行った。今回の結果より、AS および FBS のヒト上皮細胞への影響に有意な差は認められなかった。また、我々が開発した AS を用いた培養粘膜上皮細胞シートは、従来の FBS を用いた培養上皮細胞シートと同様の組織学的構造、生物学的特徴を示した。これらのデーターより、AS を用いる当手法は、従来の FBS を用いる手法に取って代わる、より安全で倫理面に配慮した培養上皮細胞シート作成法となりえる可能性が示唆された。今後は、このヒト自己血清を用いた培養粘膜上皮細胞シート作成を、実際の臨床に使用し、その安全性、生物学的効果、手術成績を評価する予定である。

E. 結論

ヒト自己血清を用いた培養粘膜上皮細胞シート作成は、従来使用されてきた FBS と同等の生物学的特性を示し、難治性眼表面疾患患者に臨床使用できる可能性が示唆された。

F. 健康危険情報

特になし

G. 研究発表

論文発表

2) 1) Onguchi T, Takano Y, Dogru M, Tsubota K, Shimazaki J. Clinical evaluation of the accuracy of intraocular pressure measurement by Tono-Pen XL in eyes with amniotic membrane patching. Am J Ophthalmol 2005; 139: 570-571.

3) Shimmura S, Shimazaki J, Omoto M, Teruya A, Ishioka M, Tsubota K. Deep lamellar keratoplasty (DLKP) in keratoconus patients using viscoadaptive viscoelastics. Cornea 2005; 24: 178-181.

4) Shimmura S, Omoto M, Den S, Bissen-Miyajima H, Tsubota K, Shimazaki J. Microkeratome-assisted phacoemulsification. J Cataract Refract Surg 2005; 31: 1699-1701.

5) Shimmura S, Miyashita H, Uchino Y, Taguchi T, Kobayashi H, Shimazaki J, et al. Microkeratome-assisted deep lamellar keratoprosthesis (DLKPro). J Ref Surg 2005; in submission.

6) Shimmura S, Miyashita H, Konomi K, Shinozaki N, Taguchi T, Kobayashi H, Shimazaki J, Tanaka J, Tubota K. Transplantation of corneal endothelium with Descemet's membrane using a hyroxyethyl methacrylate polymer as a carrier. Br J Ophthalmol 2005; 89: 134-137.

7) Dogru M, Asano-Kato N, Tanaka M, Igarashi A, Shimmura S, Shimazaki J, Okada N, Takano Y, Fukagawa K, Tsubota K, Fujishima H. Ocular surface and MUC5AC alterations in atopic patients with corneal shield ulcers. Curr Eye Res 2005; 30: 897-908.

8) Dogru M, Okada N, Asano-Kato N, Tanaka M, Igarashi A, Takano Y, Fukagawa K, Shimazaki J, Tsubota K, Fujishima H. Atopic ocular surface disease: Implications on tear function and ocular surface mucins. Cornea 2005; 24: S18-S23.

9) Higa K, Shimmura S, Shimazaki J, Tsubota K. Hyaluronic acid-CD44 interaction mediates the adhesion of lymphocytes by amniotic membrane stroma. Cornea 2005; 24 : 206-212.

10) Higa K, Shimmura S, Miyashita H, Shimazaki J, Tsubota K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. Exp Eye Res 2005; 81: 218-223.
11) Yoshida S, Shimmura S, Shimazaki J, Shinozaki N, Tsubota K. Serum-free spheroid culture of mouse corneal keratocytes. Invest Ophthalmol Vis Sci 2005; 46: 1653-1658.

学会発表

国内

1. 御宮知達也, 高野洋之, 藤島浩, 島﨑潤. 羊膜上のトノペンによる眼圧測定の評価. 第28回日本眼科手術学会, 大阪市, 2005/1/28-30.
2. 佐竹良之, 相場昌代, 村戸ドール, 角 環, 富田真智子, 比嘉一成, 榛村重人, 坪田一男, 島﨑潤. 口腔粘膜培養上皮移植後のバリヤー機能の評価. 第29回角膜カンファランス, 第21回日本角膜移植学会, 徳島市, 2005/2/17-19.
3. 川島素子, 田聖花, 御宮知達也, 大本雅弘, 榛村重人, 島﨑潤. 角膜変性症に対する深層表層移植術. 第 29 回角膜カンファランス, 第 21 回日本角膜移植学会, 徳島市, 2005/2/17-19.
4. 冨田真智子, 榛村重人, 佐竹良之, 坪田一男, 島﨑潤. アトピー性皮膚炎合併患者における角膜移植. 第 29 回角膜カンファランス, 第 21 回日本角膜移植学会, 徳島市, 2005/2/17-19.
5. 比嘉一成, 榛村重人, 宮下英之, 島﨑潤, 坪田一男. 角膜輪部上皮におけるメラノサイトの分布第 29 回角膜カンファランス, 第 21 回日本角膜移植学会, 徳島市, 2005/2/17-19.
6. 吉田悟, 榛村重人, 只野貴示枝, 島﨑潤, 坪田一男. ケラトサイトの長期、大量培養法の検討. 第 29 回角膜カンファランス, 第21回日本角膜移植学会, 徳島市, 2005/2/17-19.

H．知的財産権の出願・登録状況（予定を含む）

1． 特許取得 なし
2． 実用新案登録 なし
3． その他

厚生科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書
「粘膜上皮幹細胞移植術における基質の開発に関する研究」

分担研究者　　中村　隆宏　同志社大学再生医療研究センター　講師

研究要旨
羊膜を基質に用いた難治性眼表面疾患に対する培養粘膜上皮細胞移植術は、細胞治療による新しい再生医療として世界中で注目を集めている。現在報告されている培養粘膜上皮細胞移植術の安全性・倫理的問題点として、滅菌操作が行われていない羊膜を使用している点である。本研究では、質の高い培養粘膜上皮幹細胞移植の開発を念頭において、その基質の倫理的課題を克服するため、安全性・倫理面に配慮した滅菌操作可能な乾燥羊膜の開発を検討した。その結果、凍結乾燥させた後、γ線滅菌処理を施行した乾燥羊膜は、常温保存可能で従来の羊膜と同等の細胞生物学的特徴を保持し、乾燥羊膜を基質に用いた培養上皮シートの作成も可能であることがわかった。同時に、作成した乾燥羊膜の生体適合性を確認するため、家兎の角膜に移植した結果、極めて高い生体適合性を示すことがわかった。

A. 研究目的

難治性眼表面疾患に対する再生医療学的手法を用いた外科的再建術として、さまざまな基質を用いた培養粘膜上皮移植が開発されている。その中で、生体由来材料の羊膜は、抗炎症、瘢痕抑制、血管新生抑制などさまざまな効果を持ち、また粘膜上皮の基質として極めて有用であることがこれまで報告されている。現在使用されている羊膜は、その生物学的特性から各施設での独自の基準により清潔操作下で処理後使用されているが、滅菌操作は施行されていない。近年、種々の病原体が注目を集めているように、今後ヒト羊膜を生体材料として安全に用いるにあたっては滅菌操作が重要なポイントとなる。本研究では、培養粘膜上皮幹細胞移植術の開発を念頭において、その基質の倫理的課題を克服するため、安全性・倫理面に配慮した、滅菌操作可能な羊膜の開発を検討した。また、乾燥羊膜の培養基質としての適性を評価し、同時に家兎動物モデルでの移植実験で、その生体適合性を検討した。

B. 研究方法

1）乾燥羊膜の作成

口頭、および文書による同意を得た後、感染症フリーの妊婦より帝王切開時に羊膜を採取した。採取した羊膜は清潔操作下でEDTAに浸漬後、上皮細胞を除去した。その後、真空凍結乾燥機にて羊膜を凍結乾燥処理した。作成した乾燥羊膜は、直ちに真空パック下にてγ線滅菌処理を行った。

2）乾燥羊膜を基質に用いた培養粘膜上皮シートの作成

滅菌操作された乾燥羊膜の培養基質としての適性を検討するため、乾燥羊膜を基質に用いた培養上皮シート作

成を試みた。６週令の日本白色家兎から角膜および口腔粘膜組織を無菌的に採取して上皮細胞を分離し，細胞浮遊液を作成した．次に、カルチャーインサート上に羊膜上皮を掻爬した乾燥羊膜基質を貼付し，その上に角膜および口腔粘膜上皮細胞をそれぞれ培養し，カルチャーデッシュ上にはマイトマイシンＣ処理をしたNIH-3T3細胞を培養し，共培養を2週間行った。培地には，上皮系細胞の生育に必要な培地を使用した。すなわち，DMEM/F12混合培地に，10％ＦＢＳ，インシュリン(5mg/ml)，コレラトキシン(0.1nM)，ペニシリンーストレプトマイシン(50IU)，ヒトリコンビナント上皮細胞増殖因子(EGF)(10ng/ml)を添加したものを使用した。作成した培養粘膜上皮シートの細胞生物学的・形態学的特徴を、ケラチンによる免疫染色ならびに走査型・透過型電子顕微鏡で観察した。

