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別添１

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　総括研究報告書

研究代表者　三村　達哉

平成２１（２００９）年　３月

別添２

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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）
（総括）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　東京大学大学院医学系研究科眼科学教室　助教

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。この研究として以下のテーマを掲げる。研究機関内の臨床応用の実用化を目指して研究を行う。

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、積極的に研究を行いたいと思う。

　以下のテーマについて研究する予定である。

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）

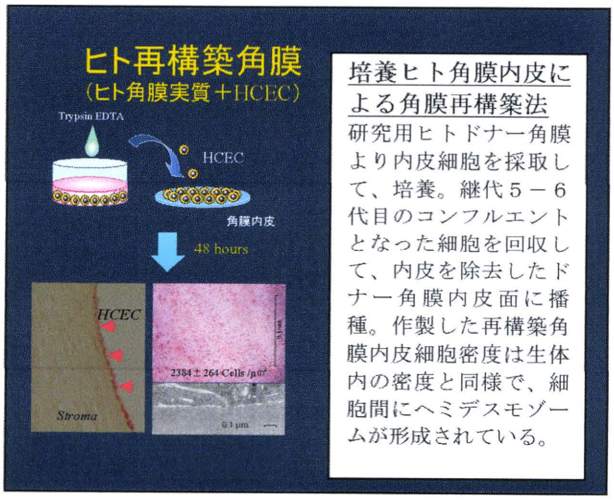
すべての研究は東京大学倫理委員会の承認を得て行う。角膜幹細胞を用いた人工角膜の人への移植を前提とした研究であるため、倫理委員会の指針、動物実験の対する指針、および研究に関与するあらゆる倫理指針を遵守する。動物の取り扱いは、苦痛を伴うものは必ず全身麻酔下に行い、両眼が失われる可能性のある場合は片眼のみに処置を行う。全ての実験において動物は the Association for Research in Vision and Ophthalmology の規約および、実験動物の飼養及び保管等に関する基準（総理府）に従って扱う。人を扱う研究では，ヘルシンキ宣言（世界医師会総会 World Medical 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。

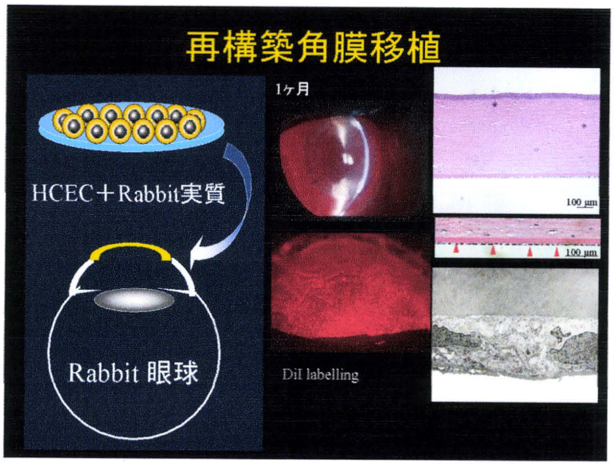


ヒト再構築角膜

（ヒト角膜実質＋HCEC）

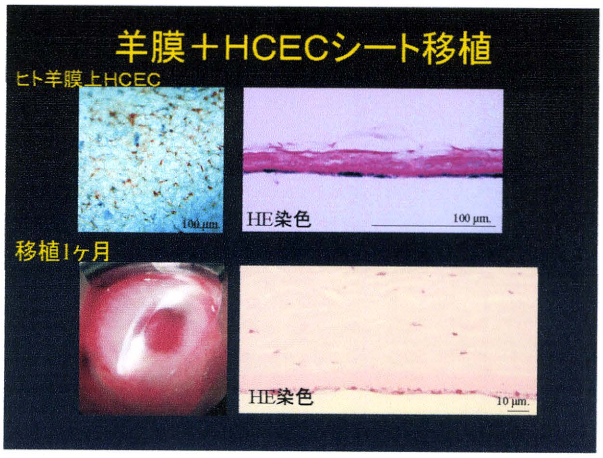
培養ヒト角膜内皮による角膜再構築法

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。



再構築角膜移植

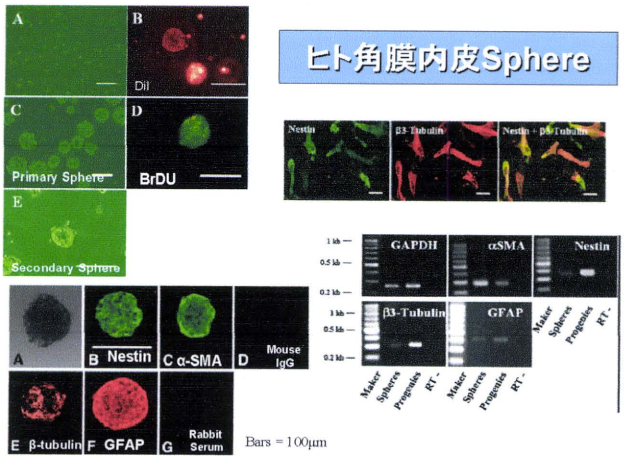
再構築角膜を家兎角膜に移植後 1 ヶ月目
角膜透明性が維持され、内皮面に蛍光標識した内皮細胞が観察される。



