検査結果, 治療への反応など多項目のdataで判断するのが望ましい。

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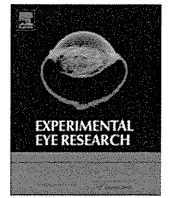
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Development of new therapeutic modalities for corneal endothelial disease focused on the proliferation of corneal endothelial cells using animal models

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ABSTRACT

This review describes our recent attempts to develop new therapeutic modalities for corneal endothelial disease using animal models including non-human primate model in which the proliferative ability of corneal endothelial cells is severely limited, as is the case in humans. First, we describe our attempt to develop new surgical treatments using cultivated corneal endothelial cells for advanced corneal endothelial dysfunction. It includes two different approaches; a "corneal endothelial cell sheet transplantation" with cells grown on a type-I collagen carrier, and a "cell-injection therapy" combined with the application of Rho-kinase (ROCK) inhibitor. Recently, it was reported that the selective ROCK inhibitor, Y-27632, promotes cell adhesion and proliferation and inhibits the apoptosis of primate corneal endothelial cells in culture. When cultivated corneal endothelial cells were injected into the anterior chamber of animal eyes in the presence of ROCK inhibitor, endothelial cell adhesion was promoted and the cells achieved a high cell density and a morphology similar to corneal endothelial cells in vivo. We are also trying to develop a novel medical treatment for the early phase of corneal endothelial disease by the use of ROCK inhibitor eye drops. In rabbit and monkey experiments using partial endothelial dysfunction models, corneal endothelial wound healing was accelerated by the topical application of ROCK inhibitor to the ocular surface, and resulted in the regeneration of a corneal endothelial monolayer with a high endothelial cell density. We are now trying to advance the clinical application of these new therapies for patients with corneal endothelial dysfunction.

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The corneal endothelium is the innermost layer of the cornea, derived from the neural crest, and plays an essential role in the maintenance of corneal transparency via its barrier and pump functions. Since the human corneal endothelium is essentially non-regenerative in vivo, endothelial cell loss due to dystrophy, trauma, or surgical intervention is followed by a compensatory enlargement of the remaining endothelial cells. Thus, there is functional reserve. However, if cell loss is too great the outcome is often irreversible corneal endothelial dysfunction. For many years penetrating kera-toplasty was the only realistic choice of surgery for visual loss due to corneal endothelial dysfunction, but it is not a risk-free treatment. To overcome the problems associated with penetrating keratoplasty, new surgical procedures (i.e. the posterior lamellar keratoplasties) which replace the endothelium without host

corneal trephination have been developed (Gorovoy, 2006; Melles et al., 2000; Price and Price, 2005; Terry and Ousley, 2001). However, irrespective of the selected keratoplasty procedure, corneal endothelial cell loss can be a long-term problem following corneal transplantation using donor tissue (Price et al., 2011; Terry et al., 2008).

The ultimate goal of our research is to develop new surgical and medical treatments for corneal endothelial disease, which provide a healthy corneal endothelium with high cell density. To achieve this we are currently focusing on the proliferation of corneal endothelial cells. Currently, our efforts are aimed at developing feasible medical treatments for the early stage of corneal endothelial dysfunction, such as those that involve the use of ROCK inhibitor eye drops. We have also tried to develop surgical treatments for advanced corneal endothelial dysfunction, such as a cultivated corneal endothelial cell sheet transplantation using a type-I collagen carrier, or a cultivated Descemet-stripping automated endothelial keratoplasty (DSAEK) surgery using a human lamellar graft in animal bullous keratopathy models. At present, we are also investigating a form of cultivated corneal endothelial

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transplantation without the use of a carrier. In this review we report our recent progress toward the development of new therapeutic modalities for corneal endothelial disease focused on the proliferation of corneal endothelial cells using animal models.

1. Cultivated corneal endothelial cell sheet transplantation in a monkey model

Although human corneal endothelial cells are mitotically inactive and are arrested at the G1 phase of the cell cycle (Joyce, 2003), they retain the capacity to proliferate *in vitro* (Engelmann et al., 1988; Miyata et al., 2001; Senoo and Joyce, 2000; Zhu and Joyce, 2004). Some groups, ours included, have worked on developing cultivated human corneal endothelial cell sheet transplantation with (Ishino et al., 2004; Mimura et al., 2004) or without (Sumide et al., 2006) carrier materials, and have demonstrated *in vivo* functionality in a rabbit model. It is known that the proliferative ability of corneal endothelial cells varies among species, and that rabbit corneal endothelial cells proliferate very well even *in vivo*. In contrast, as in humans, the ability of monkey and feline corneal endothelial cells to proliferate is severely limited (Matsubara and Tanishima, 1982; 1983; Tsuru et al., 1984; Van Horn and Hyndiuk, 1975; Van Horn et al., 1977), rendering these species as representative models for corneal endothelial cell research. To this end, our laboratory developed a corneal endothelial dysfunction model in monkeys by mechanical scraping of the endothelium followed by trypan blue staining of the denuded Descemet's membrane. Thereafter we examined the feasibility of cultivated corneal endothelial transplantation. To the best of our knowledge, endothelial research programmes using monkey models for developing new corneal therapies are not established or in widespread use in other laboratories.
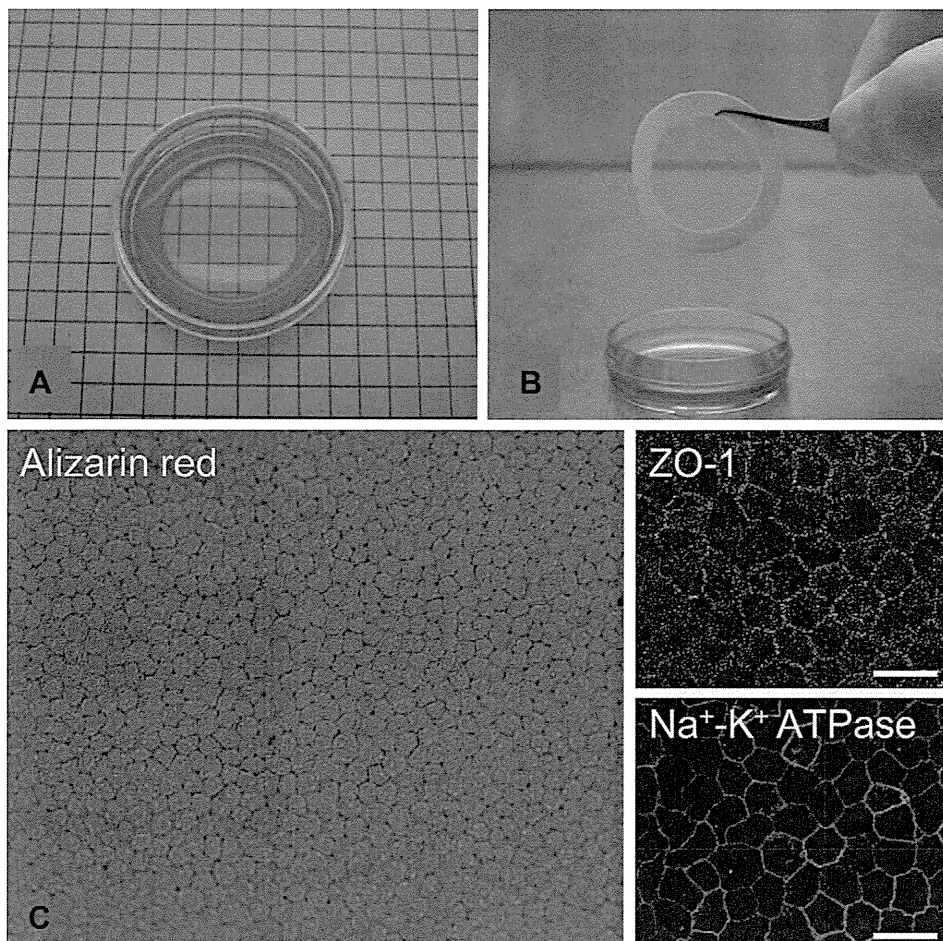
1.1. Cultivated monkey corneal endothelial sheets using collagen type-I as a carrier