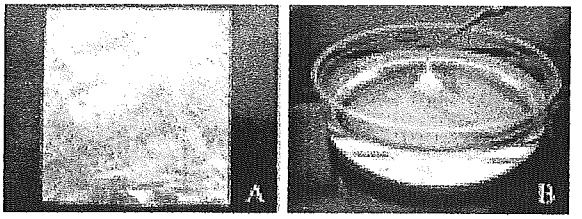
３）乾燥羊膜の生体適合性試験

作成した乾燥羊膜の角膜における生体適合性を検討する目的で、白色家兎に角膜実質ポケットを作成し、乾燥羊膜を移植した。移植後1ヶ月における角膜の透明性、組織像を評価した。

C. 研究結果

１）乾燥羊膜の作成

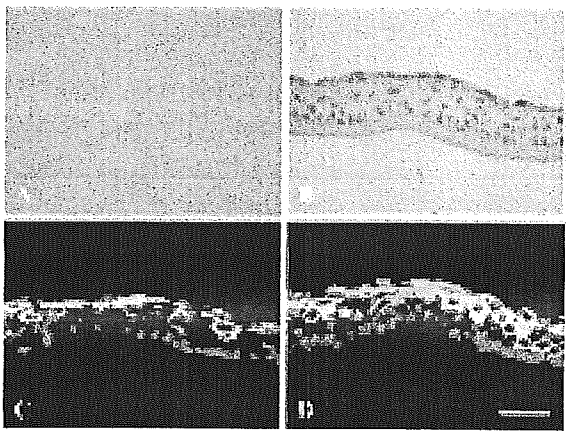
適切な条件下で作成した乾燥羊膜は半透明の様相を呈し、乾燥状態では操作性が良く、セッシを用いて保持することができ、また縫合可能であった。さらに乾燥羊膜は、湿潤状態でもその柔軟性を保持した。また、真空パック下で常温保存することが可能であることがわかった。



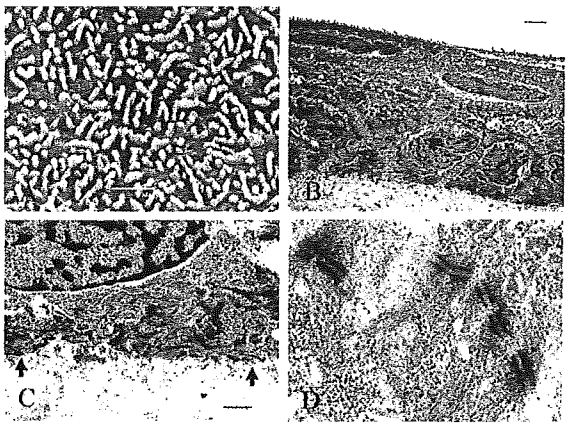
乾燥羊膜の外観（Ａ）。湿潤状態では柔軟性を示す（Ｂ）

２）培養上皮シートの作成

乾燥羊膜を培養基質に用い、家兎角膜上皮、口腔粘膜上皮を用いて培養上皮シートを作成した。培養後約5日で、上皮細胞は乾燥羊膜上でコンフレントに達した(A)。培養後2週間で、乾燥羊膜上でも4－5層の重層化した培養上皮シートを作成することが可能であった(B)。一連の過程は、従来の凍結保存羊膜を用いた場合と同等であった。次に、作成した乾燥羊膜上の培養上皮の性状を種々の細胞骨格蛋白であるケラチンの免疫染色性により評価した。培養角膜上皮シートは角膜分化型ケラチン3およびケラチン１２の免疫染色性を示した(C, D)。培養口腔粘膜上皮シートは粘膜分化型ケラチン４、１３および角膜型ケラチン３に対する免疫染色性を示した。

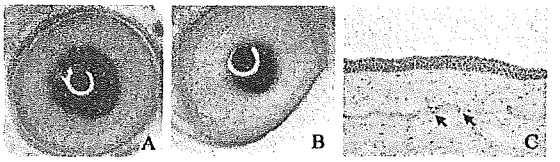


作成した培養上皮の形態学的特徴を走査型・透過型電子顕微鏡を用いて検討した。その結果、培養上皮の表層は粘膜の特徴である微絨毛を構成していた(A)。また、培養上皮細胞層は基底が円柱形を呈し、表層にむけて扁平になる正常分化の構造を示していた(B)。基底細胞と羊膜とはヘミデスモゾームにより基底膜を構成し(C)、細胞間は無数のデスモゾームにより接着構造を示した(D)。以上の結果より、乾燥羊膜を用いた培養上皮シートは、正常の粘膜上皮細胞が持つ形態学的特徴を併せ持つことがわかった。



3）生体適合性試験(動物モデル)

適正な条件下で作成した乾燥羊膜を家兎角膜実質層間ポケットに移植した。移植直後は、角膜内に乾燥羊膜が混濁ともに移植されたことがわかる(A)。移植後一ヶ月、家兎角膜は透明性を示し、移植された羊膜の混濁も改善していた(B)。その組織像では、角膜実質に浮腫や細胞浸潤を認めず、移植された乾燥羊膜の生体適合性は極めて良好であることがわかった(C)。



D. 考察

羊膜を基質に用いた培養粘膜上皮幹細胞移植術の開発において、その安全性倫理面がクリアーされた移植術を開発するため、乾燥羊膜の実際的な使用方法や生体適合性に関し検討した。今回の結果より、我々が作成した滅菌処理済みの乾燥羊膜は、従来の羊膜と同等の細胞生物学的効果を示し、動物モデルにおける移植実験でも生体適合性は良好であり、より安全で倫理面に配慮した培養粘膜上皮幹細胞移植術の基質となりえる可能性が示唆された。今後は、この凍結乾燥羊膜を用いた培養粘膜上皮幹細胞シートによる眼表面再建術を動物モデルにおける移植実験を行う予定である。また、ヒトへの前臨床試験も視野にいれ、開発を行う予定である。

E. 結論

滅菌処理を施行した凍結乾燥羊膜は、培養粘膜上皮幹細胞移植術における安全で倫理面に配慮した有用な培養基質となりえる可能性が示唆された。

F. 健康危険情報

特になし

G. 研究発表

論文発表

1．Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S. Characterization and distribution of

bone-marrow derived cells in mouse cornea. Invest Ophthalmol Vis Sci. 46; 497-503: 2005.

2．Qiao H, Hisatomi T, Sonoda K, Kura S, Sassa Y, Kinoshita S, Nakamura T, Sakamoto T, Ishibashi T. The characterization of hyalocytes: the origin, phenotype, and turnover. Br J Ophthalmol. 89; 513-517: 2005.

3．Cooper LJ, Kinoshita S, German M, Koizumi N, Nakamura T, Fullwood NJ. An investigation into the composition of amniotic membrane used for ocular surface reconstruction. Cornea 24; 722-729: 2005.

4．Kinoshita S, Nakamura T. The Promise and challenges of regenerative medicine - Corneal cells for regeneration. Ernst Schering Res Found Workshop. 54; 63-83: 2005.

5．Connon C, Kawasaki S, Liles M, Koizumi N, Yamasaki K, Nakamura T, Quantock AJ, Kinoshita S. Gene expression and immunolocalization of calcium activated chloride channel during the stratification of cultivated and developing corneal epithelium. Cell Tissue Res. 13; 1-6: 2005.

6．Inatomi T, Nakamura T, Koizumi N, Sotozono C, Kinoshita S. Current Concepts and Challenges in Ocular Surface Reconstruction using Cultivated Mucosal Epithelial Transplantation. Cornea 24; S32-8: 2005.

7．Sonoda KH, Nakao S, Nakamura T, Oshima T, Qiao H, Hisatomi T, Kinoshita S, Ishibashi T. The cellular events in normal and inflammatory conditions at cornea. Cornea. 24; S50-54: 2005.

8．中村隆宏、木下茂：実用化のはじまった再生医療—角膜再生医療．Medical Science Digest 31(4); 29-32: 2005.

9．中村隆宏、木下茂：角膜再生医療の現状 日本再生医療学会雑誌 4(2); 53-59: 2005.

１０．中村隆宏：難治性眼表面疾患に対する培養口腔粘膜上皮移植術の開発．眼紀 56; 481-487: 2005

１１．中村隆宏：眼表面の再生工学（１）臨床眼科増刊号 59(11); 163-169: 2005.

１２．中村隆宏：難治性眼表面疾患に対する外科的再建術の開発．Aging & Health 14(3); 38-39: 2005.