羊膜＋HCECシート移植

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。

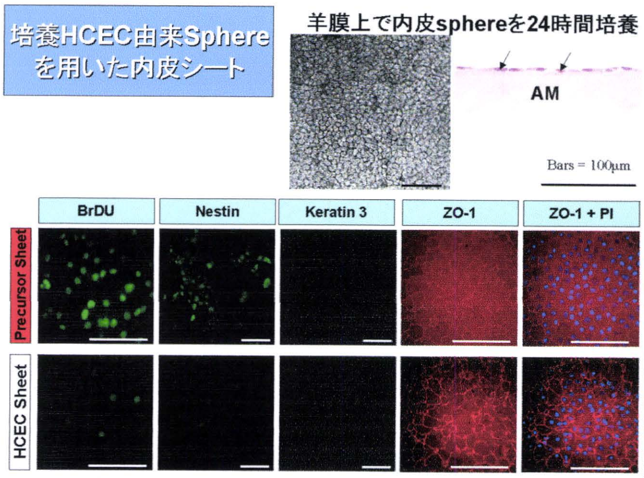
ヒト角膜内皮Sphere



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。

培養HCEC由来Sphereを用いた内皮シート

羊膜上で内皮sphereを24時間培養



ヒト角膜内皮細胞由来のスフェアーを用いた内皮シートの作成。

従来の培養内皮細胞を用いて作成していたシート(HCEC sheet)と比較して、スフェアー由来のシート(Precursor sheet)における内皮細胞は BrDUを取り込み、多くの細胞が Nestinを強く発現している。また細胞間蛋白 ZO1で染色しますと細胞の形態は良好で細胞密度もは生体内の正常ヒト角膜内皮細胞と同等であった。

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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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
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H．知的財産権の出願・登録状況

1. 特許取得　国内 3　国際特許 1
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
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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

II．研究成果の刊行に関する一覧表

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研究成果の刊行に関する一覧表（続き）

Mimura T, Usui T, Yamagami S, Funatsu H, Noma H, Honda N, Fukuoka S, Shirakawa R, Hotta H, Amano S.	Subconjunctival hemorrhage and conjunctivochalasis.	Ophthalmology		In press	2009
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Tissue engineering of corneal stroma with rabbit fibroblast precursors and gelatin hydrogels

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Purpose: To isolate fibroblast precursors from rabbit corneal stroma using a sphere-forming assay, to engineer corneal stroma with the precursors and gelatin, and to establish the therapeutic application of precursors in a rabbit corneal stroma.
Methods: In the in vitro study, a sphere-forming assay was performed to produce precursors from rabbit corneal stroma. Corneal stroma was engineered by cultivating precursors in porous gelatin for one week. In the in vivo study, the engineered corneal stromal sheet with precursors (precursor/gelatin group) or with fibroblasts (fibroblast /gelatin group) or without cells (gelatin group) was transplanted to a pocket of rabbit corneal stroma. Gene expression and extracellular matrix production were examined immunohistochemically in each group one week and four weeks after surgery.
Results: In the in vitro study, cells in the spheres were BrdU-positive, and their progeny were keratocan-positive. The study also showed that the corneas transplanted with a porous gelatin sheet did not show any opacity four weeks after transplantation in any group. In the gelatin sheet of the precursor/gelatin group, a more intense expression of type I collagen was observed relative to the other two groups four weeks after the surgery.
Conclusions: Our findings demonstrate that the transplantation of fibroblast precursors combined with gelatin hydrogel into the corneal stroma is a possible treatment strategy for corneal stromal regeneration.

Although corneal transplantation has achieved clinical success, there is a shortage of corneal donors worldwide. To solve this problem, researchers have attempted to tissue-engineer the cornea. Among the three components of the cornea (epithelium, stroma, and endothelium), tissue-engineered corneal epithelial cell sheets have been used clinically to create the corneal epithelium in patients with total limbal stem cell deficiencies [1-4]. Tissue-engineered corneal endothelial cell sheets have successfully restored corneal transparency in animal models [5-10] and are ready to be used in clinical cases. In contrast, none of tissue-engineered corneal stroma have been deemed clinically feasible, although several kinds of tissue-engineered corneal stroma have been reported [11].

For successful tissue engineering, stem cells and progenitor or precursor cells, which possess proliferative capacity and multilineage developmental potential, are proposed to be an effective cell source. Regenerative stem cells or precursors can be detected in various adult tissues with a sphere-forming assay, including the central nervous system [12], bone marrow [13], skin [14,15], retina [16], and corneal endothelium [8-10,17-20]. Stem and progenitor cells retain their regenerative potential and may promote rapid wound healing compared with differentiated fibroblasts after they are transplanted to recipients. The use of adult stem cells or precursors presents an ideal strategy for tissue regeneration and engineering [21]. Despite these many successes in the isolation and characterization of stem cells from various tissues, relatively few studies have investigated the efficacy of stem cell transplantation therapy. Using a sphere assay, we succeeded in isolating precursor cells from human and rabbit corneal stroma [20,22,23]. In this study, we have constructed a substitute for corneal stroma using fibroblast precursors and porous gelatin hydrogels in vitro by tissue engineering and have investigated the feasibility of transplantation of the engineered corneal stroma in a rabbit model.

METHODS

Sphere-forming assay: Rabbits were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Sixteen corneas were excised from eyes of eight New Zealand White rabbits weighing 2.0–2.4 kg (Saitama Experimental Animals Inc., Saitama, Japan) under deep anesthesia. The entire corneal epithelium and endothelium with Descemet's membrane were mechanically removed. The stroma was cut into small pieces approximately 1.0 mm in diameter and the pieces were incubated overnight at 37 °C in basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). Subsequently, the tissue pieces were dissociated into single

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cells with 0.2% EDTA. Dissociated single cells were used for sphere-forming assay or culture of fibroblast. The basal medium was composed of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27 (Invitrogen, Carlsbad, CA), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich), and 40 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich). The sphere-forming assay was used for primary cell culture [24,25]. Cells were cultured in the basal medium containing a methylcellulose gel matrix (0.8%; Wako, Osaka, Japan) at a density of 10 viable cells/µl in the uncoated wells of 60 mm culture dishes. For passaging, primary spheres (collected on day 7) were dissociated into single cells with 0.5% EDTA, and cells were cultured at a density of 10 cells/µl in basal medium containing 0.8% methylcellulose.

Differentiation of sphere colonies: Individual primary spheres (collected on day 7) were transferred to 13 mm glass coverslips coated with 50 µg/ml poly-L-lysine (PLL; Sigma-Aldrich, Tokyo, Japan) and 10 µg/ml fibronectin (BD Biosciences, Billerica, MA) after each glass coverslip was put in 24 wells. To promote differentiation, 1% fetal bovine serum (FBS) was added to the basal medium, and the culture was continued for another seven days.