Corneas were obtained from cynomolgous monkeys (3–5 years old: estimated comparable human age, 5–20 years) at euthanasia for other research purposes at NISSEI BILIS Co., Ltd. (Ohtsu, Japan), and KEARI Co., Ltd. (Wakayama, Japan). At all times the ARVO guidelines for the use of animals in ophthalmic research were adhered to, as were local and national ethical rules. We cultivated monkey corneal endothelial cells according to a modified protocol for human corneal endothelial cell culture (Ishino et al., 2004; Miyata et al., 2001). In brief, Descemet's membrane was stripped of intact monkey corneal endothelial cells and dissociated using Dispase II. The monkey corneal endothelial cells were cultivated on tissue culture plates coated with cell attachment reagent (FNC coating mix) in culture medium containing DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 ng/ml bFGF. Primary cultures formed confluent layers of hexagonal cells within 14 days, with an average cell density of more than 2500 cells/mm^2. After 3–5 passages on culture plates, confluent subculture cells were seeded onto rehydrated collagen type-I sheets (Fig. 1A, B) at a concentration of 5–10 × 10^2 cells/mm^2. After reaching confluence in one week, cells were kept in culture for an additional two weeks. Alizarin red staining revealed mainly hexagonal, homogeneous cells with an average density of 2240 ± 31 cells/mm^2 (mean ± S.E.) (Fig. 1C). Immunohistochemical staining of ZO-1 and Na$^+$/K$^+$-ATPase revealed that these functional proteins were located at the cell boundaries of the cultivated MCEC sheets (Fig. 1C). Examination by TEM showed a monolayer of endothelial cells similar to that seen in normal *in vivo* corneal endothelium of monkeys (Koizumi et al., 2007).
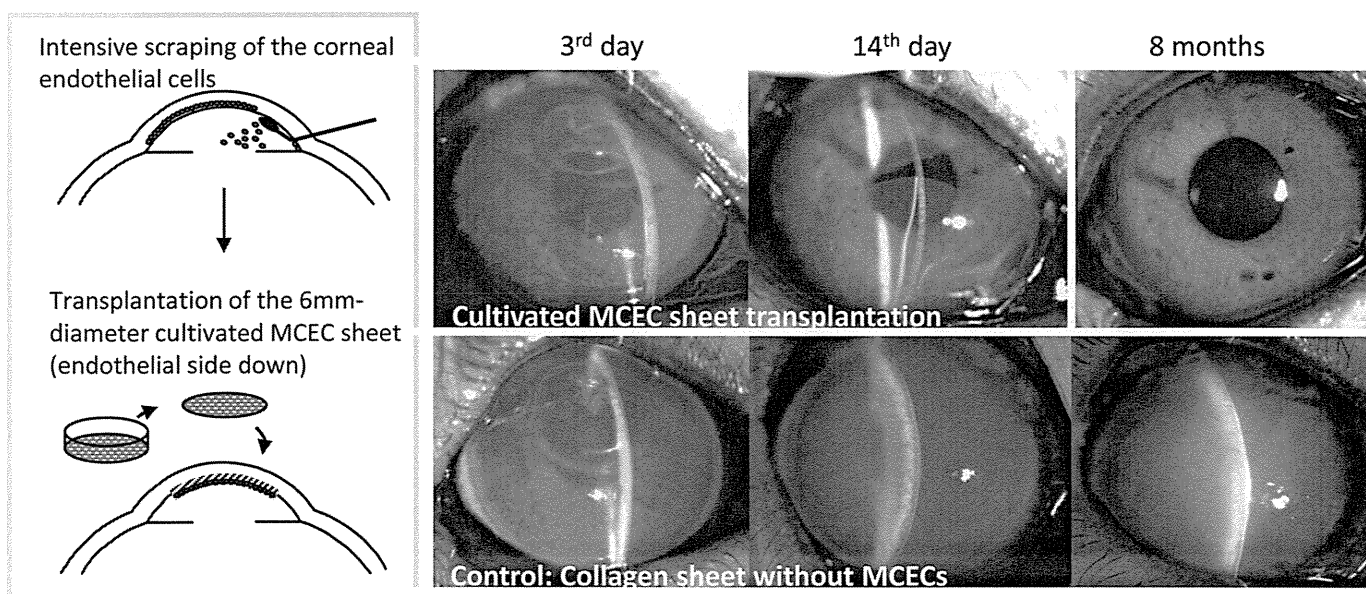
1.2. Transplantation of cultivated monkey corneal endothelial cells on type-I collagen sheets into monkey eyes

Six female cynomolgous monkeys (2.0–2.5 kg) were anesthetized intramuscularly with a mixture of ketamine hydrochloride (5 mg/kg; Sankyo, Tokyo, Japan) and xylazine (1 mg/kg, Bayer, Munich, Germany) followed by inhalation anesthesia with isoflurane. Surgery was carried out in an animal surgery room at the same levels of cleanliness as for human keratoplasty. During surgical procedures, animals were observed by veterinarians monitoring pulse, blood pressure, and partial pressure oxygen. To induce endothelial dysfunction 3-mm limbal-corneal incisions were made in the right eyes of the six monkeys, and then the corneal endothelia were removed by mechanical scraping using a 20G silicone needle, followed by 0.04% trypan blue staining to confirm that all endothelial cells were removed from Descemet's membrane. The scraped area measured at least 9 mm in diameter (the diameter of the cornea is approximately 10 mm). For the posterior graft, a 6 mm-diameter limbal-corneal incision was made and a 6-mm diameter disc of a cultivated monkey corneal endothelial cells on a sheet was brought into the anterior chamber in four eyes of four animals using a lens glide with the corneal endothelial side facing the anterior chamber. In one of the surgeries a DiI labeled cultivated monkey corneal endothelial cell sheet was used. In all cases the limbal-corneal incision was closed with 10-0 nylon interrupted sutures and the cultivated monkey corneal endothelial cell sheet attached to Descemet's membrane by air injection. As controls, a collagen sheet without monkey corneal endothelial cells was transplanted in one eye of one endothelial-dysfunctional animal, and a suspension of cultivated monkey corneal endothelial cells was injected into the anterior chamber in one eye of another. Following surgery we conducted a four-year follow up of corneal clarity (slit-lamp), corneal thickness (ultrasound pacymeter) and *in vivo* corneal endothelial assessment (non-contact specular microscopy).