２．学会発表

国内

1. 中村隆宏、関山英一、曽我部寿代、堀切智子、稲富勉、外園千恵、横井則彦、木下茂：翼状片に対する凍結乾燥羊膜移植術．第 28 回日本眼科手術学会、大阪、2005. 1. 29.
2. 稲富　勉、中村隆宏、小泉範子、外園千恵、木下　茂：自己培養口腔粘膜上皮移植と同種角膜移植による二期的手術戦略：第 28 回日本眼科手術学会、大阪、2005. 1. 28.
3. 中村隆宏、稲富勉、関山英一、曽我部寿代、堀切智子、小泉範子、外園千恵、木下茂：ヒト自己血清を用いた培養上皮移植システムの開発．第 29 回角膜カンファレンス，第 21 回日本角膜移植学会、徳島、2005. 2. 18.
4. 関山英一、中村隆宏、木下茂:眼表面粘膜上皮における血管新生関連因子発現マッピング．第 29 回角膜カンファレンス，第 21 回日本角膜移植学会、徳島、2005. 2. 18.
5. 稲富　勉、中村隆宏、小泉範子、外園千恵、木下　茂：自家培養口腔粘膜上皮移植術の適応と有効性の検討．第 29 回角膜カンファレンス，第 21 回日本角膜移植学会、徳島、2005. 2. 18.

6. 山岸哲哉，稲富勉，外園千恵，中村隆宏，小泉範子，木下茂：培養粘膜上皮移植眼に対する白内障手術戦略．第29回角膜カンファランス・第21回日本角膜移植学会，徳島，2005. 2. 18.
7. 谷岡秀敏、川崎論、山崎健太、稲富勉，小泉範子，中村隆宏，横井則彦、小室青、木下茂：臨床応用を目指したヒト結膜上皮培養シートの作製．　第１０９回日本眼科学会　京都、2005. 3. 24.
8. 小林史郎、外園千恵、中村隆宏、山崎健太、曽我部寿代、餅田千佳子、木下茂：羊膜上皮のおける成長因子発言量と部位による比較．第１０９回日本眼科学会　京都、2005. 3. 24.
9. 関山英一、中村隆宏、木下茂：眼表面粘膜上皮における血管新生関連因子発現マッピング．第１０９回日本眼科学会　京都、2005. 3. 26.

海外

1. Nakamura T. Strategy for Corneal Regeneration -Tissue Engineering and Cellular Surgery. Cardiff & KPUM meeting. Kyoto, Japan, 2005.01.25.
2. Nakamura T, Ishikawa F, Sonoda K.-H, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S. Characterization and distribution of bone marrow-derived cells in the mouse cornea. 2005 Keystone Symposia, Molecular Regulation of Stem Cells, Banff, Canada, 2005.2.12.
3. Sekiyama E, Nakamura T, Kinoshita S. Expression Mapping of Angiogenesis Related Factors in Human Ocular Surface Mucosal Epithelium. 2005 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Fort Lauderdale, Florida, USA, 2005.5.4.
4. Connon CJ, Nakamura T, Quantock AJ , Kinoshita S. The Persistence of Transplanted Amniotic Membrane in Corneal Stroma. 2005 Annual Meeting of the ARVO (Association for Research in Vision and Ophthalmology), Fort Lauderdale, Florida, U.S.A., 2005.5.5.
5. Tanioka H, Kawasaki S, Yamasaki K, Inatomi T, Koizumi N, Nakamura T, Yokoi N, Komuro A, Kinoshita S. Establishment of a Cultivated Human Conjunctival Epithelium as an Alternative Tissue Source for Autologous Corneal Epithelia Transplantation. 2005 Annual Meeting of the ARVO (Association for Research in Vision and Ophthalmology), Fort Lauderdale, Florida, U.S.A., 2005.5.5.

H．知的財産権の出願・登録状況（予定を含む）

１．　特許取得

特願2005-8381「羊膜を用いたシート状組成物及びその作製方法」（フィブリン糊）
栗原英二、羽室淳爾、中村隆宏

２．　実用新案登録　　なし

３．　その他

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S.	Characterization and distribution of bone-marrow derived cells in mouse cornea.	Invest Ophthalmol Vis Sci	46	497-503	2005
Qiao H, Hisatomi T, Sonoda K, Kura S, Sassa Y, Kinoshita S, Nakamura T, Sakamoto T, Ishibashi T.	The characterization of hyalocytes: the origin, phenotype, and turnover.	Br J Ophthalmol	89	513-517	2005
Connon C, Kawasaki S, Liles M, Koizumi N, Yamasaki K, Nakamura T, Quantock AJ, Kinoshita S.	Gene expression and immunolocalization of calcium activated chloride channel during the stratification of cultivated and developing corneal epithelium.	Cell Tissue Res	13	1-6	2005
中村隆宏 木下茂	実用化のはじまった再生医療—角膜再生医療	Medical Science Digest	31(4)	29-32	2005
中村隆宏 木下茂	角膜再生医療の現状	日本再生医療学会雑誌	4(2)	53-59	2005
中村隆宏	難治性眼表面疾患に対する培養口腔粘膜上皮移植術の開発	眼紀	56	481-487	2005
中村隆宏	眼表面の再生工学（1）	臨床眼科増刊号	59(11)	163-169	2005
中村隆宏	培養口腔粘膜上皮移植	あたらしい眼科	22(12)	1647-1648	2005

Asano-Kato N, Fukagawa K, Okada N, Dogru M, Tsubota K, Fujishima H.	Tryptase increases proliferative activity of human conjunctival fibroblasts through protease-activated receptor-2.	Invest Ophthalmol Vis Sci	46(12)	4622-4626	2005
Okada N, Fukagawa K, Takano Y, Dogru M, Tsubota K, Fujishima H, Matsumoto K, Nakajima T, Saito H.	The implications of The upregulation of ICAM-1/VCAM-1 expression of corneal fibroblasts on the pathogenesis of allergic keratopathy.	Invest Ophthalmol Vis Sci	46(12)	4512-4518	2005
Shimmura S, Miyashita H, Konomi K, Shinozaki N, Taguchi T, Kobayashi H, Shimazaki J, Tanaka J, Tsubota K.	Transplantation of corneal endothelium with Descemet's membrane using a hyroxyethyl methacrylate polymer as a carrier.	Br J Ophthalmol	89(2)	134-137,	2005
Shiraishi K, Tsuzaka K, Yoshimoto K, Kumazawa C, Nozaki K, Abe T, Tsubota K, Takeuchi T.	Critical role of the fifth domain of E-cadherin for heterophilic adhesion with alphaEbeta7, but not for homophilic adhesion.	J Immunol	175(2)	1014-1021	2005
Yoshida S, Shimmura S, Shimazaki J, Shinozaki N, Tsubota K.	Serum-free spheroid culture of mouse corneal keratocytes.	Invest Ophthalmol Vis Sci	46(5)	1653-1658	2005
Tokumaru S, Sayama K, Yamasaki K, Shirakata Y, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Yang L, Yoshimura A, Hashimoto K.	SOCS3/CIS3 negative regulation of STAT3 in HGF-induced keratinocyte migration.	Biochem Biophys Res Commun	327	100-5	2005

Ishii K, Harada R, Matsuo I, Shirakata Y, Hashimoto K, Amagai M	In vitro keratinocyte dissociation assay for evaluation of the pathogenicity of anti-desmoglein 3 IgG autoantibodies in pemphigus vulgaris.	J Invest Dermatol	124	939-46	2005
Shirakata Y, Kimura R, Nanba D, Iwamoto R, Tokumaru S, Morimoto C, Yokota K, Nakamura M, Sayama K, Mekada E, Higashiyama S, Hashimoto K.	Heparin-binding EGF-like growth factor accelerates eratinocyte migration and skin wound healing.	J Cell Sci	118	2363-2370	2005
Tohyama M, Dai X, Yamasaki K, Shirakata Y, Hanakawa Y, Tokumaru S, Yahata Y, Yang L, Nagai H, Takashima A, Hashimoto K.	dsRNA-mediated innate immunity of epidermal keratinocytes.	Biochem Biophys Res Commun	335	505-11	2005
Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H, Yang L, Higashiyama S, Yoshimura A, Sugai M, Hashimoto K.	Induction of keratinocyte migration via transactivation of the EGF receptor by the antimicrobial peptide LL-37.	J Immunol	175	4662-8	2005

Shushakova N, Tkachuk N, Dangers M, Tkachuk S, Park JK, Zwirner J, Hashimoto K, Haller H, Dumler I.	Urokinase-induced activation of the gp130/Tyk2/Stat3 pathway mediates a pro-inflammatory effect in human mesangial cells via expression of the anaphylatoxin C5a receptor.	J Cell Sci	118	2743-53	2005
Komine M, Kakinuma T, Kagami S, Hanakawa Y, Hashimoto K, Tamaki K.	Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin.	J Invest Dermatol	125	491-8	2005

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以降は雑誌/図書等に掲載された論文となりますので、

「研究成果の刊行に関する一覧表」をご参照ください。

Characterization and Distribution of Bone Marrow–Derived Cells in Mouse Cornea

Takahiro Nakamura,[1] Fumihiko Ishikawa,[2] Koh-hei Sonoda,[3] Toshio Hisatomi,[3] Hong Qiao,[3] Jun Yamada,[1] Mitsuhiro Fukata,[2] Tatsuro Ishibashi,[3] Mine Harada,[2] and Shigeru Kinoshita[1]

Purpose. Bone marrow (BM)–derived stem cells are thought to possess extensive differentiation capacity. The present study was conducted to investigate the characteristics and distribution of these cells in the normal mouse cornea.