Culture of corneal fibroblast: Isolated fibroblasts were counted with a hemocytometer. Dissociated single primary fibroblasts were plated at a density of 5.0×10^4 cells/ml in a 60 mm tissue culture dish and cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) for seven days.

Immunocytochemistry of keratocan in spheres and progenies: Immunocytochemical examination of the seven-day spheres and their progeny was performed after seven days of adherent culture on the glass coverslips. Cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline (PBS) for 10 min. After washing in PBS, the cells were incubated for 30 min with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS, which contained 0.3% Triton X-100 (BSA/TBST; Rohm & Haas, Philadelphia, PA) to block nonspecific binding. Next, the cells were incubated for 2 h at room temperature with the following primary antibodies diluted in BSA/PBST: goat anti-keratocan polyclonal antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-conjugated mouse anti-5-bromo2'-deoxyuridine (BrdU)/fluorescence mAb (1:100; Roche Diagnostics, Basel, Switzerland). After being washed in PBS, the cells were reacted for 1 h at room temperature with fluorescence-labeled goat anti-rabbit IgG (Alexa Fluor 594, 1:400; Molecular Probes, Eugene, OR) as the secondary antibodies for the anti-keratocan antibody. Finally, fluorescence was detected by observation under a fluorescence microscope (model BH2-RFL-T3 and BX50; Olympus, Tokyo, Japan).

Preparation of gelatin hydrogels and electron microscopic observation: Porous gelatin hydrogels were prepared through the chemical cross-linking of aqueous gelatin solution with glutaraldehyde according to the method described elsewhere [26,27]. Briefly, an aqueous gelatin solution mixed with glutaraldehyde was cast into a polypropylene dish followed by the cross-linking reaction at 4 °C for 12 h. The hydrogel samples were stirred in 100 mM aqueous glycine solution at 37 °C for 1 h and washed with double-distilled water. Following the washing with sterilized saline, the hydrogel samples were trephined with a 5.0 mm diameter trephine (Kai Medical, Gifu, Japan). The structure of the gelatin hydrogel was observed using scanning electron microscopy.

Sphere or fibroblast seeding onto gelatin hydrogels and cell culture: Ten primary fibroblast spheres (50 µm or more in diameter) cultured for seven days or 100,000 primary cultured fibroblasts were applied to the gelatin hydrogels after which the gelatin hydrogels were placed in 5.0 mm diameter wells and centrifuged at 1,000 rpm (176x g) for 10 min to promote the attachment of cells to gelatin hydrogels. The tissue-engineered samples were then maintained in the basal medium containing 10% FBS in a 24 well culture dish for seven days after which the medium containing debris was removed. The primary spheres (50 µm of the diameter) contained approximately 300 cells. In some experiments, primary spheres were labeled with a fluorescent cell tracker (CM-DiI; C-7000; Molecular Probes) to trace their localization as described elsewhere [7].

Transplantation of gelatin hydrogels with corneal fibroblast precursors: A lamellar dissection was made in 24 eyes of 24 rabbits (Figure 1A,B). The engineered corneal stroma was implanted into a mid-stroma corneal pocket without suture fixation. Twenty-four rabbits were divided into three groups, the gelatin group (n=8) transplanted with gelatin hydrogels alone, the fibroblast/gelatin group (n=8) transplanted with fibroblasts cultured on gelatin hydrogels, and the precursor/gelatin group (n=8) transplanted with fibroblast precursors cultured on gelatin hydrogels.

Histological examination and immunohistochemistry of extracellular matrix: One week (n=2 in each group) or four weeks (n=6 in each group) after transplantation, the rabbits were sacrificed with an overdose of intravenous pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan). Each cornea was excised and bisected. The divided corneas or the seven-day cultured gelatin hydrogel sheets for transplantation were fixed in 4% paraformaldehyde, embedded in OCT compound (Tissue-Tek®; Miles Laboratories, Naperville, IL), and then frozen at −20 °C. Cryostat sections were cut into pieces 6 µm thick and stained with hematoxylin and eosin. For immunohistochemical staining, the sections were treated with 3% hydrogen peroxide in PBS (Sigma-Aldrich) for 15 min and then rinsed in PBS. After incubation with normal goat serum for 10 min at room temperature, tissue sections were incubated with primary antibodies (goat anti-rabbit type I collagen, type IV collagen, laminin, and vimentin polyclonal