After surgery, the monkey corneal endothelial cell sheet was attached to Descemet's membrane and remained attached in all experimental eyes (Fig. 2, 3rd day). In the two control eyes (i.e. sheet only, and cell-injection) severe corneal edema was observed after surgery. In the postoperative day 5–14 period in the operative group the monkey corneal endothelial cell sheets became detached from Descemet's membrane and dropped into the anterior chamber in all of three eyes. Nevertheless, these corneas achieved full clarity (Fig. 2, 14th day), which was maintained at least up to eight months after surgery (Fig. 2, 8 months). These experiments revealed that whereas irreversible corneal edema and neovascularization, similar to that seen in advanced bullous keratopathy in humans, occurred following endothelial scraping, eyes which received cultivated monkey corneal endothelial cell sheet transplantation recovered their clarity and became less edematous with time. Ours is the first study to investigate the feasibility of cultivated corneal endothelial sheet transplantation in a primate allograft model in which corneal endothelial cells have low *in situ* proliferative potential. Interestingly, in the successful post-surgery animals corneal endothelial cells more than 2000 cells/mm^2 were observed by specular microscopy six months postoperatively. In some additional experiments, we found DiI labeled donor corneal endothelial cells on the host Descemet's membrane outside of the sheet transplantation area in the early postoperative period (Koizumi et al., 2008). This was unexpected, and the mechanism of wound healing was not as we initially envisaged; i.e. we did not expect to see migration or proliferation of monkey corneal endothelial cells in the eye. This finding lead us to speculate that, once cultivated *in vitro*, monkey corneal endothelial cells might recover their proliferative ability and are able to migrate onto the host Descemet's membrane and proliferate *in vivo*. This provides us with

Fig. 1. Cultivated monkey corneal endothelial cell sheet on a collagen type-I carrier. (A, B) Primary culture of monkey corneal endothelial cells subcultured on a collagen type-I sheet. The cultivated corneal endothelial sheet is transparent and easy to handle. (C) Alizarin red staining of the cultures reveals mainly hexagonal, homogeneous cells with a density of 2800 cells/mm^2. The cultivated monkey corneal endothelial cells on a collagen type-I sheets expressed ZO-1 and Na$^+$/K$^+$-ATPase at their lateral cell membranes (green). Propidium iodide was used to visualize the cell nuclei (red). (Scale bars: 50 μm). (Reprinted with some modification from Koizumi et al. (2007) with permission from the Association for Research in Vision and Ophthalmology). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Schematic image of the surgical procedure and slit-lamp photographs after cultivated monkey corneal endothelial cell sheet transplantation. In cultivated monkey corneal endothelial cell sheet transplanted eyes, the sheet was attached to Descemet's membrane on the 3rd day and a clear cornea was recovered by two weeks. The eyes remain clear up to the most recent observation, even though the sheet was detached from the posterior cornea.

a potential new concept for the treatment of corneal endothelial dysfunction, which involves not just transplantation of a cultivated corneal endothelial sheet, but the transplantation of endothelial cells which have the renewed ability to proliferate *in vivo*. Our long-term observation using non-contact specular microscopy suggest that corneal endothelial cell proliferation was stopped when the cells reached confluence probably due to contact-inhibition (Koizumi et al., 2008).
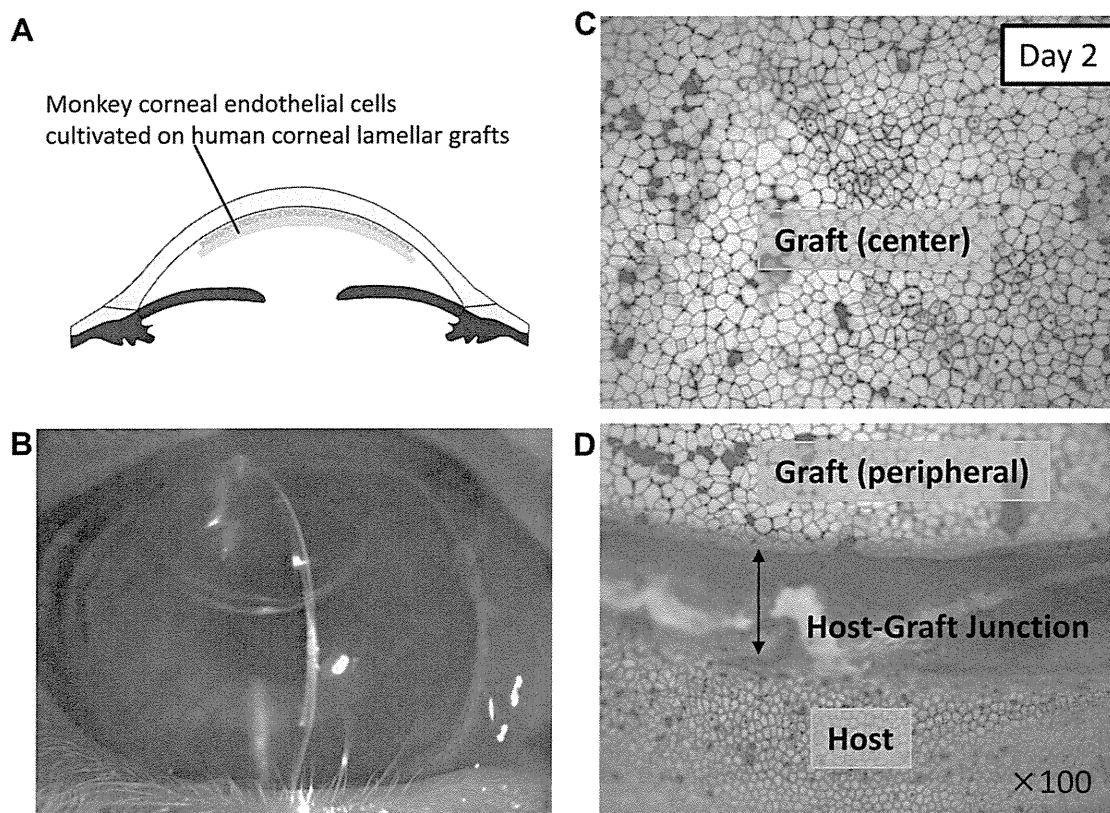
1.3. Cultivated-DSAEK surgery in an animal models

