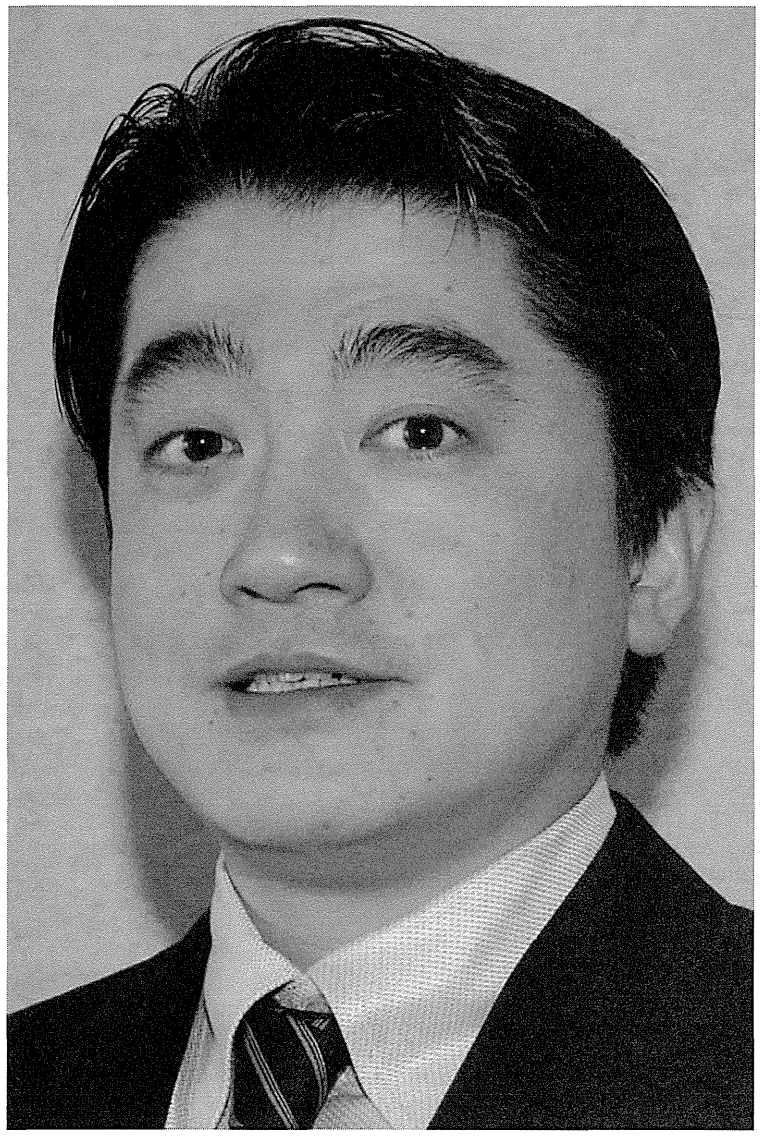
REFERENCES

1. Thiel HJ, Behnke H. [A hitherto unknown subepithelial hereditary corneal dystrophy]. *Klin Monbl Augenheilkd* 1967; 150(6):862–874.
2. Weiss JS, Moller HU, Lisch W, et al. The IC3D classification of the corneal dystrophies. *Cornea* 2008;27(Suppl 2):S1–83.
3. Küchle M, Green WR, Volcker HE, Barraquer J. Reevaluation of corneal dystrophies of Bowman's layer and the anterior stroma (Reis-Bücklers and Thiel-Behnke types): a light and electron microscopic study of eight corneas and a review of the literature. *Cornea* 1995;14(4):333–354.
4. Mashima Y, Yamada M, Oguchi Y. [Autosomal dominant inherited corneal dystrophies associated with TGFBI mutation]. *Nippon Ganka Gakkai Zasshi* 2001;105(10):659–672.
5. Munier FL, Frueh BE, Othenin-Girard P, et al. BIGH3 mutation spectrum in corneal dystrophies. *Invest Ophthalmol Vis Sci* 2002;43(4):949–954.
6. Munier FL, Korvatska E, Djemai A, et al. Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. *Nat Genet* 1997;15(3):247–251.
7. Okada M, Yamamoto S, Tsujikawa M, et al. Two distinct kerato-epithelin mutations in Reis-Bucklers corneal dystrophy. *Am J Ophthalmol* 1998;126(4):535–542.
8. Ellies P, Renard G, Valleix S, Boelle PY, Dighiero P. Clinical outcome of eight BIGH3-linked corneal dystrophies. *Ophthalmology* 2002;109(4):793–797.
9. Amm M. [Photo-therapeutic keratectomy (PTK)—a successful treatment for Thiel-Behnke dystrophy and its recurrence]. *Ophthalmologe* 1999;96(8):489–493.
10. Fagerholm P. Phototherapeutic keratectomy: 12 years of experience. *Acta Ophthalmol Scand* 2003;81(1):19–32.
11. Sorour HM, Yee SB, Peterson NJ, et al. Recurrence of chromosome 10 Thiel-Behnke corneal dystrophy (CDB2) after excimer laser phototherapeutic keratectomy or penetrating keratoplasty. *Cornea* 2005;24(1):45–50.
12. Ellies P, Bejjani RA, Bourges JL, Boelle PY, Renard G, Dighiero P. Phototherapeutic keratectomy for BIGH3-linked corneal dystrophy recurring after penetrating keratoplasty. *Ophthalmology* 2003;110(6):1119–1125.
13. Paparo LG, Rapuano CJ, Raber IM, Grewal S, Cohen EJ, Laibson PR. Phototherapeutic keratectomy for Schnyder's crystalline corneal dystrophy. *Cornea* 2000;19(3):343–347.
14. Hafner A, Langenbucher A, Seitz B. Long-term results of phototherapeutic keratectomy with 193-nm excimer laser for macular corneal dystrophy. *Am J Ophthalmol* 2005;140(3): 392–396.
15. Dinh R, Rapuano CJ, Cohen EJ, Laibson PR. Recurrence of corneal dystrophy after excimer laser phototherapeutic keratectomy. *Ophthalmology* 1999;106(8):1490–1497.
16. Das S, Langenbucher A, Seitz B. Delayed healing of corneal epithelium after phototherapeutic keratectomy for lattice dystrophy. *Cornea* 2005;24(3):283–287.