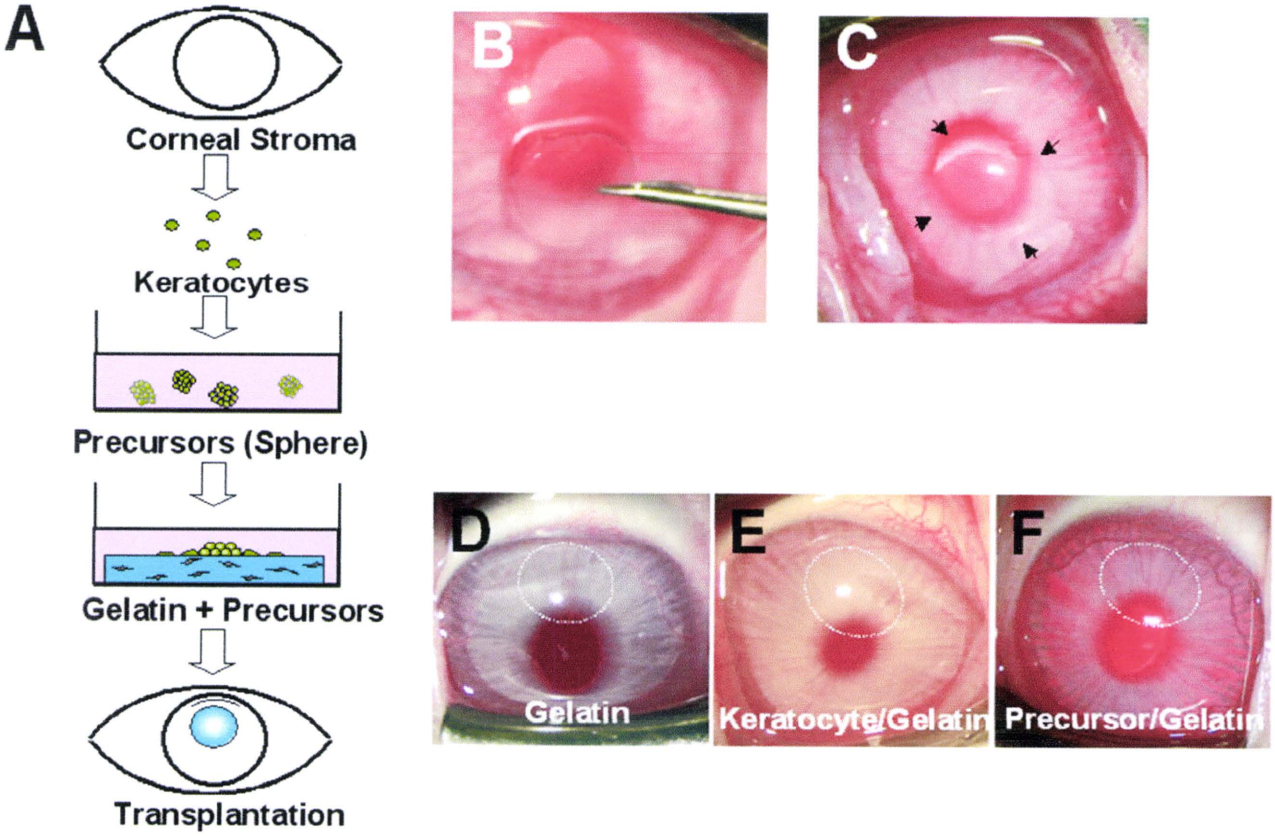


Figure 1. Schematic illustration and clinical findings. **A**: Fibroblast precursors were isolated from the rabbit corneal stroma using a sphere-forming assay. Corneal stroma was engineered by cultivating precursors in porous gelatin for one week. The engineered corneal stromal sheet with precursors was transplanted in a pocket of rabbit corneal stroma. **B**,**C**: Gelatin hydrogels (gelatin group), gelatin hydrogels with corneal fibroblasts (fibroblast/gelatin group), or gelatin hydrogels with corneal fibroblast precursors (precursor/gelatin group) were implanted into the corneal stroma (indicated by arrows in **C**). **D-F**: Representative photographs of corneas four weeks after transplantation in each group are shown. No corneal opacity and no rejection were observed in any group four weeks after transplantation (indicated by dotted white circles in **D-F**).

antibodies; all from Santa Cruz Biotech) at a concentration of 3 µg/ml overnight at 4 °C. Immunoreactivity was detected by the streptavidin-biotin-peroxidase method using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan). The final reaction product was visualized using 3,3′-diaminobenzidine tetrahydrochloride.

Immunohistochemistry of CD34 and nestin: In the precursor/gelatin group, frozen sections (8 µm) were stained with mouse anti-CD34 mAb (1:100; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) or mouse anti-nestin mAb (1:400; BD Biosciences, San Jose, CA) for 2 h and incubated with fluorescence-labeled goat anti-mouse IgG (Alexa Fluor 488, 1:200; Molecular Probes) for 30 min at room temperature. After several washings with PBS, the sections were coverslipped using anti-fading mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and were observed under the fluorescein microscope.

RESULTS

Isolation of sphere colonies and secondary sphere formation: Corneal stroma was disaggregated into single cells, which were cultured for seven days. A photograph of a representative sphere is shown in Figure 2A. Many cells within the sphere colonies were positive for BrdU, indicating active DNA synthesis (Figure 2B). The cells from primary spheres primarily differentiated into fibroblast-like cells (Figure 2C). Secondary spheres were generated from dissociated primary spheres (Figure 2D). The primary spheres' diameters are larger than those of the secondary spheres as shown in Figure 2A,D. Additionally, replating to generate secondary sphere colonies was less efficient than the generation of primary spheres [23]. These results indicate that the precursor cells had a limited proliferative capacity.

Immunocytochemical analysis of extracellular matrix in gelatin hydrogels: Scanning electron microscopy demonstrated porous structure of the gelatin hydrogels (Figure 3A). Corneal fibroblasts or fibroblast precursors were