Another concept for corneal endothelial repair is the use of donor posterior stromal tissue as a carrier for cultivated corneal endothelial cells. We examined the feasibility of using human corneal lamellar graft tissue as a carrier for the cultivation of corneal endothelial cells. Descemet's membrane, with an intact corneal endothelium, was removed from human corneal tissue obtained from an American eye bank (SightLife, Seattle, WA) for research purposes. Corneal lamellar grafts (150–200 μm thick and 8 mm in diameter) were made from the posterior stroma using a Moria microkeratome. They were preserved in the freezer at $-20\ ^\circ$C for four weeks before being seeded with monkey corneal endothelial cells (2×10^5 cells/graft) and cultivated for three weeks. Under general anesthesia, the corneal endothelium and Descemet's membrane were removed by scraping with a 20G silicone needle, and the lamellar grafts with monkey corneal endothelial cells were transplanted onto the posterior cornea of one monkey and one rabbit using a Busin glide in a similar procedure to DSAEK (Fig. 3A). The allograft in the monkey eye was performed for the long-term observation of the surgical outcome; the xenograft in the rabbit was performed for the short-term (up to 48 h) evaluation of donor endothelial cell damage during the graft insertion process. Histological examination showed that confluent monkey corneal endothelial cells were established on the human corneal lamellar graft at a density of 2240 cells/mm², and the protein expression of ZO-1 and Na⁺/K⁺-ATPase was confirmed by immunohistochemistry. The day after surgery, the graft was well attached to the host corneal stroma and mild corneal edema was observed (Fig. 3B). Histological examination of the rabbit eye with alizarin red staining showed no donor endothelial damage due to the graft insertion (Fig. 3C). A long-term observation in the monkey model indicated that the cornea recovered its clarity by postoperative week two. Pre-experimental corneal thickness of the monkey was 473 μm, and the corneal thickness one week postoperatively was 1042 μm, which decreased to 600 μm at the eight month time point. One month after surgery the cornea was clear and remained so seven months later (Fig. 4). No signs of rejection were detected with the use of minimal immunosuppressive treatment (steroid ointment applied once daily for one month). Control eyes from which corneal endothelial cells were scraped showed severe bullous keratopathy after surgery, which did not recover during the observation period. By non-contact specular microscopy, polygonal cells were observed at a density of 2178 cells/mm² at two months and 1841 cells/mm², 8 months after surgery (Fig. 4D). Though our results are still preliminary, they suggest the possibility of cultivated corneal endothelial cell transplantation using a corneal lamellar graft.

2. Cell-injection therapy using a selective Rho-kinase (ROCK) inhibitor

Direct transplantation of cultivated corneal endothelial cells onto the posterior cornea by "cell-injection into the anterior

A

Monkey corneal endothelial cells cultivated on human corneal lamellar grafts

B

C Day 2

Graft (center)

D Graft (peripheral)

Host-Graft Junction

Host ×100

Fig. 3. Cultivated monkey corneal endothelial transplantation (cultivated-DSAEK) in a rabbit corneal endothelial dysfunction model. Using a microkeratome, a human corneal lamellar graft was created onto which monkey corneal endothelial cells were cultivated for 3 weeks. The graft was successfully transplanted into rabbit eyes. Donor monkey corneal endothelial cells were detected both at the center and peripheral part of the graft were not damaged by the graft insertion process. In addition, donor monkey corneal endothelial cells were clearly distinguished from host (rabbit) corneal endothelial cells with acellular area (host-graft junction).
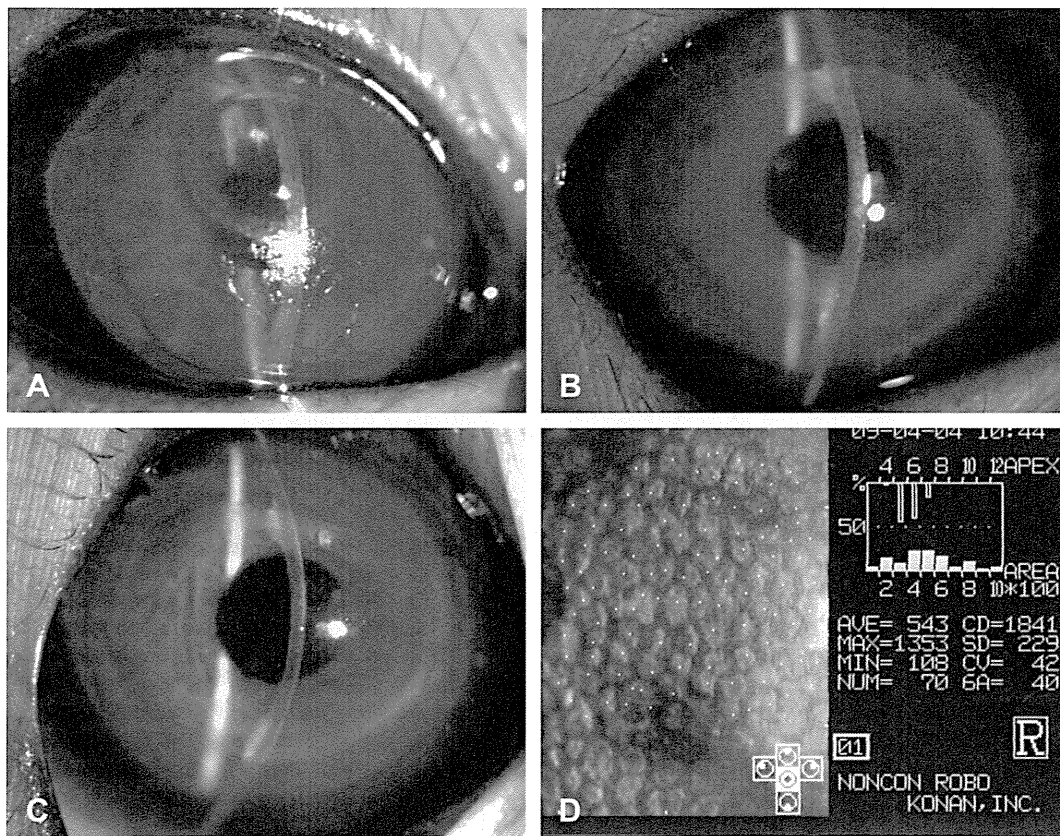
Fig. 4. Cultivated-DSAEK in a monkey corneal endothelial dysfunction model. DSAEK graft composed of monkey corneal endothelial cells cultivated on a human lamellar graft was transplanted into endothelially denuded monkey eyes. The graft was well-attached to the host corneal stroma 24 h after transplantation (A). One month after surgery, the cornea became clear (B) and remained so for up to 8 months (C). Corneal endothelial cells at a density of 1841 cells/mm^2 were observed (D).

chamber" has been considered an ideal method of reconstructing the corneal endothelial layer of patients with endothelial dysfunction. To develop an effective method to deliver cultivated corneal endothelial cells to the posterior cornea, magnetic attachment of iron-powder (Mimura et al., 2003; Mimura et al., 2005a) or superparamagnetic microspheres (Patel et al., 2009) incorporated in cultivated corneal endothelial cells has been attempted. These approaches work in a rabbit transplantation model or an organ culture model of the human eye, but have not yet been clinically applied. Now, we are trying to develop a cell-injection therapy combined with the use of a ROCK inhibitor which promotes corneal endothelial cell adhesion onto the posterior cornea.