Methods. BM cells and BM-derived hematopoietic stem/progenitor cells (HSCs) from enhanced GFP (eGFP) transgenic mice (lin^-, Sca-1^+) were intravenously transplanted into irradiated wild-type C57BL/6 mice. At 4 to 6 months after transplantation, the mice were killed, and their whole corneas examined by histologic and immunohistochemical methods (CD11c, CD11b, and CD45).

Results. At 2 weeks after BM cell transplantation, GFP^+ cells gradually migrated into the cornea from the limbal area. At 2 to 6 months, they were distributed over the entire cornea. In cross sections of whole cornea, GFP^+ cells comprised 27.3% ± 11.1% (BM) and 24.0% ± 8.01% (HSC) of total cells in the peripheral corneal stroma. In the center of the corneal stroma, GFP^+ cells were 7.58% ± 2.63% (BM) and 8.06% ± 1.76% (HSC) of total cells. Immunohistochemistry showed that GFP^+ $CD11c^+$, $CD11b^+$, $CD11c^-$, and $CD11b^-$ cells occupied the entire corneal stroma.

Conclusions. The present study provides direct evidence of the distribution of BM-derived cells in the mouse cornea. Immunohistochemical study showed that some of these cells are BM-derived antigen-presenting cells such as dendritic cells and macrophages. Some elements of BM-derived cells may continue to exist in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2005;46:497–503) DOI:10.1167/iovs.04-1154

Adult somatic stem cells have been isolated from several tissue sources including neurons,[1,2] retina,[3] corneal limbal epithelium,[4,5] and bone marrow (BM).[6–8] It had been thought that somatic stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. However, recent studies suggest that tissue-specific stem cells can differentiate into lineages other than their tissue of origin and that, with respect to the developmental potential of different adult cell types, there is far more plasticity than previously thought. Particular attention has been focused on the plasticity of BM-derived stem cells. They are reported to possess extensive differentiation capacities and can differentiate into several epithelial types such as liver, lung, and skin.[9] Furthermore, BM-derived mesenchymal stem cells can differentiate in vitro not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endoderm characteristics.[10] These findings suggest that BM-derived stem cells may have the ability to transdifferentiate into a variety of tissues, including those of the eye.

Normal corneal tissue is located in the anterior segment of the eye, and it participates in several major functions. It is the gateway into the eye of visual images and plays a critical role in maintaining corneal transparency and avascularity. It is composed of three layers: the corneal epithelium, stroma, and endothelium. Corneal epithelial stem cells exist in the basal cell layer of the limbal region[4,5] and in the transitional zone between the cornea and conjunctiva. They are supported by the limbal vascular arcade. Little is known about stem cells of the corneal stroma and endothelium, and the origin of these cells is not well understood.

From an immunologic point of view, the normal avascular cornea was thought to be an immune-privileged site without functional antigen-presenting cells (APCs) and largely devoid of BM-derived cells. Therefore, higher success rates would be expected with corneal than other organ transplants. This notion has lost favor since the demonstration of large numbers of resident BM-derived cells of different lineages—for example, macrophages and dendritic cells—in both the epithelium and stroma of the normal cornea.[11–13] Until now, indirect evidence obtained by immunohistochemical studies has shown these cells to be present and important questions, such as the original cell type and the physiological and functional significance of these progenitors, remain unanswered.

We are the first to attempt the characterization and clarification of the distribution of BM-derived cells in the normal mouse cornea. In the current study, we sought to acquire a direct demonstration by transplanting BM cells from enhanced green fluorescence protein (eGFP) transgenic mice using our unique protocol.[14–16] We transplanted GFP-labeled BM cells and hematopoietic stem/progenitor cells (HSCs) into syngeneic C57BL/6 (wild-type) mice and found BM-derived cells distributed in the mouse cornea. We then evaluated the characteristics of these BM-derived cells by immunohistochemical studies.

Materials and Methods

Experimental Animals

The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental

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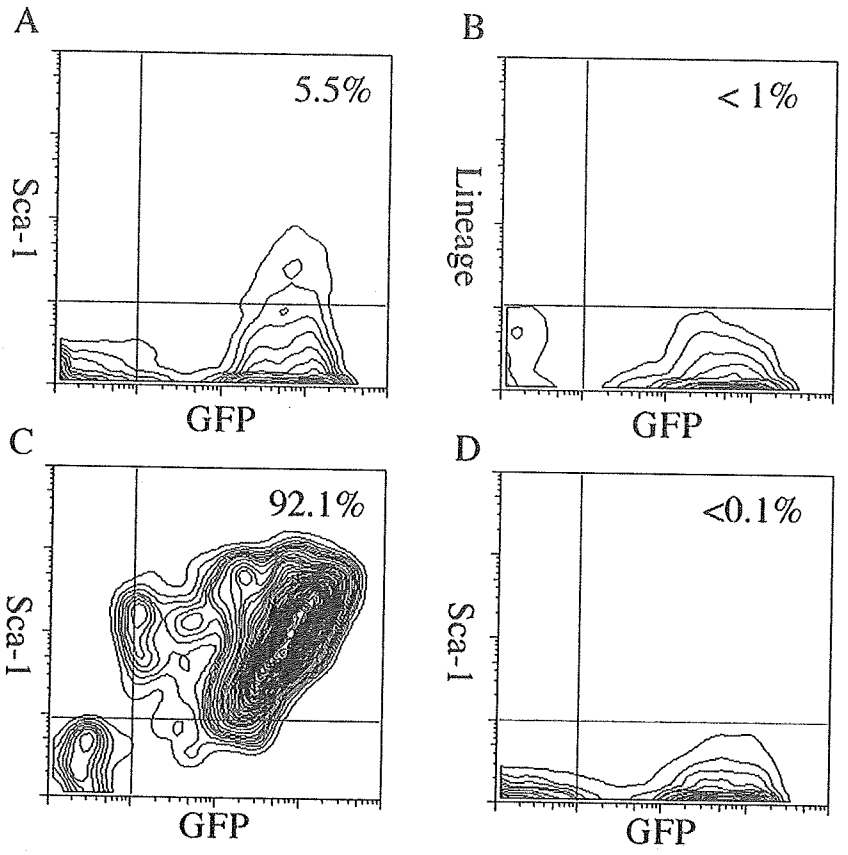

FIGURE 1. Enrichment of hematopoietic stem cells confirmed by flow cytometry. Original BM cells (**A**). After lineage depletion (**B**). Positive (**C**) and negative (**D**) selection for Sca-1$^+$ cells. After negative and positive selection, the lin$^-$ Sca-1$^+$ cell purity of all GFP$^+$ cells exceeded 95%. Each percentage represents the amount of double-positive cells among all nucleated cells.

procedures were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine and Kyushu University. Adult (n = 3) and newborn (n = 6) C57BL/6 mice were the recipients of BM cell and HSC transplants, respectively. BM cells were obtained from mice that transgenically express GFP, driven by the chicken β-actin promoter.[17]

Bone Marrow Transplantation

To observe directly the migration of BM-derived cells into the mouse cornea, we used BM cell transplantation.[16] Female eGFP mice (8–10 weeks old) were killed by cervical dislocation while under deep ether anesthesia, and BM cells were obtained by flushing the femurs with sterile phosphate-buffered saline (PBS). The BM cells were washed several times in sterile PBS, filtered twice through a nylon mesh (pore size, 70 μm), counted, and resuspended in PBS at 5×10^7 cells/mL. To generate chimeric mice, all BM cells (6×10^6 to 1×10^7) derived from eGFP transgenic mice were intravenously injected into 8-week-old C57BL/6 recipients that had been lethally x-irradiated with 9 Gy. Their eyes were protected with lead shields to prevent radiation retinopathy. These BM cell transplant recipients were then maintained under special pathogen-free conditions, and successful BM cell transplantation was confirmed by the identification of GFP$^+$ cells in peripheral blood at 2 weeks after transplantation. The corneas of three mice were carefully studied by fluorescence biomicroscopy until 6 months after transplantation. We also used these corneas for histologic and immunohistochemical studies.