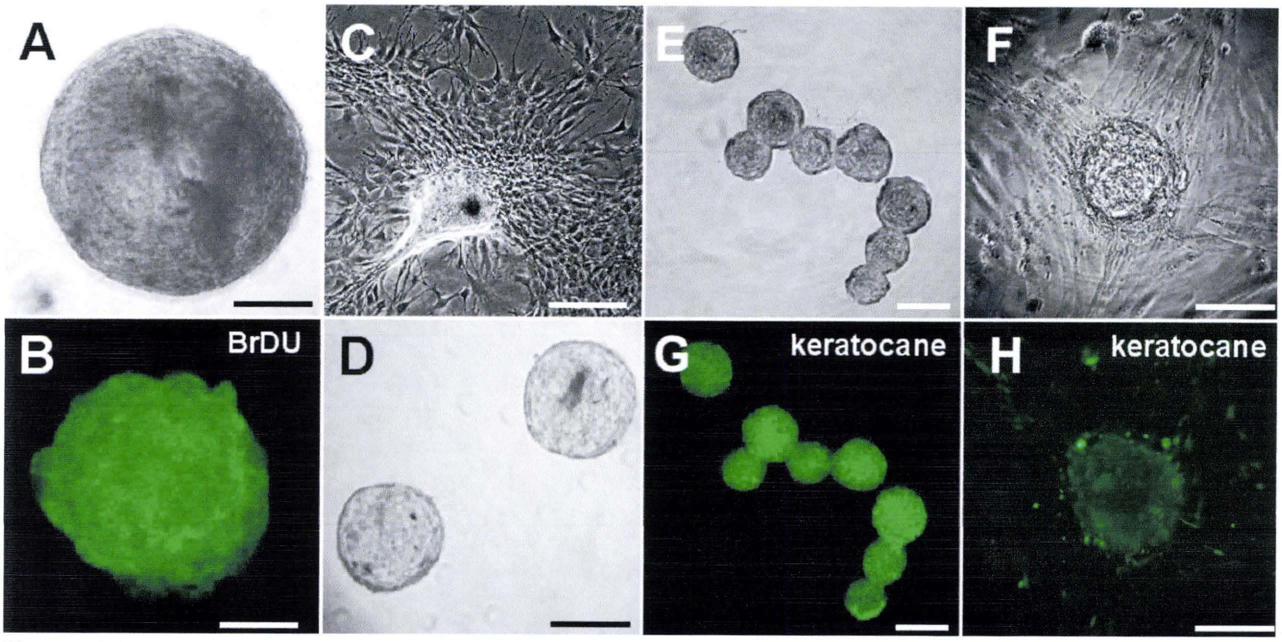


Figure 2. Sphere formation from rabbit corneal fibroblasts. **A**: A representative day 7 sphere from rabbit corneal fibroblasts had a diameter of approximately 300 μm. **B**: Each primary sphere was positive for BrdU on day 7. **C**: The differentiated progeny from the primary sphere showed a typical fibroblast-like morphology. **D**: Secondary spheres were generated from dissociated primary spheres. **E** and **F**: Day 7 spheres were positive for keratocan. **G** and **H**: Progenies derived from the sphere are positive for keratocan. Scale bar=100 μm.

cultured on porous gelatin hydrogels for seven days, and the expression of vimentin, a marker of mesenchymal cells, as well as extracellular matrix (ECM) production were evaluated. The positive staining with vimentin suggests that the progenitor cells were not converted into inflammatory cells. Vimentin expression was more intense in porous gelatin hydrogels with fibroblast precursors than it was in those with corneal fibroblasts (Figure 3B). Weak expression of laminin was also seen in the gelatin hydrogels with fibroblast precursors while no expression was observed in those with corneal fibroblasts (Figure 3B). Little expression of types I and IV collagen was detected in the porous gelatin hydrogels with corneal fibroblasts or fibroblast precursors (Figure 3B).

Clinical observation after surgery: The therapeutic use of precursors derived from corneal fibroblasts was investigated in a rabbit model (Figure 1A-C). As shown in the representative anterior segment photographs from the gelatin group (transplanted with gelatin hydrogels alone, Figure 1D), the fibroblast/gelatin group (transplanted with gelatin hydrogels with corneal fibroblasts, Figure 1E), and the precursor/gelatin group (transplanted with gelatin hydrogels with corneal fibroblast precursors, Figure 1F), the corneas were clear and showed no edema and no cell infiltration of the stroma in all of the groups. No apparent immunological reactions were observed during the follow-up period. No side effects including an increase in intraocular pressure, corneal neovascularization, corneal ulcer, or corneal infection were detected during the observation period.

Histological examination and analysis of extracellular matrix production with immunohistochemistry: The corneas were excised from the eyes either one week or four weeks after transplantation. The expressions of vimentin and ECM molecules such as laminin, type I collagen, and type IV collagen were more intense in the precursor/gelatin group than the other groups after one week (Figure 4A). Their expression increased after four weeks in the precursor/gelatin group (Figure 4B).

Expression of progenitor cell markers in the transplanted gelatin hydrogels: We examined the expression of CD34, which is a well known hematopoietic stem/progenitor cell marker and is also expressed by all differentiated keratocytes [28], and nestin, a neural progenitor cell marker in the precursor/gelatin group, four weeks after transplantation. Many CD34-positive cells were detected in and around the transplanted gelatin hydrogels (Figure 5A). Nestin-positive cells were also observed in the transplanted gelatin hydrogels (Figure 5B). No CD34-positive or nestin-positive cells were seen in the other groups in the transplanted gelatin hydrogels (data not shown).

DISCUSSION

We established the therapeutic application of precursors in a rabbit corneal stroma using fibroblast precursors and gelatin hydrogels as the substitute carrier of corneal stroma. Cultured precursors derived from adult rabbit stroma retain the essential fibroblast morphology. The transplanted gelatin