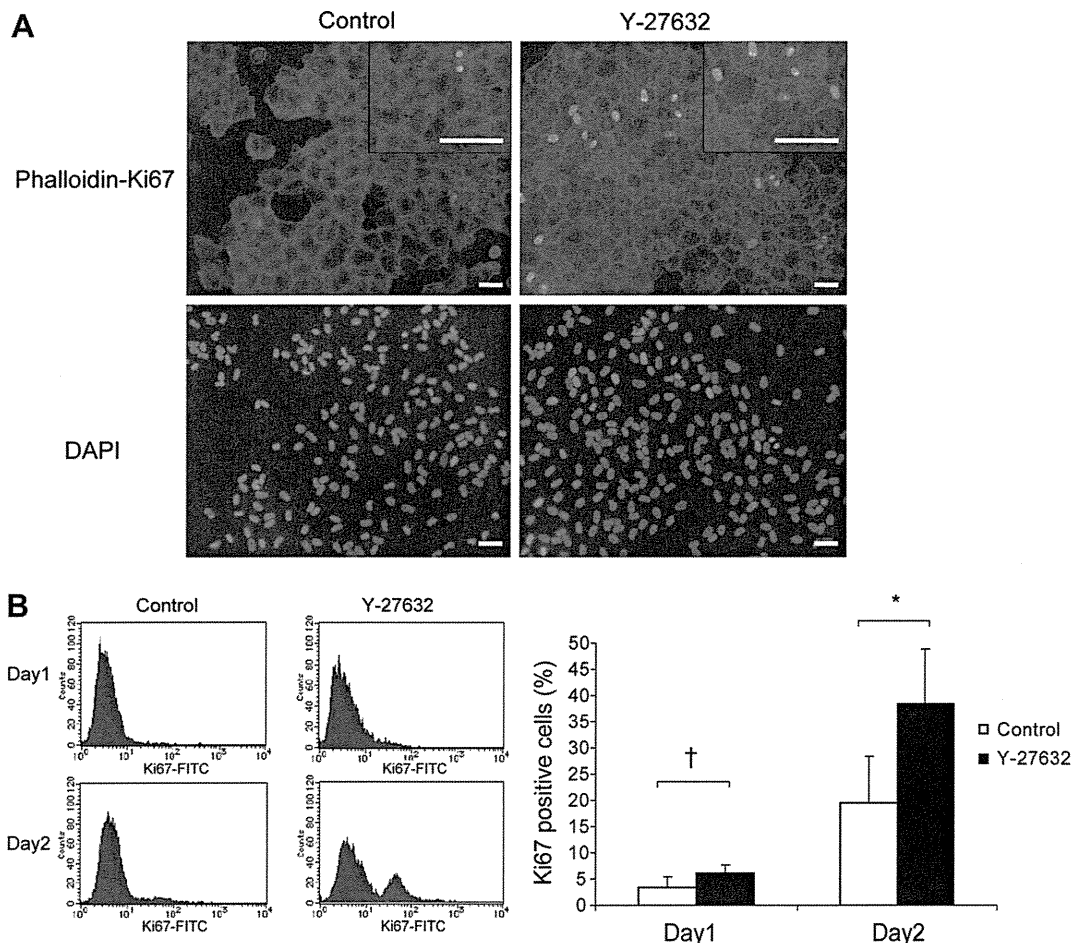### 2.1. ROCK inhibitor and corneal endothelial cells in vitro

Rho-kinase (ROCK) is a serine/threonine kinase, which serves as a target protein for Rho and has been initially characterized as a mediator of the formation of RhoA-induced stress fibers and focal adhesions. The Rho/ROCK pathway is involved in regulating the cytoskeleton, cell migration, cell proliferation, and apoptosis (Coleman et al., 2004; Hall, 1998; Olson et al., 1995; Riento and Ridley, 2003). In the cornea field, it is reported that ROCKs are involved in corneal epithelial differentiation, cell-cycle progression, and cell–cell adhesion (Anderson et al., 2002; Anderson and SundarRaj, 2001; SundarRaj et al., 1998). ROCKs also influence the phenotype of stromal cells, their cytoskeleton reorganization, and cell–matrix interactions (Anderson et al., 2004; Harvey et al., 2004; Kim et al., 2006; Kim and Petroll, 2007; Lakshman et al., 2007; Petroll et al., 2004). In terms of the corneal endothelium, the

Rho/ROCK pathway has an influence on the wound healing and barrier function (D'Hondt et al., 2007; Satpathy et al., 2005; 2004).

In 2007, our collaborators reported that a selective ROCK inhibitor, Y-27632, diminished the dissociation-induced apoptosis of human embryonic stem cells (Watanabe et al., 2007). We subsequently examined the effect of Y-27632 on primate corneal endothelial cells in vitro and found that the inhibition of Rho/ROCK signaling by Y-27632 inhibited dissociation-induced apoptosis and promoted the adhesion and proliferation of monkey corneal endothelial cells (Okumura et al., 2009) (Fig. 5). We are now applying commercially available Y-27632 purchased from Wako Pure Chemical Industries (Osaka, Japan) to human corneal endothelial cells in culture, as well as developing cell-injection therapies. We have no commercial interest with the use of Y-27632 of this project.

2.2. Cell-injection therapy combined with ROCK inhibitor in animal models

Rabbit corneal endothelial cells were cultured as previously described and 2 x 10^5 cells were injected into the anterior chambers of rabbit eyes from which host corneal endothelial cells had been scraped off. Cells were injected with or without 100 μM of ROCK inhibitor, Y-27632. The eye of each animal was kept in the face-down position for 3 h following injection (Mimura et al., 2005b), and it was found that when Y-27632 was present the donor cells became nicely attached onto the host Descemet's membrane and the host cornea recovered its transparency. This attachment was not so advanced in the cell-injected eyes without the inclusion of Y-27632. Histological examination confirmed that cell adhesion

Fig. 5. ROCK inhibitor (Y-27632) promotes the proliferation of monkey corneal endothelial cells. (A) Double-immunostaining of Ki67 and actin fibers; passaged monkey corneal endothelial cells were cultured for 48 h and stained successively with Ki67 and phalloidin. Ki67 (green), actin (red), and DAPI (blue). Insets are higher magnification. Scale bars 250 μm. (B) Ki67 positive cells were analyzed by flow cytometry. Monkey corneal endothelial cells were subcultured for 1 or 2 days, and stained successively with Ki67. The numbers of Ki67 positive cells were significantly elevated in the presence of Y-27632 on both day 1 and 2 ($^{†}P < 0.05$, $^{*}P < 0.01$). Data are expressed as the mean $\pm$ SE ($n = 6$). (Reprinted from Okumura et al. (2009) with permission from the Association for Research in Vision and Ophthalmology). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

was enhanced by Y-27632, and that the healthy polygonal mono-layer was reconstructed in the cell-injected eyes with this selective ROCK inhibitor. Unlike in the Y-27632-treated eyes, corneal edema persisted in the cell-injected eyes without Y-27632 and most of the endothelial cells showed fibroblastic changes with elongated cell shapes (in submission). Stratification was also detected by phal-loidin staining. Repeated experiments in monkeys with longer observation periods (in submission) have confirmed that the procedure results in a high density of corneal endothelial cells formed into healthy polygonal monolayers.

## 3. Eye drop treatment for corneal endothelial disease

A pure medical treatment for corneal endothelial disease has been sought for a long time by ophthalmologists and patients. It has been reported that human corneal endothelial cells in organ cultured corneas proliferate in response to wounding, as they are if released from contact-inhibition by EDTA (Senoo et al., 2000). It has also been reported by our group that connexin43 knockdown by siRNA promotes corneal endothelial proliferation and wound healing in a rat corneal endothelial injury model (Nakano et al., 2008). However, to the best of our knowledge no pharmacolog-ical agent is in use clinically for the treatment of corneal endothelial dysfunction.