17. Pogorelov P, Langenbucher A, Kruse F, Seitz B. Long-term results of phototherapeutic keratectomy for corneal map-dot-fingerprint dystrophy (Cogan-Guerry). *Cornea* 2006;25(7): 774–777.
18. O'Brart DP, Gartry DS, Lohmann CP, Patmore AL, Kerr Muir MG, Marshall J. Treatment of band keratopathy by excimer laser phototherapeutic keratectomy: surgical techniques and long term follow up. *Br J Ophthalmol* 1993;77(11): 702–708.
19. Hahn TW, Sah WJ, Kim JH. Phototherapeutic keratectomy in nine eyes with superficial corneal diseases. *Refract Corneal Surg* 1993;9(2 Suppl):S115–118.
20. Hersh PS, Spinak A, Garrana R, Mayers M. Phototherapeutic keratectomy: strategies and results in 12 eyes. *Refract Corneal Surg* 1993;9(2 Suppl):S90–95.
21. Kozobolis VP, Siganos DS, Meladakis GS, Pallikaris IG. Excimer laser phototherapeutic keratectomy for corneal opacities and recurrent erosion. *J Refract Surg* 1996;12(2): S288–290.
22. John ME, Van der Karr MA, Noblitt RL, Boleyn KL. Excimer laser phototherapeutic keratectomy for treatment of recurrent corneal erosion. *J Cataract Refract Surg* 1994;20(2): 179–181.
23. Marcon AS, Rapuano CJ. Excimer laser phototherapeutic keratectomy retreatment of anterior basement membrane dystrophy and Salzmann's nodular degeneration with topical mitomycin C. *Cornea* 2002;21(8):828–830.
24. Thomann U, Meier-Gibbons F, Schipper I. Phototherapeutic keratectomy for bullous keratopathy. *Br J Ophthalmol* 1995; 79(4):335–338.
25. Sharma N, Prakash G, Sinha R, Tandon R, Titiyal JS, Vajpayee RB. Indications and outcomes of phototherapeutic keratectomy in the developing world. *Cornea* 2008;27(1): 44–49.
26. Fujita A, Hieda O, Nakajima N, Kinoshita S. [The VF-14 index of functional visual impairment in patients with corneal disease treated by phototherapeutic keratectomy]. *Nihon Ganka Gakkai Zasshi* 2005;109(11):736–740.
27. Fukuoka H, Kawasaki S, Yamasaki K, et al. Lattice corneal dystrophy type IV (p.Leu527Arg) is caused by a founder mutation of the TGFBI gene in a single Japanese ancestor. *Invest Ophthalmol Vis Sci* 2010;51(9):4523–4530.
28. Hayashi H, Maeda N, Ikeda Y, Nishida K, Watanabe H, Tano Y. Sclerotic scattering illumination during phototherapeutic keratectomy for better visualization of corneal opacities. *Am J Ophthalmol* 2003;135(4):559–561.
29. Bücklers M. Uber eine weitere familiäre Hornhaut-dystrophie (Reis). *Klin Monatsbl Augenkeilkd* 1949;114:386–397.
30. Kobayashi A, Sugiyama K. In vivo laser confocal microscopy findings for Bowman's layer dystrophies (Thiel-Behnke and Reis-Bücklers corneal dystrophies). *Ophthalmology* 2007; 114(1):69–75.
31. Miller A, Solomon R, Bloom A, Palmer C, Perry HD, Donnenfeld ED. Prevention of recurrent Reis-Bücklers dystrophy following excimer laser phototherapeutic keratectomy with topical mitomycin C. *Cornea* 2004;23(7):732–735.