Hematopoietic Stem Cell Transplantation

To characterize BM-derived stem/progenitor cells in the mouse cornea, we performed HSC transplantation.[14,15] BM cells were harvested from femurs and tibias of 8- to 12-week-old eGFP mice. Single-cell suspensions of donor cells were prepared by repeated serial passage through a 23-gauge needle. To deplete mature hematopoietic cells, the BM cells were incubated with lineage-specific antibodies (B220, CD3, Gr-1, Mac-1, and TER 119) for 30 minutes at 4°C. After washing with PBS containing 2% fetal bovine serum, the cells were incubated with sheep anti-rat immunomagnetic beads (Dynabeads M-450 coupled to sheep anti-rat IgG; Dynal, Great Neck, NY). Cells not bound to the immunobeads were further purified for Sca-1$^+$ cells. The purity of lineage$^-$ cells was higher than 92% in all experiments. After negative selection of mature hematopoietic and immune cells, positive selection of Sca-1$^+$ cells was performed as just described. After negative and positive selection, the purity of lin$^-$ Sca-1$^+$ cells of all the eGFP$^+$ cells exceeded 95% (Fig. 1).[14,15] To obtain high cell purity, samples were applied twice to columns in each experiment. The resultant 10^4 lin$^-$ Sca-1$^+$ cells were transplanted into C57BL/6 mice within 2 days of their birth. The HSC transplant recipients were maintained under special pathogen-free conditions for 4 weeks. Successful HSC transplantation was confirmed by the identification of GFP$^+$ cells in the peripheral blood at 4 weeks after transplantation. At 4 to 5 months after HSC transplantation, six mice were used for histologic and immunohistochemical studies.

Antibodies

The primary antibodies (all from BD-PharMingen, San Diego, CA) used in this study were purified hamster anti-mouse CD11c (clone HL3), purified rat anti-mouse CD45 (clone 30-F11), and RPE-conjugated rat anti-mouse CD11b (clone M1/70). Secondary antibodies were Cy3-conjugated goat anti-hamster IgG and Cy3-conjugated donkey anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA).

Immunohistochemistry

Immunohistochemical studies of markers for APCs were performed by using a previously reported method[11–13] and a modified version of our method.[18,19] Briefly, freshly excised corneas were fixed for 60 minutes at 4°C in 4% paraformaldehyde, extensively washed with PBS, fast frozen in liquid nitrogen, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek II; Miles Laboratories, Elkhart, IN).

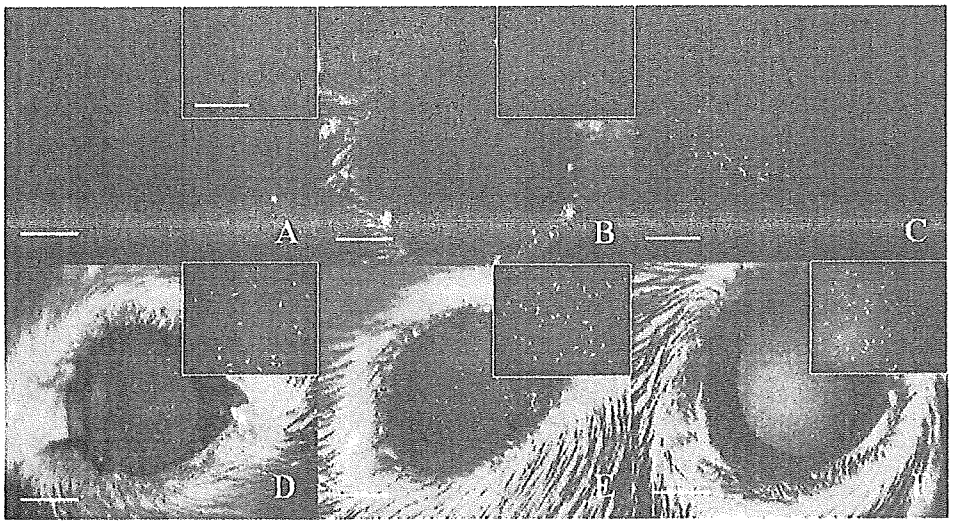

FIGURE 2. Representative time-course slit lamp photographs of murine eyes after BM cell transplantation. (**A**) One week, (**B**) 2 weeks, and (**C**) a high magnification of (**B**) at the limbal area; (**D**) 1, (**E**) 2, and (**F**) 6 months after transplantation. *Boxes* contain data from the center of the cornea. In the early stages (within the first week) after BM cell transplantation, we observed no GFP^+ cells in the recipient mouse cornea (**A**). Within 2 weeks, there was intense staining for GFP^+ cells in the periphery of the cornea (**B**, **C**). Within 2 months, GFP^+ cells appeared to migrate into the center of the cornea. Their numbers increased in both the periphery and center of the cornea (**D**, **E**). Starting at 2 months after BM transplantation, cell density reached a plateau that persisted up to 6 months (**F**). Scale bars: (**A**, **B**, **D**–**F**) 1 mm; (**C** and *insets*) 250 μm.

Cryostat sections (7 μm in thickness) were placed on gelatin-coated slides, air-dried, and rehydrated in PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with both anti-Fc receptor mAb (CD16/32; BD PharMingen, San Diego, CA) and 2% bovine serum albumin (BSA) for CD11c and CD11b and with 2% BSA and 10% donkey serum for CD45 at room temperature for 30 minutes. Then the sections were incubated at room temperature for 1 hour with the primary antibody and washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. The controls were incubated with the appropriate normal rat and hamster IgG (Dako, Kyoto, Japan) at the same concentration as, but without, the primary antibody. After staining with the primary antibody (CD11c, CD45), the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Cy3-conjugated goat anti-hamster IgG, and Cy3-conjugated donkey anti-rat IgG. After several washes with PBS, the sections were coverslipped using antifade mounting medium, with or without propidium iodide (PI; Vectashield; Vector Laboratories) and examined under a confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Quantitative Evaluation

For statistical assessment of corneal cell distribution and characterization, four different fields and six different sections of each cornea were analyzed (24 areas/eye). For analytical purposes, each cornea was divided into central and peripheral areas. The central area was defined as the area within 1 mm of the center and the peripheral area as that within a 1- to 1.5-mm radial distance from the center.

RESULTS

Migration of BM Cells into the Cornea

In the early stages (first week) after BM cell transplantation, there were no GFP^+ cells in the recipient mouse cornea (Fig. 2A). Within 2 weeks of transplantation, some GFP^+ cells appeared in the periphery of the cornea. However, only a small number of GFP^+ cells were present in the center of the cornea (Figs. 2B, 2C). Within 2 months, the number of GFP^+ cells in both the periphery and center of the cornea gradually increased. From 2 months after BM cell transplantation, the cell density reached a relative plateau that persisted up to 6 months (Figs. 2D–F). Our quantitative analysis of GFP^+ cells in the mouse cornea is summarized in Figure 3.

Distribution of BM Cells and HSCs

To determine whether there were BM-derived GFP^+ cells in the recipient cornea, we performed histologic analysis under a dual-channel fluorescence microscope. Cross-sections of recipient corneas showed that most of the GFP^+ cells were distributed in the peripheral corneal stroma and that cell density gradually decreased toward the center (Fig. 4A–D). In the entire corneal epithelium, we noted only a small number of

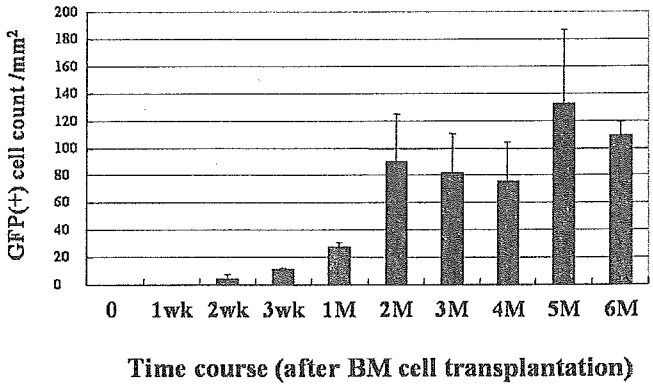

FIGURE 3. Quantitative analysis of GFP^+ cells in the mouse cornea at the indicated times after BM cell transplantation. During the first 2 months, the number of GFP^+ cells gradually increased. Thereafter, cell density reached a relative plateau that persisted up to 6 months after transplantation.