3.1. ROCK inhibitor eye drop treatment in in vivo animal models

With the purpose of developing a pharmacological treatment for corneal endothelial dysfunction, we examined the effect of Y-27632 ROCK inhibitor eye drops on corneal endothelial cells using an animal corneal endothelial injury model. The target of the pharmacological treatment is the early phase of corneal endothelial disease in patients such as those with Fuchs' dystrophy, or those with corneal endothelial damage induced by intraocular surgeries who nevertheless retain some healthy corneal endothelial cells.

First, we made a partial endothelial injury by transcorneal freezing using a 7 mm diameter stainless-steel cryo-probe in rabbits. After injury, 10 mM of Y-27632 diluted in 50 μl of phosphate-buffered saline was applied topically in one eye of each animal six times daily for 2 days, while PBS was applied in the other eye as a control. In the Y-27632-treated eyes less corneal edema was observed by slit-lamp microscopy and ultrasound pachymetry. Histology showed that the mean wound area of Y-27632-treated eyes was significantly smaller than that of control eyes (Fig. 6). These results demonstrate that the topical administration of selec-tive ROCK inhibitor, Y-27632, as an eye drop has the potential to enhance corneal endothelial wound healing (Okumura et al., 2011).

To establish the application of ROCK inhibitor eye drop in a clinical setting we have recently conducted a similar experiment
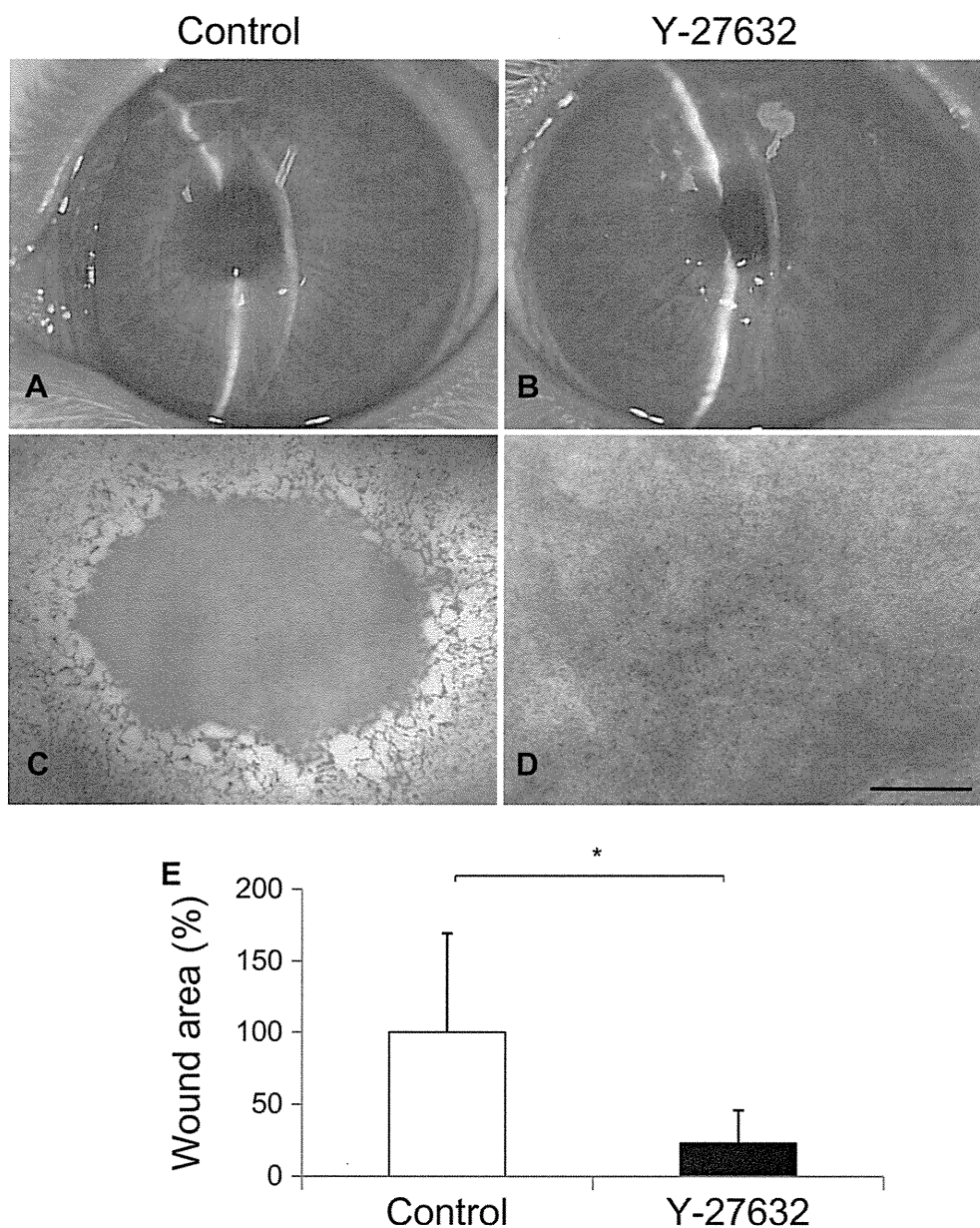
Fig. 6. Effects of ROCK inhibitor Y-27632 eye drops in a rabbit model. The center of the corneal endothelium was damaged by transcorneal freezing, after which Y-27632 was applied topically for 2 days. Slit-lamp microscopy revealed that corneal transparency was higher in the Y-27632 group compared to the control group (A, B). Alizarin red staining shows that corneal endothelial wound healing was promoted in the Y-27632 group compared to the control group (D, E). The mean wound area of the Y-27632 group was significantly smaller than that of the control group after 48 h (23.1 ± 22.9% as a ratio of control; *$P < 0.05$) (E). Scale bar: 500 μm. (Reprinted from Okumura et al. (2009) with permission from the British Journal of Ophthalmology). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using a partial corneal endothelial dysfunction model in monkey. Early indications show that the topical application of Y-27632 following cryo-injury enables the corneal endothelium to retain a high cell density in cynomolgus monkeys during a 1-month observation period, with reproducibility confirmed in six eyes (in submission).

4. Discussion: toward the clinical application of new therapies

There are a numbers of research papers which report protocols for human corneal endothelial cell culture, however, it is still difficult for us to consistently expand usable amounts of human corneal endothelial cells which retain a healthy morphology and high cell density. Recently, we have used a selective ROCK inhibitor, Y-27632, in our human corneal endothelial cell culture and it has improved the culture results. Based on these findings, we are now planning to apply cell-injection therapy using human cultivated corneal endothelial cells combined with Y-27632 to advanced corneal endothelial dysfunction patients in clinical setting. In line with ethical considerations, endothelial cell expansion has great potential to be helpful in the reconstruction of the posterior cornea with possibilities for genetically-engineered endothelial cells or HLA matched corneal endothelial cells to help avoid the allograft rejection.

Regarding the Y-27632 eye drop treatment, we have obtained the approval of the Institutional Review Board of Kyoto Prefectural University of Medicine and have started a clinical pilot study of

ROCK inhibitor eye drop treatment for bullous keratopathy and have confirmed its safety and ability to recover corneal endothelial cell density in some patients with specific conditions. Currently, we are accumulating evidence regarding the mechanism of ROCK inhibitor eye drops; our current understanding is that it works by stimulating proliferation of the patients' corneal endothelium (unpublished data).