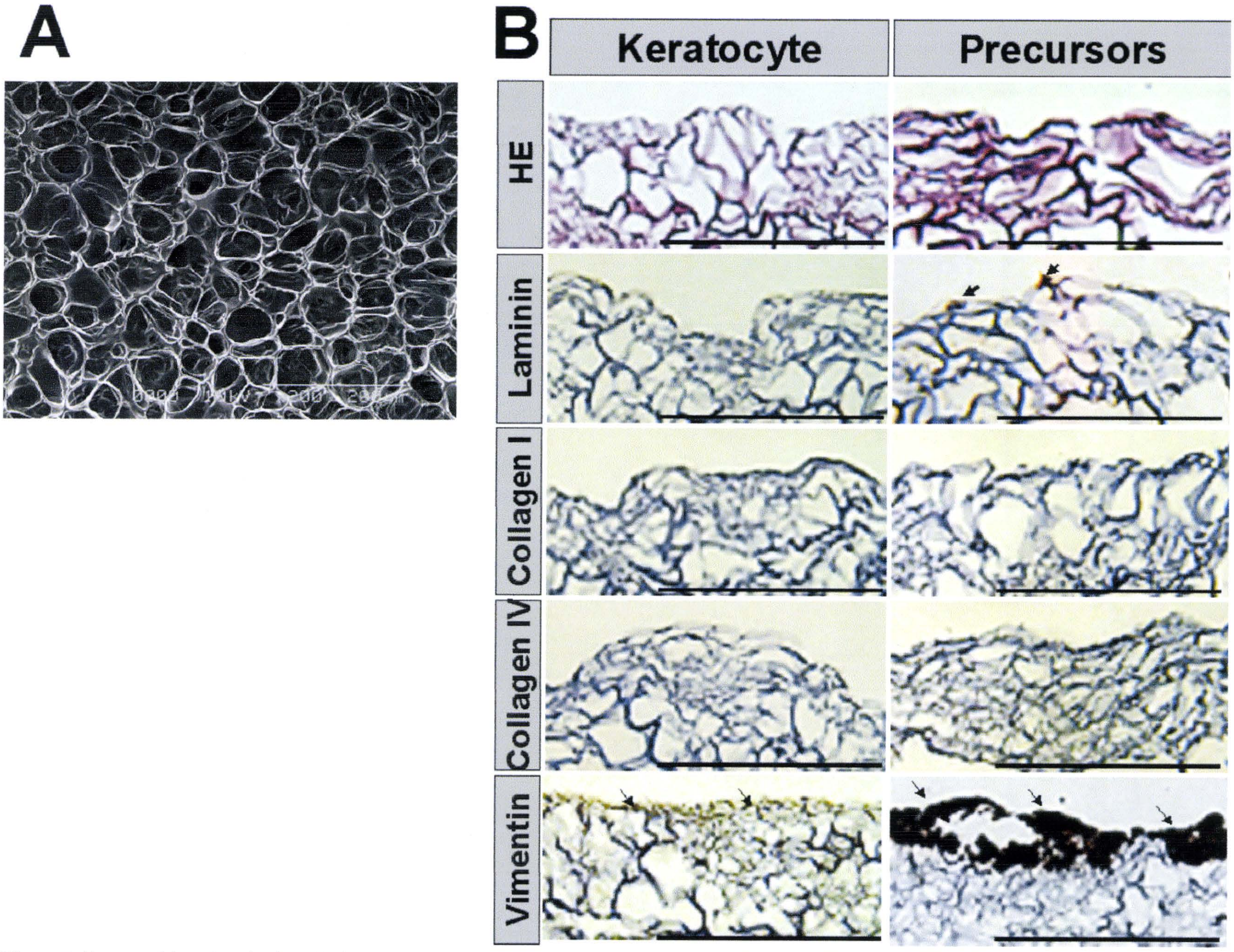
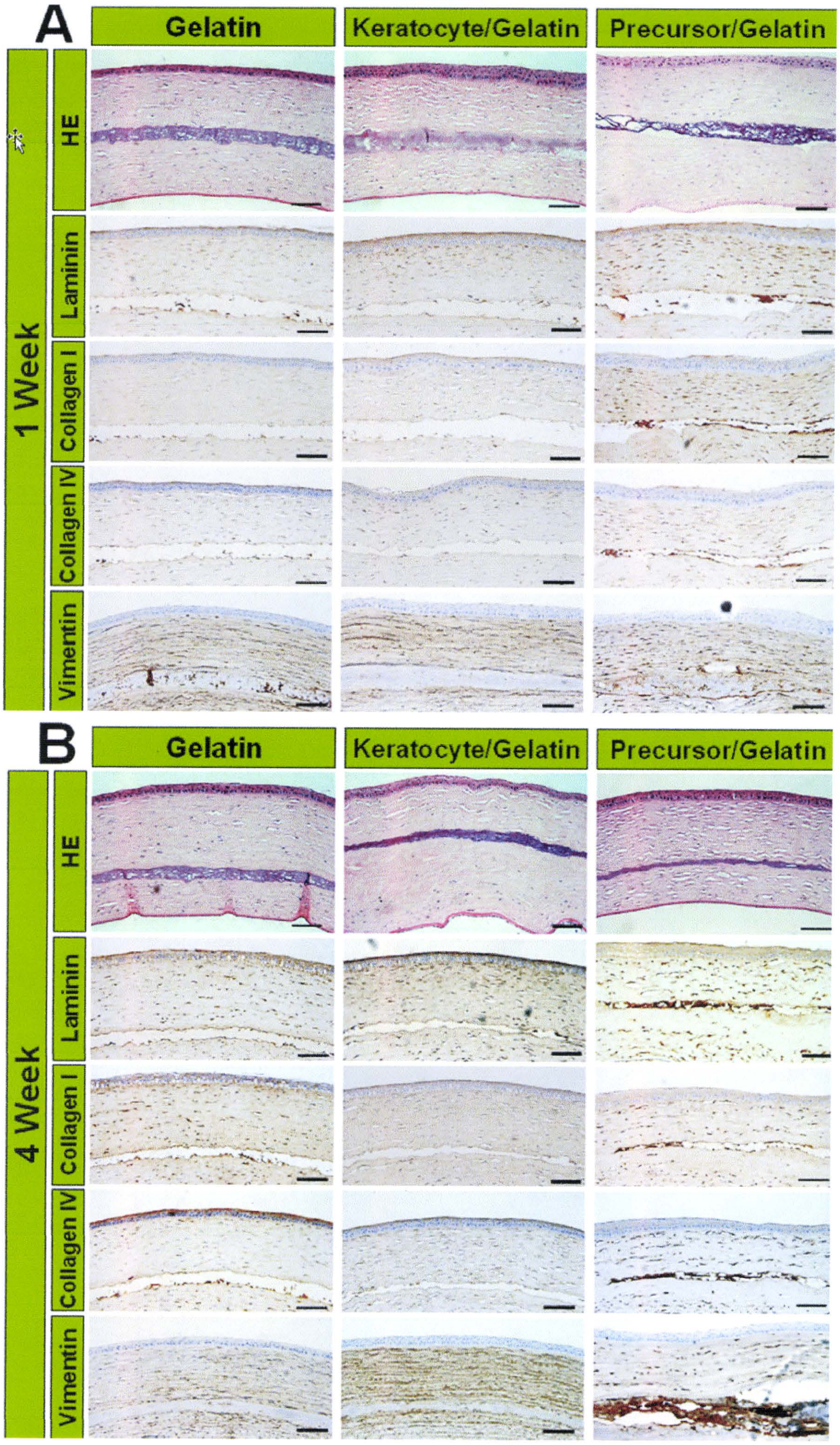


Figure 3. Immunohistochemical analysis of extracellular matrix in porous gelatin hydrogels with corneal fibroblasts or fibroblast precursors. Corneal fibroblasts or fibroblast precursors were seeded onto porous gelatin hydrogels and cultured for one week. **A**: Scanning electron microscopy revealed a porous structure for the gelatin hydrogels. **B**: Hematoxylin and eosin staining and immunohistochemical analysis of vimentin and ECM in porous gelatin hydrogels seven days after seeding of corneal fibroblasts or fibroblast precursors are shown. Vimentin staining was more intense in the gelatin hydrogels with corneal fibroblast precursors than in those with corneal fibroblasts (arrows). Other ECM components such as laminin, type I collagen, and type IV collagen are not expressed in the gelatin hydrogels with corneal fibroblasts or fibroblast precursors before transplantation except for a weak expression of laminin in the gelatin hydrogels with corneal fibroblast precursors (arrow). Scale bar=200 μm in **B**.

hydrogels with fibroblast precursors induced the production of ECM by the host stroma.