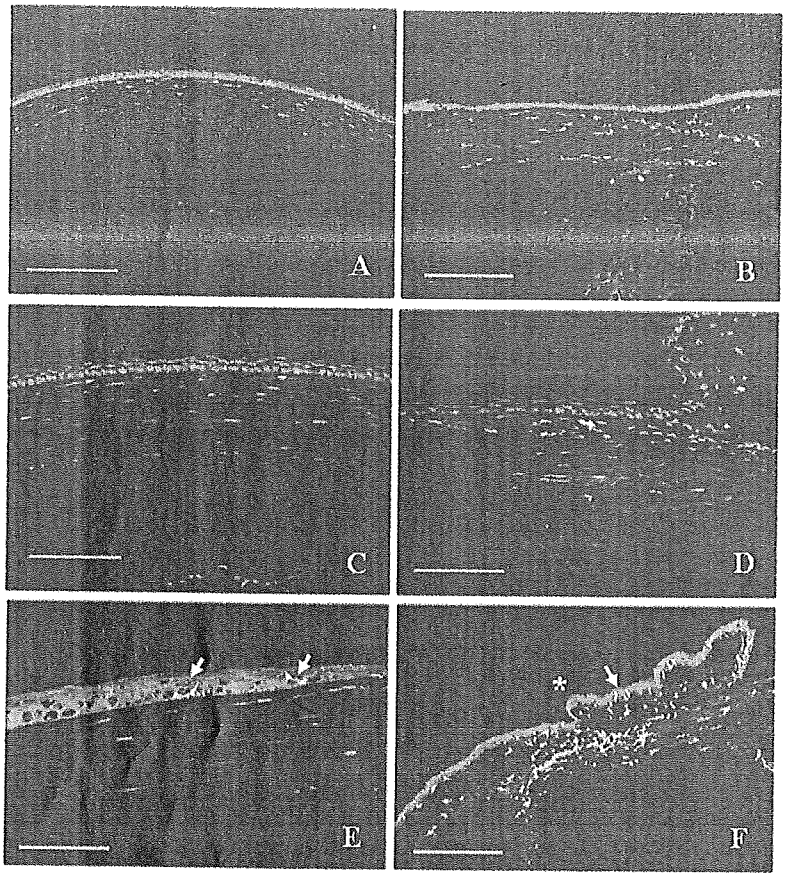

FIGURE 4. Representative cross sections of recipient corneas (A, B, E, BM transplantation; C, D, F, HSC transplantation) show that most of the GFP^+ cells were distributed in the peripheral corneal stroma and that the cell density gradually decreases toward the center (A, C, central area; B, D, peripheral area). Only a small number of GFP^+ cells were observed throughout the epithelium (E, *arrows*). In contrast, in conjunctival epithelium, several GFP^+ cells were noted (F, *arrow*; area to the right of the conjunctiva *). Cell nuclei were stained with PI (*red*). Scale bars: (A, B, F) 200 μm; (C, D, E) 100 μm.

these cells (Fig. 4E), whereas in the conjunctival epithelium we observed several GFP^+ cells (Fig. 4F). The percentage of GFP^+ cells per section was calculated as the number of GFP^+ cells divided by the total number of PI^+ cells $\times$ 100. In the peripheral cornea of mice receiving BM cell transplants, GFP^+ cells were 2.03% $\pm$ 1.87% (epithelium) and 27.3% $\pm$ 11.1% (stroma). At the center of the cornea, they were 0.93% $\pm$ 0.65% (epithelium) and 7.58% $\pm$ 2.63% (stroma). By contrast, in the peripheral corneas of mice transplanted with HSC, GFP^+ cells were 0.78% $\pm$ 0.51% (epithelium) and 24.0% $\pm$ 8.01% (stroma). At the center of the cornea, they were 0.58% $\pm$ 0.4% (epithelium) and 8.06% $\pm$ 1.76% (stroma; Fig. 5). The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

Immunohistochemical Analysis

To characterize BM-derived GFP^+ cells in corneal tissue, primarily the corneal stroma, we used fluorescence immunohistology with antibodies to the leukocyte markers CD11c, CD11b, and CD45. Negative control sections, incubated with normal rat and hamster IgG but without the primary antibody, exhibited no discernible specific immunoreactivity over the entire region.

$CD11c^+$ or $CD11b^+$ indicate cells coexpressing GFP and CD11c or GFP and CD11b, respectively. The percentage of $CD11c^+$ or $CD11b^+$ cells was calculated by dividing the respective number of cells by the total number of GFP^+ cells $\times$ 100. In the corneal peripheral stroma of BM cell recipients, we observed 19.4% $\pm$ 9.93% $CD11c^+$ cells and 38.7% $\pm$ 16.3% $CD11b^+$ cells. In the central stroma, 15.3% $\pm$ 8.94% were $CD11c^+$ cells and 48.7% $\pm$ 13.1% were $CD11b^+$ cells. In the corneal peripheral stroma of HSC recipients, there were 35.7% $\pm$ 14.0% $CD11c^+$ cells and 56.7% $\pm$ 22.4% $CD11b^+$ cells. In the central stroma, 41.5% $\pm$ 17.8% were $CD11c^+$ cells and 53.7% $\pm$ 13.9% were $CD11b^+$ cells (Figs. 6 , 7, 8). Most GFP^+ cells in the cornea were immunostained with CD45 in both BM- and HSC-recipients (Fig. 9). Asterisks in Figure 8 indicate statistically significant difference between $CD11c^+$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

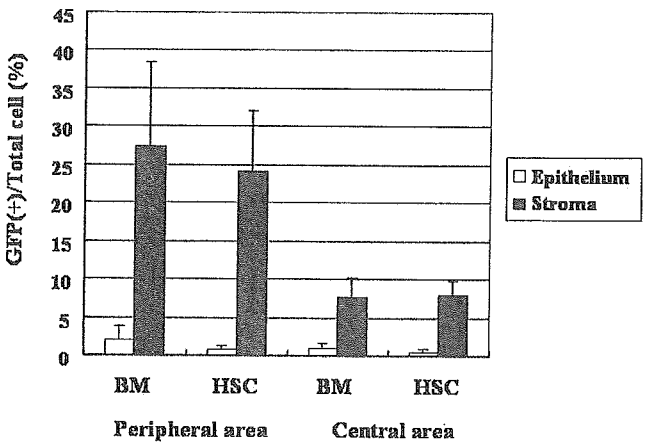

FIGURE 5. Distribution of GFP^+ cells in the cornea of mice transplanted with BM cells or HSCs. The percentage of GFP^+ cells per section was calculated as the number of GFP^+ cells divided by the total number of PI^+ cells plus GFP^+ cells $\times$ 100. Most of the GFP^+ cells were distributed in the peripheral corneal stroma. Cell density gradually decreased toward the center. In the entire area covered by epithelium, there were only a few GFP^+ cells. The differences between epithelium and stroma in each category were statistically significant (Mann-Whitney test; $P < 0.01$).

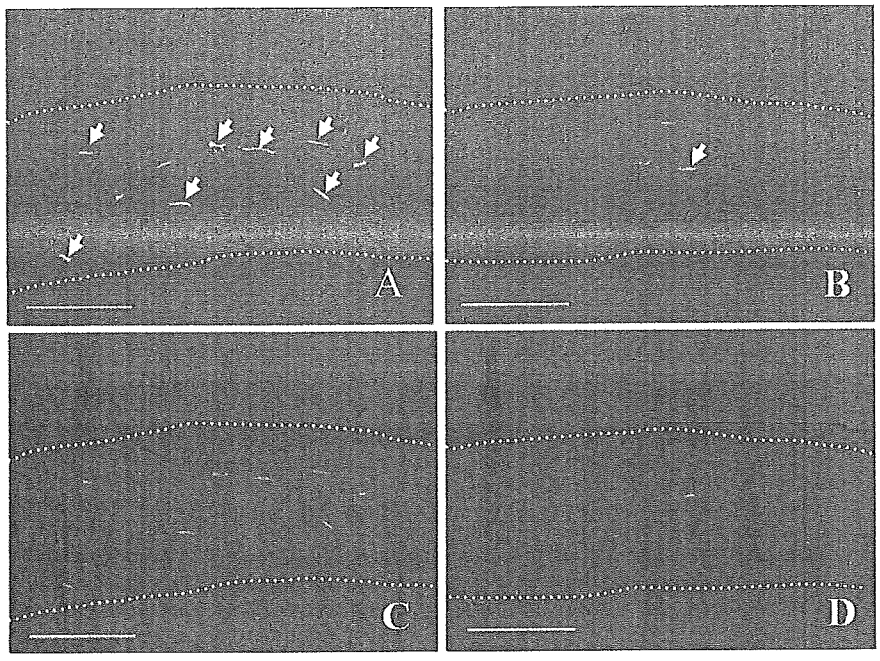

FIGURE 6. Representative immunohistochemical staining for CD11c (*red*) in the peripheral (**A**, **C**) and central (**B**, **D**) cornea of mice receiving HSC transplants. Some GFP^+ cells (*green*) immunostained with CD11c (*arrows*). Note the presence of GFP^+CD11c^- cells throughout the transplant-recipient cornea. GFP (**A**, **B**), CD11c (**C**, **D**). *Dotted lines*: perimeter of whole corneal tissue. Scale bars, 100 μm.