Given the burden on individuals and healthcare providers as a result of corneal endothelial dysfunction, the discovery and introduction into clinical practice of new pharmacological agents which are safe and effective is highly desirable. Such an achievement will potentially reduce the over-reliance on corneal transplantation and improve the quality of life and vision for many.

Acknowledgments

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Stem Cells, Tissue Engineering, and Hematopoietic Elements

ROCK Inhibitor Converts Corneal Endothelial Cells into a Phenotype Capable of Regenerating *In Vivo* Endothelial Tissue

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Corneal endothelial dysfunction accompanied by visual disturbance is a primary indication for corneal transplantation. We previously reported that the adhesion of corneal endothelial cells (CECs) to a substrate was enhanced by the selective ROCK inhibitor Y-27632. It is hypothesized that the inhibition of ROCK signaling may manipulate cell adhesion properties, thus enabling the transplantation of cultivated CECs as a form of regenerative medicine. In the present study, using a rabbit corneal endothelial dysfunction model, the transplantation of CECs in combination with Y-27632 successfully achieved the recovery of corneal transparency. Complications related to cell injection therapy, such as the abnormal deposition of the injected cells as well as the elevation of intraocular pressure, were not observed. Reconstructed corneal endothelium with Y-27632 exhibited a monolayer hexagonal cell shape with a normal expression of function-related markers, such as ZO-1, and Na$^+$/K$^+$-ATPase, whereas reconstruction without Y-27632 exhibited a stratified fibroblastic phenotype without the expression of markers. Moreover, transplantation of CECs in primates in the presence of the ROCK inhibitor also achieved the recovery of long-term corneal transparency with a monolayer hexagonal cell phenotype at a high cell density. Taken together, these results suggest that the selective ROCK inhibitor Y-27632 enables cultivated CEC-based therapy and that the modulation of Rho-ROCK signaling activity serves to enhance cell engraftment for cell-based regenerative medicine. *(Am J Pathol 2012, 181:268–277; http://dx.doi.org/10.1016/j.ajpath.2012.03.033)*

Corneal endothelial dysfunction is a major cause of severe visual impairment, because corneal endothelial cells maintain corneal transparency through their barrier and Na$^+$-K$^+$ transport system. Highly effective surgical techniques to replace corneal endothelium (eg, Descemet's stripping endothelial keratoplasty) have been developed,[1,2] aimed at replacing penetrating keratoplasty for overcoming pathological dysfunctions of corneal endothelial tissue. Several research groups, including ours, have devoted an intensive amount of effort in an attempt to establish new treatment methods suitable for a practical clinical intervention to repair corneal endothelial dysfunctions.[3–6] Because corneal endothelium is composed of a monolayer and is technically difficult to transplant into the anterior chamber as a structurally flexible cell sheet, those research teams cultured corneal endothelial cells (CECs) on substrates such as collagen sheets and amniotic membrane.

The injection of cultivated cells has been reported for the treatment of a number of organs associated with degenerative diseases such as the heart,[7] vessels,[8] pancreas,[9] and cartilage.[10] In regard to corneal endothelium, it is known that injected cultured CECs appear to be washed off by aqueous humor flow, thus resulting in the poor adhesion of those injected cells onto the corneal tissue. To develop an effective method for delivering cultivated CECs to the posterior cornea, the magnetic attachment of iron powder or superparamagnetic microspheres incorporated in the cultivated CECs has been attempted. This method has been shown to work in a

rabbit transplantation model[11,12] and in an organ culture model of the human eye[13]; however, these methods have yet to be applied in the clinical setting.

Cell adhesion is known to be mediated through transmembrane adhesion molecules linked to the intracellular cytoskeleton. In addition to the structural function, these adhesion molecules reportedly serve as a platform for the interplay with the surrounding environments.[14,15] Rho GTPase proteins are key modulators of cytoskeletal dynamics that occur after cell adhesion.[16–18] It has been reported that Rho GTPases induce a specific type of actin cytoskeleton through mediating downstream effectors mDia and Rho-associated kinase (ROCK), and that they regulate a variety of cellular functions.[19] Cell adhesion, motility, and cell morphogenesis are thought to be determined by the balance between mDia and ROCK activities.[19] We recently reported that the adhesion of CECs to a substrate was enhanced by inhibiting Rho/ROCK signaling.[20] This finding coincides well with those of other studies that demonstrated that Rho-ROCK signaling negatively regulates the integrin-mediated adhesion of monocytes, and that the inhibition of ROCK by a selective ROCK inhibitor upregulates adhesion.[17,18] These features have led us to hypothesize that the inhibition of ROCK signaling may provide a way to manipulate the cell adhesion property of cultivated corneal endothelium to the extent practical for regenerative medicine.

In this current study, in two animal models (rabbit and primate) of corneal endothelial dysfunctions, the transplantation of cultivated CECs in combination with ROCK inhibitor Y-27632 successfully achieved the recovery of corneal transparency. Inhibition of the ROCK signaling manipulated the adhesion property of the cultivated CECs. Moreover, the injected CECs functioned sufficiently well to reconstruct the corneal endothelium with an appropriate cell density, morphology, and expression of function-related markers. This novel treatment strategy may provide a new therapeutic modality for corneal-endothelium–associated pathological dysfunctions.

Materials and Methods

Materials

Rabbit eyes were purchased from Funakoshi Corporation (Tokyo, Japan). Alizarin red S stain and selective ROCK inhibitor Y-27632 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and basic fibroblast growth factor (bFGF), Vybrant DiI cell-labeling solution, Alexa Fluor 594-conjugated phalloidin, Alexa Fluor 488-conjugated phalloidin, Alexa Fluor 488-conjugated goat anti-mouse IgG, anti-vinculin antibody, ROCK1 Stealth RNAi (HSS109291, HSS109292, and HSS109293), ROCK2 Stealth RNAi (HSS114106, HSS114107, and HSS114108), Stealth RNAi negative control medium GC #2, and Lipofectamine RNAiMAX were purchased from Life Technologies (Carlsbad, CA). Dispase II was purchased from Roche Applied Science (Penzberg, Germany). FNC Coating Mix was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). Ki-67 monoclonal antibody, propidium iodide (PI), and Cytochalasin D were purchased from Sigma-Aldrich Co. (St. Louis, MO). ZO-1 polyclonal antibody was purchased from Zymed Laboratories (South San Francisco, CA). α–Smooth muscle actin (α-SMA) monoclonal antibody was purchased from Thermo Fisher Scientific (Kalamazoo, MI). Na^+/K^+-ATPase monoclonal antibody was purchased from Upstate Biotech (Lake Placid, NY). DAPI was purchased from Vector Laboratories (Burlingame, CA). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI).

Animal Experiment Approval

In all experiments, animals were housed and treated in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit experiments were performed at Doshisha University (Kyoto, Japan) according to the protocol approved by that university's Animal Care and Use Committee (approval no. 0831). The monkey experiments were performed at the Research Center for Animal Life Science at Shiga University of Medical Science (Otsu, Japan) according to the protocol approved by that university's Animal Care and Use Committee (approval no. 2008-10-5).