Biosketch

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羊膜移植の適応と効果

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要 約

目 的：羊膜移植の適応と効果を明らかにする．

対象および方法：1998年4月から2008年3月までの10年間に京都府立医科大学眼科で羊膜移植を施行した304眼の疾患，術式と術後経過をレトロスペクティブに検討した．

結 果：疾患は翼状片145眼，瘢痕性角結膜上皮症93眼，腫瘍性疾患22眼，遷延性上皮欠損15眼，結膜弛緩症12眼，緑内障11眼，その他が6眼であった．術後1年以上の経過観察を行った翼状片99眼の再発率は6.1%であった．瘢痕性角結膜上皮症の内訳は眼類天疱瘡30眼，化学外傷・熱傷29眼，Stevens-Johnson症候群23眼，その他11眼であり，93眼中88眼(94.6%)で癒着解除と結膜嚢再建を得た．腫瘍性病変は良性腫瘍12眼，悪性腫瘍10眼であり，腫瘍切除後の再建に羊膜を用いた．全例において羊膜に起因する合併症を認めなかった．

結 論：羊膜移植は翼状片の再発抑制や眼表面再建に有用である．(日眼会誌 116：374-378，2012)

キーワード：羊膜移植，翼状片，瘢痕性角結膜上皮症，眼表面再建

Indications and Surgical Outcomes of Amniotic Membrane Transplantation

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Abstract

Purpose：To evaluate the indications and surgical outcomes of amniotic membrane transplantation (AMT) for ocular-surface disease.

Subjects and Methods：This study involved 304 AMTs performed at Kyoto Prefectural University of Medicine between April 1998 and March 2008. Preoperative diagnoses, clinical features, surgical procedures and postoperative outcomes were analyzed retrospectively.

Results：Of 304 cases, 145 cases had a pterygium (48 primary, 82 recurrent, and 15 pseudo-pterygium). The recurrence rate at one year was 6.1% among the 99 cases of pterygium followed for at least one year postoperatively. Ninety-three cases had severe ocular surface diseases including ocular pemphigoid (30), chemical or thermal burn (29), Stevens-Johnson syndrome (23), and others (11)；AMT and epithelial transplantation was combined in 64 cases, and successful ocular-surface reconstruction was obtained in 88 cases (94.6%). Neoplasia was observed in 22 cases (12 benign, 10 malignant). The ocular-surface was successfully reconstructed in all cases by AMT combined with complete tumor resection. Other preoperative diagnoses included persistent epithelial defects (PED) (15), conjunctival chalasis (12) and uncontrollable glaucoma (11). No cases experienced any AMT-related complication.