DISCUSSION

The cornea is a transparent, avascular tissue, with integrity maintained by various factors derived from the tear film and aqueous humor. Although the normal cornea does not contain vessels, there is indirect immunohistochemical evidence that it is endowed with a significant number of resident BM-derived APCs.[11–13] Hamrah et al.[11] reported that corneal epithelium contains major histocompatibility complex (MHC) class II-negative Langerhans' cells and corneal stroma a large number of resident BM-derived cells of different lineages. These cells were not only macrophages but also $CD11c^+$ dendritic cells. Brissette-Storkus et al.[12] also documented that the normal murine corneal stroma contains a significant number of $CD45^+$ leukocytes and that most of these cells are monocytes or macrophages. However, to date, there has been no direct demonstration of their existence. BM-derived stem cells, such as hematopoietic- and mesenchymal stem cells, have extensive differentiation capacity.[8,9] We considered two possible mechanisms of BM-derived cell differentiation: One is that BM-derived stem cells that have differentiated into APCs such as Langerhans' cells or macrophages migrate into corneal tissue. Alternatively, BM-derived stem cells transdifferentiate into corneal cells such as corneal keratocytes, and function in the cornea. We examined these possibilities using our unique protocol and found that some BM-derived cells were definitely distributed in the cornea. We also determined that these cells are partially of BM-derived APC lineage, a finding that directly confirms the cell origin of

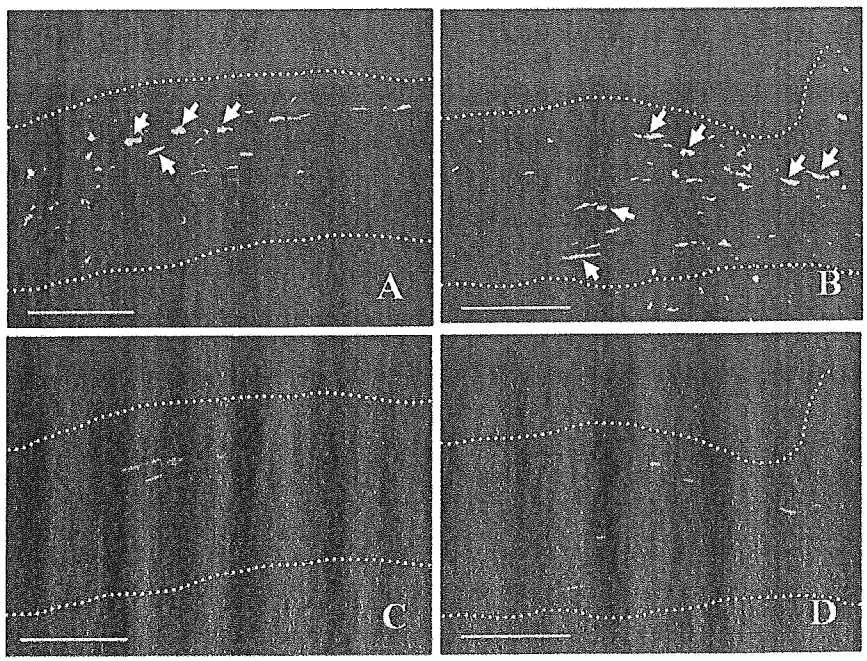

FIGURE 7. Representative immunohistochemical staining for CD11b (*red*) in the cornea of mice receiving BM cell (**A**, **C**) or HSC (**B**, **D**) transplants. Some GFP^+ cells (*green*) immunostained with CD11b (*arrows*). Note the GFP^+CD11b^- cells dispersed throughout the transplant-recipient cornea. GFP (**A**, **B**), CD11b (**C**, **D**). *Dotted lines*: whole corneal tissue. Scale bars, 100 μm.

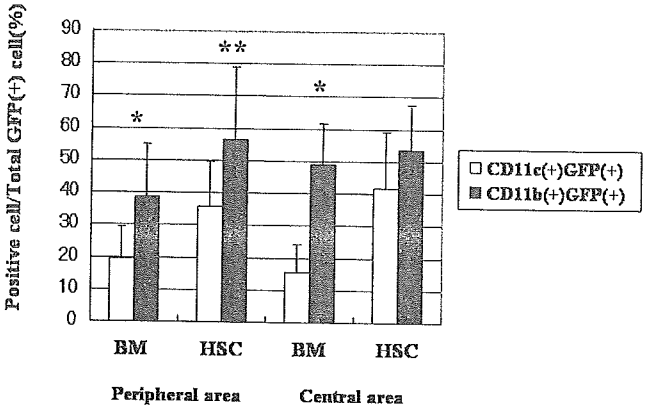

FIGURE 8. Quantitative analysis of the immunohistochemistry for CD11c and CD11b in the cornea of mice receiving transplants of BM cells or HSCs. The percentage of positive cells per section was calculated as the number of cells that coexpressed GFP and CD11c or GFP and CD11b, divided by the total number of GFP^+ cells × 100. The number of $CD11c^+$ cells in both the peripheral and central areas of the cornea was greater in mice receiving transplants of HSCs than in those receiving BM cells. Approximately 50% of GFP^+ cells were immunostained with CD11b in both BM cell and HSC recipients. There were statistically significant differences between $CD11c^+$ and $CD11b^+$ (Mann-Whitney test; $^*P < 0.01$, $^{**}P < 0.05$).

a significant number of resident dendritic cells in the corneal tissue.

Our observations in corneas receiving GFP-labeled BM cell transplants are of particular interest. Ours is the first report on the time course of the migration of GFP-labeled BM cells into the cornea. Within 2 months after BM cell transplantation, the density of GFP-labeled cells gradually increased; thereafter, cell density was comparatively stable, and finally, at 6 months, it reached a plateau. These findings led us to the interesting hypothesis that BM-derived cells continuously migrate into corneal tissue and contribute to corneal integrity. At present, we do not know the longevity of GFP-labeled BM cells in the mouse cornea. Using other experimental protocols, further cell biological study is needed to clarify this point.

There have been no reports on the distribution of hematopoietic stem/progenitor cells (not bone marrow cells) in the mouse cornea. Although the type of transplantation necessary to obtain these data is very difficult, our group has mastered the technique by using a unique protocol that facilitates our long-term observation of the eyes of transplant-recipient mice.

Our study demonstrates that most of the GFP^+ cells were distributed in the corneal stroma: Approximately 25% were found in the periphery and 7% in the center. In contrast, a small number, approximately 1%, were found in the corneal epithelium. The distribution rates of GFP^+ cells were similar in mice receiving with BM cells and HSCs. These results suggest that cells migrating into the corneal tissue may be definite populations of BM cells, such as HSCs or undifferentiated progenitor cells.

Based on our immunohistochemical results, we divided GFP^+ cells in the corneal tissue into four groups: GFP^+CD11c^+, GFP^+CD11b^+, GFP^+CD11c^-, and GFP^+CD11b^- cells. GFP^+CD11c^+ cells (approximately 40% in the HSC transplantation experiment) are thought to express the dendritic cell phenotype[20–22] and GFP^+CD11b^+ cells (approximately 55% in HSCs) either the dendritic cell or macrophage phenotype.[23] Using a protocol similar to ours, Espinosa-Heidmann et al.[24] found that BM-derived progenitor cells contributed to experimental choroidal neovascularization. When they used the F4/80 antibody (monocyte marker), they observed $GFP^+F4/80^+$ cells in the limbus, ciliary body, and normal choroid and sclera, suggesting a high turnover and recruitment rate of infiltrated macrophages. Based on their findings and our observations, we postulate that some of the GFP^+ cells in the mouse cornea are BM-derived APCs.

Some of the GFP^+ cells were negative for cell-surface markers for APCs (CD11c and CD11b), and their origin is unclear. Corneal stroma is composed of both corneal keratocytes and a variety of extracellular matrices comprising collagen subtypes. In our experience, the morphology of GFP^+ cells in the corneal stroma and of corneal keratocytes is very similar. If BM-derived stem cells terminally transdifferentiate into corneal keratocytes, they can be expected eventually to lose surface CD45 expression. We posit that our immunologic experiment did not detect immature $Sca\text{-}1^+$ cells in the mouse cornea (data not shown), suggesting that transplanted hematopoietic stem/progenitor cells first homed to BM and engrafted in the recipient mice, and then provided mature BM-derived cells in the cornea. Based on our present results we cannot unequivocally claim that BM-derived GFP^+ cells can transdifferentiate into corneal cell phenotypes or neurons. Therefore, morphologic and immunohistochemical studies are under way to examine extracellular matrices and cell-surface markers that are uniquely synthesized by corneal keratocytes.

Several technical and conceptual issues deserve consideration in the interpretation of our results. It is important to note that even in eGFP mice significantly fewer than 100% of the cells express GFP. As this may be due to cell-cycle dependent expression of GFP, we suggest that our results underestimate the potential contribution of BM-derived cells in the mouse cornea. We are currently investigating whether the findings we made with our animal model are applicable to humans. Therefore, we are studying the distribution of BM-derived cells in human corneas.