Cell Culture of Rabbit and Monkey CECs

Ten rabbit eyes were used for the rabbit CECs (RCECs) culture. Eight corneas from four cynomolgus monkeys (3 to 5 years of age; estimated equivalent human age: 5 to 20 years) housed at the Nissei Bilis Co. (Otsu, Japan) and the Keari Co. (Wakayama, Japan), respectively, were used for the monkey CECs (MCECs) culture. The RCECs and MCECs were cultivated as described previously.[3,20] Briefly, Descemet's membrane with CECs was stripped and incubated in 0.6 U/mL of Dispase II to release the CECs. After a 60-minute incubation at 37°C, the CECs obtained from individual corneas were resuspended in culture medium and plated in one well of a six-well plate coated with cell attachment reagent (FNC Coating Mix). All primary cell cultures and serial passages of CECs were performed in growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL bFGF. CECs were cultured in a humidified atmosphere at 37°C in 5% CO_2. The culture medium was changed every 2 days. When cells reached confluency in 10 to 14 days, they were rinsed in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, and passaged at ratios of 1:2 to 1:4. Cultivated CECs derived from both rabbit and monkey corneas at passages 3 through 5 were used for all experiments. To confirm the cultivation of the CECs, the morphology and density of the cultivated cells were compared with normal *in vivo* rabbit CECs examined using a noncontact specular microscope (Noncon Robo, SP-8800; Konan Medical, Nishinomiya,

Japan) and stained with Alizarin red. In some experiments, to investigate the fate of the injected CECs *in vivo*, the CECs were labeled with fluorescein by use of the Vybrant DiI cell-labeling solution according to the manufacturer's protocol.

Rabbit Corneal Endothelial Dysfunction Model

To create rabbit corneal endothelial pathological dysfunction models, the lenses of both eyes of 12 Japanese white rabbits were removed under general anesthesia by use of the Alcon Series 20000 Legacy Surgical System (Alcon, Fort Worth, TX) to deepen the anterior chamber. Next, the corneal endothelium of each of those eyes was mechanically scraped with a 20-gauge silicone needle (Soft Tapered Needle; Inami, Tokyo, Japan) from Descemet's membrane as described previously.[3,4] The scraped area was then confirmed by 0.04% trypan blue staining during surgery. In the preliminary experiments, we confirmed that Descemet's membrane was intact, the mechanically scraped area had no cells on Descemet's membrane, and that residual CECs were detected in only a 500- to 600-μm area at the edge of Descemet's membrane.

Injection of Cultivated CECs into the Rabbit Eyes

To evaluate the injection of cultivated CECs with ROCK inhibitor, RCECs at a density of 2.0 × 10⁵ cells were suspended in 200 μl DMEM supplemented with 100 μmol/L of Y-27632 and then injected into the anterior chamber of the eyes of the above-described corneal endothelial dysfunction rabbit model. RCECs with Y-27632 were injected into the right eyes of six rabbits, and RCECs without Y-27632 were injected into the right eyes of the other six rabbits. After the injection, the eyes of those 12 rabbits were kept in the face-down position for 3 hours under general anesthesia. The left eyes of those 12 rabbits in which the corneal endothelium was removed mechanically were used as a control. One rabbit injected with RCECs with Y-27632 and one rabbit injected with RCECs without Y-27632 were euthanized 3 hours after injection for histological examination. The corneal appearance of the other 10 rabbits was examined daily by use of a slit-lamp microscope for the first week, and then once every 2 days for the following 2 weeks. Those 10 rabbits were then euthanized for histological examination. Corneal thickness was determined by use of an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan), and the mean of 10 measured values was then calculated (up to a maximum thickness of 1200 μm, the instrument's maximum reading). Intraocular pressure was measured by use of a pneumatonometer (30 Classic; Reichert, NY).

Histological Examination of Rabbit Eyes After CEC Injection

Sections (6-μm) of corneal specimens obtained from the 10 rabbits euthanized 2 weeks after injection were embedded in OCT compound and then fixed in 4% formaldehyde. Differential interference contrast (DIC) images

and fluorescence images of DiI-labeled cells were obtained by use of a fluorescence microscope (TCS SP2 AOBS; Leica Microsystems, Welzlar, Germany). For flat-mount examinations, whole corneal specimens were fixed in 4% formaldehyde and incubated in 1% bovine serum albumin (BSA) to block any nonspecific binding. To evaluate the effect of Y-27632 on the adhesion property of the cells, corneas obtained from the 2 rabbits euthanized 3 hours after injection were examined by actin staining performed with a 1:400 dilution of Alexa Fluor 488–conjugated phalloidin. Actin staining was used to evaluate the cellular morphology. The cell nuclei were then stained with PI. To investigate the phenotype of the reconstructed corneal endothelium obtained from the 10 rabbits euthanized 2 weeks after injection, immunohistochemical analyses of actin, α-SMA, ZO-1, Na⁺/K⁺-ATPase, DiI, and Ki-67 were performed. α-SMA was used to evaluate the fibroblastic change. ZO-1, a tight-junction–associated protein, and Na⁺/K⁺-ATPase, the protein associated with pump function, were used for function related markers of CECs. The α-SMA, ZO-1, and Na⁺/K⁺-ATPase staining were performed with a 1:200 dilution of α-SMA monoclonal antibody, ZO-1 polyclonal antibody, and Na⁺/K⁺-ATPase monoclonal antibody, respectively. Ki-67 (a cell-proliferation–related maker) staining was performed using a 1:400 dilution of anti-mouse Ki-67 antibody. For the secondary antibody, a 1:2000 dilution of Alexa Fluor 488–conjugated goat anti-mouse IgG was used. Cell nuclei were then stained with DAPI, and the slides were inspected by fluorescence microscopy.

Effect of Y-27632 on MCECs in Culture

MCECs were cultured at a density of 2.5 × 10⁴ cells/cm² on Lab-Tek Chamber Slides (NUNC A/S, Roskilde, Denmark). Actin staining was performed with 1:400-diluted Alexa Fluor, as described above, after 24 hours of seeding, and vinculin staining was performed using 1:200-diluted vinculin after 3 hours of seeding. The number of attached MCECs was evaluated by use of CellTiter-Glo Luminescent Cell Viability Assay performed according to the manufacturer's protocol. The MCECs were seeded with a different concentration of Y-27632 at the density of 1.0 × 10³ cells onto 96-well plates, and the number of adhered MCECs at 24 hours after seeding was then measured by use of a Veritas Microplate Luminometer (Promega). In addition to ROCK signaling inhibition, to evaluate the effect of inhibition of actin polymerization on CECs adhesion, MCECs were seeded with a different concentration of cytochalasin D at the density of 1.0 × 10³ cells onto 96-well plates, and the number of adhered MCECs at 24 hours after seeding was then measured. Five samples were prepared for each group.

To determine the adhesion property of the MCECs onto the basement membrane, the cells were seeded onto rabbit corneas in which the corneal endothelium was mechanically denuded and the basement membranes were exposed. The cells were seeded at the density of 2.5 × 10⁴ cells/cm² suspended in culture medium supplemented with or without 10 μmol/L Y-27632. Actin staining was performed at 3 hours after seeding in the same