Conclusions：AMT proved effective for preventing the recurrence of pterygium and for ocular-surface reconstruction in patients with severe ocular-surface disease or ocular-surface neoplasia.

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Key words：Amniotic membrane transplantation, Pterygium, Severe ocular surface disease, Ocular-surface reconstruction

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I 緒　言

羊膜は子宮と胎盤の最内層を覆う半透明の薄い膜で，羊膜上皮組織とその下の基底膜，緻密層，海綿層の4層構造をとる．免疫組織化学的にはⅣ型コラーゲンが羊膜の基底膜のみならず，緻密層，海綿層にも分布し，これが上皮伸展のための良好な基質になりうると考えられている[1]．また，羊膜には血管成分がないため拒絶反応が起こりにくいとされている．羊膜の基底膜は，生体内組織でも最も厚い基底膜とされ，皮膚熱傷後の被覆や臍ヘルニアの修復，人工膣，腹部手術の際の癒着防止などに利用されてきた[2][3]．

眼科領域では，1940年にハンガリーの de Rötth が人工膣形成術にヒントを得て，主に化学外傷に由来した眼瞼癒着に対し羊膜移植を行った報告がある[4]．その後，1995年に Kim と Tseng が，家兎眼を用いて眼表面再建における羊膜移植の有用性を報告した[5]．日本では，1996年に，Tsubota らにより，眼類天疱瘡，Stevens-Johnson 症候群に対する眼表面再建に羊膜が初めて用いられ，その有用性が示された[6]．現在では羊膜移植は，再発翼状片などの難治性眼表面疾患に広く用いられるようになった．また，羊膜移植に起因していると思われる合併症として，羊膜移植術後の methicillin-resistant *Staphylococcus aureus* 感染が Hori らにより報告されている[7]．今回，京都府立医科大学眼科で1998年以後の10年間に羊膜移植を実施した全症例の原疾患および臨床経過を解析し，その適応と効果，合併症について検討した．

II 対象および方法

対象は1998年4月から2008年3月までの10年間に京都府立医科大学眼科にて羊膜移植を施行した304眼であり，原疾患と手術目的，また10年間の羊膜移植眼数の年次推移を調査した．京都府立医科大学眼科では大学倫理委員会(倫理委)の承認を受けて，1998年4月から羊膜移植を開始した．全身合併症のない帝王切開予定の妊婦で，3か月以内の血清検査でB型肝炎，C型肝炎，梅毒，ヒト免疫不全ウイルスが陰性である者を羊膜ドナーとした．羊膜の採取ならびに臨床使用について，帝王切開前に文書による同意を得たうえで，帝王切開時に胎盤につながる羊膜を採取した．クリーンベンチで羊膜を洗浄し，3×3 cm に細切し，滅菌チューブ内に入れて−80℃で保存した．倫理委からの指示により，採取から3か月以内に限定して羊膜の臨床使用を行った．その後，採取後1年以内の期間内に凍結保存羊膜に汚染がないことを確認できたため倫理委への追加申請を行い，2007年10月からは採取後1年以内の使用としている[8]．

疾患別に術前所見，術式と術後経過についてレトロスペクティブに検討し，術後1年以上経過観察した翼状片99眼については，術前の翼状片範囲，瞼球癒着，複視，再発について解析した．翼状片の術後に，羊膜移植を行った強膜上に再び病的結膜の侵入を認めたものを「再発」と定義した．また瞼球癒着については，術前に存在した癒着が解除できて安定した所見を得たものを「結膜嚢再建」と定義した．瘢痕性角結膜上皮症については，疾患別症例数，および上皮移植の併用についても検討した．また，羊膜に起因する合併症の有無を検討した．