Adult vertebrate corneal stroma is composed primarily of collagen type 1 fibrils, smaller amounts of other ECM proteins, and keratocytes. Therefore, both cells and a stratified complex of the ECM, which form a scaffold for precursors, are necessary to engineer a three-dimensional corneal stroma. Porous scaffolds used in tissue engineering contribute to cell proliferation and differentiation in a suitable environment as well as the maintenance of structure and composition in the injured tissues. Furthermore, three-dimensional porous scaffolds provide a larger surface for cell attachment, migration, and proliferation compared with two-dimensional scaffolds, which facilitate contact inhibition in confluent cells. Because corneal fibroblast proliferation is substrate-dependent, it is preferable to increase the surface area of the culture substrate. Several three-dimensional substrates such as collagen have been designed to demonstrate their capacity for proliferative enhancement [29-31]. Their long-term safety, stability, and efficacy in vivo have been adequately established in humans. However, the significant drawback of collagen is its poor biodegradation and bioabsorption. The biodegradation of biomaterials and their biocompatibility are probably their most desired properties because the insoluble biomaterials develop biological reactions in vivo resulting in tissue opacity. Gelatin, a denatured type of collagen, possesses


A
Gelatin
Keratocyte/Gelatin
Precursor/Gelatin
1 Week
HE
Laminin
Collagen I
Collagen IV
Vimentin
B
Gelatin
Keratocyte/Gelatin
Precursor/Gelatin
4 Week
HE
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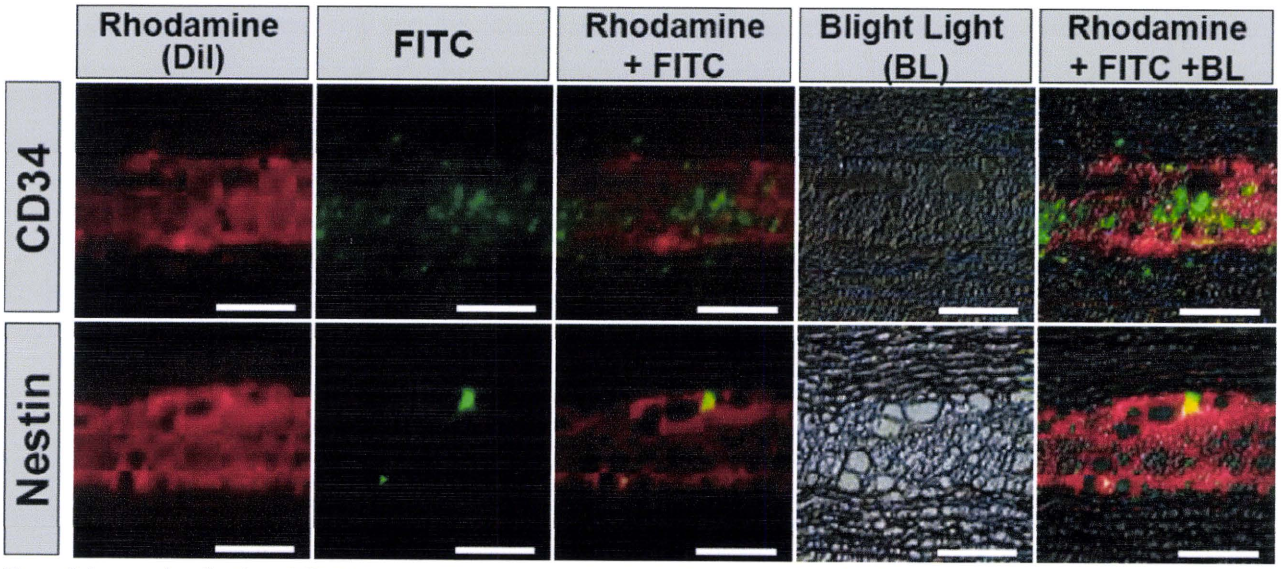

Figure 5. Immunolocalization of CD34-positive or nestin-positive cells within the transplanted DiI-positive precursors in the precursor/gelatin group four weeks after transplantation of gelatin hydrogels with corneal fibroblast precursors. Bright light (black and white, background), rhodamine (red, the transplanted DiI-labeled corneal fibroblast precursors in the gelatin hydrogels), and FITC (green, CD34- or nestin-positive cells) are superimposed with Adobe Photoshop software. Whole transplanted gelatin hydrogels are shown in light red by many DiI-positive corneal fibroblast precursors. A few CD34-positive cells or nestin-positive spindle cells are scattered within the gelatin hydrogels. Scale bar=100 µm.

most of the properties of an ideal scaffold and has been applied clinically as an implant material [32]. We used a biodegradable porous gelatin hydrogel, which is effective in facilitating cell migration and the delivery of oxygen and nutrients to the migrated cells, as a carrier of corneal fibroblast precursors. In the previous in vivo degradation tests in the conjunctival sac of mice, the residual radioactivity in carrier gels ranged from 50.7% to 54.7% on day 1 and from 11.2% to 18.4% on day 7. [33]. In monkey skull defect models, the gelatin hydrogel with a water content of 93.8 wt% did not degrade and remained at the skull defect site 12 weeks after application [34]. In the current study, the hydrogel did not degrade in the corneal stroma four weeks after transplantation. The rate of hydrogel degradation in the corneal stroma may be slow compared with those of the conjunctival sac of mice and skull defect models of the monkey.