In conclusion, ours is the first study that presents direct evidence for the migration into the cornea of GFP-labeled BM-derived cells. We provide immunohistochemical evidence that some of the migrating cells were BM-derived cells such as

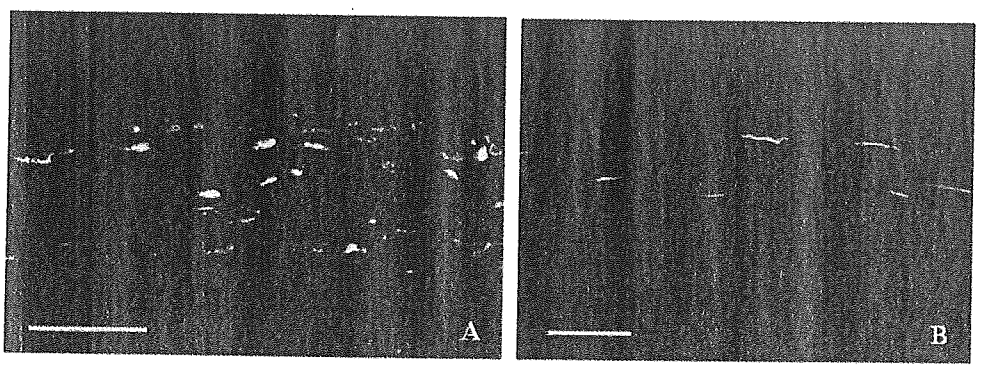

FIGURE 9. Representative immunohistochemical staining for CD45 (*red*) in the cornea of HSC-recipient mice. (**A**) Peripheral, (**B**) central retina. Most GFP^+ cells were immunostained with CD45 (*yellow*). Scale bars: (**A**) 100 μm; (**B**) 50 μm.

dendritic cells and macrophages. Cell biology studies will determine the lineage(s) of the other cells.

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References

1. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. 1992;255:1707-1710.
2. Gage FH. Mammalian neural stem cells. *Science*. 2000;287:1433-1438.
3. Tropepe V, Coles BL, Chiasson BJ, et al. Retinal stem cells in the adult mammalian eye. *Science*. 2000;287:2032-2036.
4. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol*. 1986;103:49-62.
5. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57:201-209.
6. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 1997;276:71-74.
7. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science*. 2000;287:1442-1446.
8. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
9. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multilineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;105:369-377.
10. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;418:41-49.
11. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci*. 2002;43:639-646.
12. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci*. 2002;43:2264-2271.
13. Hamrah P, Liu Y, Zhang Q, Dana MR. The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci*. 2003;44:581-589.
14. Ishikawa F, Livingston AG, Wingard JR, Nishikawa S, Ogawa M. An assay for long-term engrafting human hematopoietic cells based on newborn NOD/SCID/beta2-microglobulin(null) mice. *Exp Hematol*. 2002;30:488-494.
15. Ishikawa F, Livingston AG, Minamiguchi H, Wingard JR, Ogawa M. Human cord blood long-term engrafting cells are CD34+ CD38−. *Leukemia*. 2003;17:960-964.
16. Hisatomi T, Sakamoto T, Sonoda KH, et al. Clearance of apoptotic photoreceptors: elimination of apoptotic debris into the subretinal space and macrophage-mediated phagocytosis via phosphatidylserine receptor and integrin alphavbeta3. *Am J Pathol*. 2003;162:1869-1879.
17. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. "Green mice" as a source of ubiquitous green cells. *FEBS Lett*. 1997;407:313-319.
18. Nakamura T, Nishida K, Dota A, et al. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2001;42:549-556.
19. Nakamura T, Nishida K, Dota A, Kinoshita S. Changes in conjunctival clusterin expression in severe ocular surface disease. *Invest Ophthalmol Vis Sci*. 2002;43:1702-1707.
20. Leenen, PJ, Radosevic, K, Voerman, JS, et al. Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J Immunol*. 1998;160:2166-2173.
21. Vremec, D, Zorbas, M, Scoolay, R, et al. The surface phenotype of dendritic cells purified form the mouse thymus and spleen: investigations of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med*. 1992;176:47-58.
22. Metlay JP, Witmer-Pack MD, Agger R, et al. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med*. 1990;171:1753-1771.
23. Ross GD, Vetvicka V. CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. *Clin Exp Immunol*. 1993;92:181-184.
24. Espinosa-Heidmann DG, Caicedo A, Hernandez EP, Csaky KG, Cousins SW. Bone marrow-derived progenitor cells contribute to experimental choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2003;44:4914-4919.

EXTENDED REPORT

The characterisation of hyalocytes: the origin, phenotype, and turnover

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Aim: To determine the characterisation of hyalocytes: the origin, phenotype, and turnover in the rodent.
Methods: To characterise the ultrastructure and distribution of hyalocytes, transmission and scanning electron microscopy was performed in rat eyes. Immunophenotypical analysis was performed by either anti-ED1 or ED2 antibodies. To examine the origin of the hyalocytes, the chimeric mice were created and were used to transplant the bone marrow (BM) cells from enhanced green fluorescent protein (EGFP) transgenic mice. The turnover of hyalocytes was examined at 0, 4, 6, 7, and 12 months after BM transplantation.
Results: Hyalocytes were distributed especially in the vitreous cortex and had an irregular shape with a spherical granule. Immunophenotypical studies demonstrated that most of the hyalocytes in rat eyes expressed ED2 but not ED1. In the chimeric mice, the hyalocytes were GFP negative right after BM transplantation. Interestingly, more than 60% of hyalocytes were replaced within 4 months and approximately 90% within 7 months after BM transplantation.
Conclusions: The rodent hyalocytes were shown to express tissue macrophage marker, were derived from BM, and totally replaced within 7 months. These data provide the characterisation of hyalocytes in physiological conditions, especially their origin, distribution, and turnover, and may contribute to the better understanding of the pathogenesis of vitreoretinal disease.

The existence of cells in the peripheral or cortical region of the vitreous body abutting the inner surface of the retina has been described.[1 2] These cells, cuurently known as hyalocytes, are located in the vitreous cavity at an average distance of 50 μm from the inner surface of the retina and are concentrated anteriorly in the vitreous base and posteriorly in the vicinity of the optic papilla.[3 4] Morphological studies have demonstrated that hyalocytes belong to the monocyte/macrophage lineage but differ significantly from other tissue macrophages.[5 5–8] The hyalocytes express monocyte/macrophage cell marker but do not express CD68, glial fibrillary acidic protein (GFAP), cellular retinaldehyde binding protein, and cytokeratin.[9–11] Although these results may indicate that hyalocyte is derived from monocyte/macrophage but not from retinal cells such as glial cells, and retinal pigment epithelium, there is no definitive evidence of the origin and the regeneration of the hyalocytes. We recently investigated a possible physiopathological role of hyalocytes in eyes.[12] We demonstrated that hyalocytes could participate not only in proliferative vitreous diseases but also in immunological disorders. However, the characterisation of hyalocytes under physiological or pathological conditions remains unclear.

Recently, enhanced GFP (EGFP) transgenic mice were generated; the tissues of EGFP mice are green under excitation light.[13–15] GFP fluorescence can easily be detected by flow cytometry or fluorescence microscopy without any cofactor for light emission or any specific staining procedures.[16 17] Experiments using EGFP transgenic mice allow us to track cellular movement in an in vivo model. We thus created chimeric mice by transplanting bone marrow (BM) from EGFP transgenic mice into irradiated wild type (WT) mice. If hyalocyte is derived from haematopoietic progeny, green cells under excitation light will increase in the vitreous cavity of chimeric mice.

In order to investigate the nature of the vitreous in a preliminary study on the morphology, distribution, and immunocytochemical characterisation of hyalocytes within the rodent vitreous, we examined various aspects of hyalocytes. Additionally, the origin and the kinetics of hyalocytes were investigated by using the chimeric mice.

MATERIALS AND METHODS

Experimental animals

WT C57BL/6 female mice and Brown Norway rats (postnatal 8 weeks) were obtained from SLC Japan (Shizuoka, Japan). EGFP transgenic female mice (B6 background)[15] were kindly provided by Dr Masaru Okabe (Osaka University, Japan). All animals were treated humanely and were housed in specific pathogen free conditions at Kyushu University.

Transmission electron microscopy (TEM)

The rats were anaesthetised with an intraperitoneal injection of pentobarbital and their eyes were enucleated and the posterior segments fixed in 1% glutaraldehyde and 1% paraformaldehyde in sterile phosphate buffered saline (PBS). They were then post-fixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon. Ultrathin sections were cut from blocks and mounted on copper grids.

Scanning electron microscopy (SEM)

The removed eye posterior segments were fixed as described in TEM. After dehydration, the retinas were saturated in t-butyl alcohol, and critical point drying was performed (Eiko, Tokyo). The tissue was then placed on stubs by means of self adhesive carbon tabs and sputtered with gold of 20 nm

Abbreviations: BM, bone marrow; EGFP, enhanced green fluorescent protein; FACS, flow cytometry analysis; GFAP, glial fibrillary acidic protein; ILM, inner limiting membrane; PBS, phosphate buffered saline; PI, propidium iodide; SEM, scanning electron microscopy; TEM, transmission electron microscopy