表1　羊膜移植症例の疾患別内訳

疾患名	手術件数	%
翼状片	145	48
瘢痕性角結膜上皮症	93	30
腫瘍性疾患	22	7
遷延性上皮欠損	15	5
結膜弛緩症	12	4
緑内障	11	4
その他	6	2
計	304	100

III 結　果

1998年4月から2008年3月までの10年間において羊膜移植を304眼に実施した．原疾患は，翼状片145眼，瘢痕性角結膜上皮症93眼，腫瘍性疾患22眼，遷延性上皮欠損15眼，結膜弛緩症12眼，緑内障11眼，その他が6眼であった(表1)．眼数はのべ手術件数であり，12眼(翼状片4眼，瘢痕性角結膜上皮症5眼，腫瘍性疾患3眼)において各1回，1眼(遷延性上皮欠損)で10回実施した再手術を含む．術式は，羊膜を基質として用いた症例が多数であったが，Stevens-Johnson 症候群の涙液減少症例に対して涙点閉塞のための代用実質(stuff)として使用し，遷延性上皮欠損症例に対しては被覆を目的として使用した．1998年から2008年3月までの年次推移は表2，図1のとおりであり，全体として増加傾向にあった．特に翼状片，瘢痕性角結膜上皮症，腫瘍性疾患は，術後の安定した成績もあり，症例数が増加していった．

羊膜移植症例の約半数を占めた翼状片145眼の内訳は初発翼状片48眼，再発翼状片82眼，偽翼状片15眼であり，再発翼状片の術前再発回数は1〜9回(平均2.3回)であった．手術時の併用療法として，高齢で結膜下結合組織が疎であるなどの症例を除き，ほぼ全例でマイトマイシンCを用いた．

術後1年以上経過を追えた翼状片99眼中，術前に翼状片の範囲が瞳孔領を超える症例は15眼(15%)，術前に瞼球癒着を認めた症例は29眼(29%)，複視を認めた症例は22眼(22%)であった．99眼のうち，1年以内に6眼に再発を認め，93眼(93.9%)で再発を生じなかった(図2)．再発した6眼はすべて角膜への侵入を認めない軽度の再発であり，再手術を要したのは1眼のみであった．

表 2 年度別疾患別羊膜移植実施件数

年度	1998	'99	2000	'01	'02	'03	'04	'05	'06	'07	計
翼状片	5	0	0	5	17	13	25	33	27	20	145
瘢痕性角結膜上皮症	12	7	3	3	2	3	11	10	22	20	93
腫瘍性疾患	0	0	1	0	1	2	3	1	8	6	22
遷延性上皮欠損	1	2	2	0	0	0	0	1	6	3	15
結膜弛緩症	0	2	0	6	1	0	1	0	0	2	12
緑内障	0	0	3	1	5	2	0	0	0	0	11
その他	0	1	1	1	0	1	2	0	0	0	6
計	18	12	10	16	26	21	42	45	63	51	304

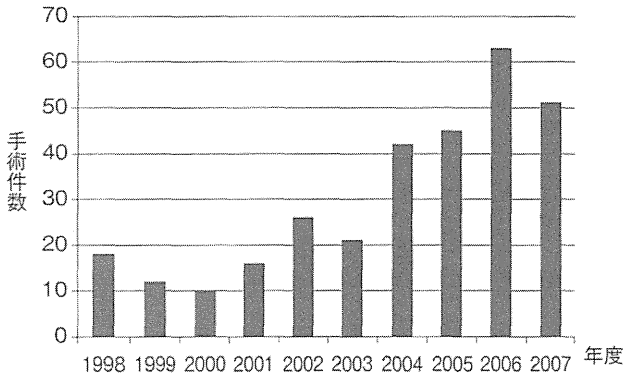

図 1 羊膜移植手術件数の年次推移.