Each primary sphere (50 µm in diameter) contained approximately 300 cells. Therefore 10 spheres contain approximately 3,000 cells. Despite the total cell number within 10 corneal fibroblast spheres being approximately 30 fold less than 100,000 corneal fibroblasts, the porous gelatin hydrogels that incorporated 10 corneal fibroblast spheres cultured ex vivo for seven days showed more intense immunostaining for vimentin than those incorporating 100,000 corneal fibroblasts cultured for seven days, which indicates that the corneal fibroblast precursors had superior proliferative potential on the gelatin hydrogels compared with corneal fibroblasts. Where weak expression of laminin and collagens were detected in gelatin hydrogels that incorporated corneal fibroblasts precursors, these ECM components were barely detected in the gelatin incorporating corneal fibroblasts ex vivo before transplantation. These results indicate that the gelatin hydrogel itself had no ability to induce tissue regeneration in vitro or ex vivo.

We have previously produced spheres from human [22] and rabbit [23] corneal stroma. The individual spheres and their progeny expressed mesenchymal and neuronal lineage marker proteins. Moreover, they expressed keratocan that were used as keratocyte-specific markers [35-37], suggesting that fibroblast progenitors and progenies have an essential lineage of keratocytes. Immunohistochemical findings on day 7 and 28 showed that the expression of type I collagen, type IV collagen, laminin, and vimentin were strongly positive in the transplanted gelatin hydrogels of the precursor/gelatin group while they were faint in the gelatin and fibroblast/gelatin groups. Gelatin hydrogels alone function to induce ECM production to some extent in vivo, but the efficacy is not as high as that of the gelatin hydrogels with precursors.

In the ex vivo experiments, the expression of ECMs were low in all groups while the in vivo experiments showed that vimentin expression in transplanted gelatin hydrogels was higher in precursor/gelatin groups compared with the other two groups. In Figure 4A, the corneal stroma as a whole (even tissue far away from the transplanted tissue) had stronger expression of vimentin and ECM in the precursor/gelatin group than the other groups. Also in Figure 4A, vimentin expression in the gelatin group was stronger than that in the fibroblast/gelatin group. These suggest that ECM and vimentin arise from the host and not the transplanted cells.

Furthermore, expression of ECM and vimentin increased four weeks after transplantation in all groups compared with just one week. The gelatin only also promotes ECM secretion. Both precursors and gelatin may promote the production of ECM derived from the host stroma and not the transplanted cells.

Immunofluorescence microscopy of the precursor/gelatin group on day 28 revealed that the CD34-positive cells and nestin-positive cells were localized to the transplanted gelatin hydrogels. This indicates that fibroblast precursors with a greater self-renewal potential continue to proliferate even after transplantation and can supply fibroblasts necessary for the regeneration of the host stroma. The nestin-positive cells may also contribute to induction of nerve regeneration.

The combined transplantation of corneal fibroblast precursors with gelatin hydrogels into a corneal stromal pocket has several advantages over penetrating keratoplasty of full-thickness donor cornea. Most complications associated with open-sky surgery such as expulsive hemorrhage and the risks of wound dehiscence would be eliminated. Several postoperative complications such as a postoperative corneal irregular astigmatism, wound leakage, corneal infection, vascularization, and persistent epithelial defect can be avoided. Allograft rejection is a leading cause of the failure of conventional full thickness corneal allografting with local and/or systemic immunosuppressants. Histologically, no apparent inflammatory reaction including immunological rejection was detected in the current corneal fibroblast precursor allotransplantation. This finding suggests that the transplanted precursors can survive in the corneal stroma without rejection.

One of the major limitations for clinical application of corneal fibroblast precursor transplantation is the availability of donor fibroblast precursors in sufficient quantities. This study demonstrated that the required number of spheres per cornea was 10 spheres to promote the expression of ECM. In the human cornea, the number of digested stromal cells from individual corneas was 1.1×10^5, and the number of spheres grown from one cornea was 1,566±211 [22], which are adequate quantities for transplantation. These results suggest the feasibility of transplantation of autologous fibroblast precursor derived from a small piece of stroma. The main weakness of this study may be the relatively short observation period. Further long-term investigation including side effects, rejection, corneal opacity, infection incidence, and histological observation is necessary. Additionally, our tissue engineering based on the ECMs, which were not abundant in normal cornea, does not represent restoration of a true stromal ECM. Further investigation is necessary to show stromal-specific matrix molecules such as keratan sulfate or keratocan in the transplanted gelatin hydrogels.

As for the in vivo physiologic function of the transplanted cornea, we could not perform the corneal sensitivity test in the animal model because we cannot evaluate the subjective corneal sensitivity of an animal using a Cochet-Bonnet esthesiometer, which is the only corneal sensitivity test. Although we had no idea of corneal stress test, we found that all groups had no obvious corneal weakness and there was no significant difference in intraocular pressure among the three groups. As for corneal nerves in the host tissues, a microscopic examination of the frozen section and a confocal microscopic examination of whole mount cornea showed no distinguished corneal nerve in the host cornea after transplantation because it is generally difficult to observe the corneal nerve. Further examinations about corneal physiologic function after precursor transplantation may be necessary.

In summary, we established a method for three-dimensional tissue engineering of the substitute for corneal stroma using fibroblast precursors and gelatin hydrogels. Corneal fibroblast precursors-based corneal stromal regeneration combined with gelatin hydrogel is a promising new therapy to promote fibroblast adherence and ECM deposition after corneal fibroblast precursor transplantation. This therapeutic tissue engineering is applicable to any type of cell in regenerative medicine. The transplantation of corneal fibroblast precursors into a corneal stromal pocket proved to be a simple and effective treatment strategy for corneal regeneration, which may replace conventional full-thickness corneal grafting and compensate for the worldwide shortage of donor corneas.

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