瘢痕性角結膜上皮症 93 眼の内訳は，眼類天疱瘡 30 眼，化学外傷・熱傷 29 眼，Stevens-Johnson 症候群 23 眼，その他 11 眼であり，このうち 64 眼で上皮移植を併用した．上皮移植の術式は輪部移植・角膜上皮形成術 20 眼，培養角膜上皮シート移植 3 眼，培養口腔粘膜上皮シート移植 41 眼であった．これらの症例では，癒着解除を行った後に羊膜を移植し，瘢痕性の角膜混濁部に上皮移植を行った．93 眼中 88 眼で癒着解除と結膜嚢再建を得た．

腫瘍性病変は，結膜母斑，メラノーシスなどの良性腫瘍 12 眼，扁平上皮癌などの悪性腫瘍が 10 眼であった．腫瘍切除後の組織欠損部に羊膜を移植し，全例で良好な眼表面再建を得た．

一方で，緑内障，遷延性上皮欠損，結膜弛緩症については，年次推移において症例数の増加を認めなかった．

緑内障では，濾過胞形成の困難な末期緑内障 11 眼に羊膜移植併用の線維柱帯切除術を施行した．羊膜を用いることにより 7 眼で安定した濾過胞の形成を得た．

遷延性上皮欠損では 6 例 15 眼に羊膜が用いられていた．上皮修復が得られずに 10 回の羊膜移植を要した 1 例が含まれていた．

結膜弛緩症では，円蓋部挙上型 12 眼に施行した．全例で結膜嚢円蓋部および，下方涙液メニスカスの良好な再建を得た．その後，結膜切開を短縮できる術式の改良により羊膜を用いる必要がなくなったため，施行例は 12 眼のみとなった．

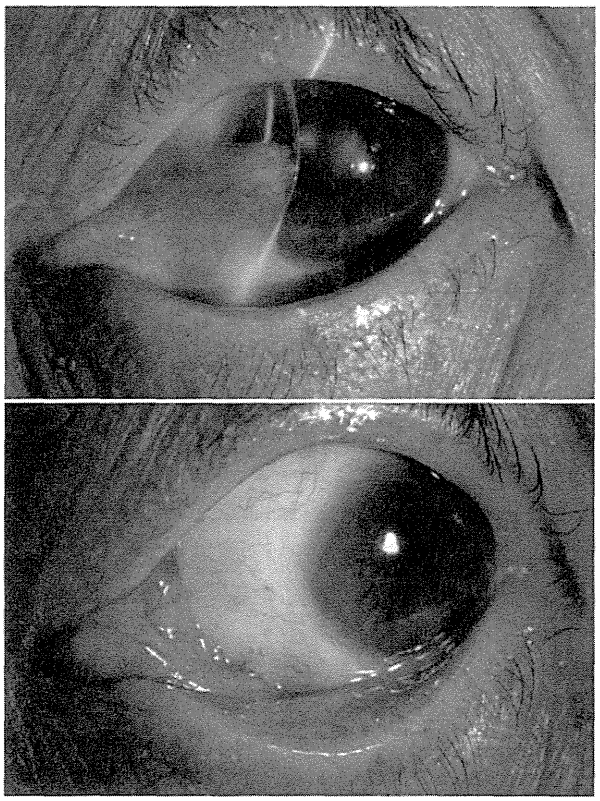

図 2 翼状片に対する羊膜移植併用翼状片切除術.
上：術前．再発翼状片であり，強い瞼球癒着を伴う．
下：術後．再発を認めず，眼表面は良好である．

羊膜移植術を行った全症例にて，羊膜に起因する感染症，拒絶反応などの合併症を認めなかった．

Ⅳ 考 按

羊膜移植使用症例数は 10 年間で徐々に増加していった．これは，羊膜が角膜上皮の再生のみならず，結膜の再建においても有効な治療材料であり，幅広い疾患に対して用いられるようになったためと考えられる．

10 年間に羊膜移植を実施した 304 眼のうち，最も多かった疾患は翼状片であり，145 眼に用いられた．羊膜

移植は，羊膜という新しい基質を供給することにより，結膜下組織の線維化を抑制し，結膜上皮の正常な分化を促すと考えられている[9]．さらに，羊膜には癒着防止作用もあり，瞼球癒着の再発を抑制する．翼状片は結膜下線維芽細胞の増殖性変化を生じて，角膜輪部のバリアーを越えて角膜上に腫瘍状に増殖したものであり，再発翼状片の場合は初発翼状片よりさらに強い結膜下組織の異常増殖と輪部バリアー機能の低下を伴う[9]．瞳孔領を越えて広範囲に及ぶ翼状片の症例や，瞼球癒着，術前複視を伴うような再発翼状片は，切除のみでは再発の可能性が高く，また自家結膜移植では組織欠損範囲を十分に被覆できない．このような症例において羊膜移植を用いると，組織欠損部を十分被覆でき，また上述したような癒着防止効果を期待できる．Shimazakiらは再発翼状片18例を含む27例の翼状片に対して，羊膜移植併用の手術を行い，良好な再建を得たと報告している[10]．福岡らは，再発翼状片手術において，羊膜を使用することは，結膜嚢再建および瘢痕抑制の両面から有効かつ安全であると報告した[11]．当科10年間の手術例においても羊膜使用の翼状片手術の成績は良好であり，再手術を要した症例は1例のみであった．

次いで症例数の多かった疾患は，瘢痕性角結膜上皮症であった．Tsubotaらは1996年に，眼類天疱瘡，Stevens-Johnson症候群における眼表面再建に羊膜を使用し，14眼中12眼で術後の良好な再建が認められたと報告した[6]．今回の著者らの検討でも眼類天疱瘡，化学外傷・熱傷，Stevens-Johnson症候群といった瘢痕性角結膜上皮症93眼中88眼で術後良好な再建を得た．瘢痕性角結膜上皮症に対する羊膜移植の効果としては，線維化の抑制，角膜上皮の増殖分化促進，抗炎症・新生血管抑制作用，創傷治癒促進効果があるといわれている[12]．

結膜腫瘍に対しては，腫瘍切除後に広範囲の結膜欠損を認めた症例に用いた．羊膜を用いることにより，広範囲の切除と良好な再建が可能となった．1997年にTsengらは，眼類天疱瘡など16眼に羊膜を使用した結膜再建術の報告をしている[13]．そのなかで，悪性黒色腫など5眼の結膜腫瘍切除後の結膜再建に羊膜を使用し，良好な再建を得たと報告し，その後も同様の症例報告がみられる[14)~16)]．

遷延性上皮欠損に対して当科では，治療用ソフトコンタクトレンズ(SCL)装用など他の治療で軽快を得られなかった症例のみを対象として羊膜移植を実施した．2007年，Sawらのイギリス国内における233例の羊膜使用症例の検討では，遷延性上皮欠損に対する使用が最も多かった[17]が，改善の乏しい症例が多いことが指摘されている．また，LetkoらはSCL装用や瞼板縫合術施行でも改善の認められなかった遷延性上皮欠損症例30眼に羊膜移植術を施行し，13例で完治を得られず，羊膜移植は遷延性上皮欠損の治療の第一選択ではないと報告した[18]．著者らも，遷延性上皮欠損への羊膜移植の効果は，SCL装用とほぼ同等であり，SCLは脱落のリスクがある半面，装用が容易であり，どちらを選ぶかは症例ごとに主治医が判断するのでよいと考える．

緑内障に対しては，難治症例において羊膜移植が濾過胞形成に有用であることが報告されている．Fujishimaらは，全層角膜移植術後などの難治性緑内障14眼に対する線維柱帯切除術において羊膜移植を併用し，良好な濾過胞が維持されて，術後の良好な眼圧コントロールが可能であったと報告した[19]．久保らは，複数回の緑内障手術後の難治性緑内障眼と全層角膜移植術後の眼圧維持が困難であった症例の2例に対して羊膜を用いて線維柱帯切除術を行い，良好な術後成績を得ている[20]．また樋野らは，緑内障末期かつ重症の偽眼類天疱瘡に対して，羊膜移植併用線維柱帯切除術を施行し，羊膜移植併用により濾過胞の維持に成功して良好な眼圧維持が可能となったことを報告した[21]．羊膜移植は，癒着や瘢痕を伴う難治緑内障症例の濾過胞形成に有用と思われる．ただし，それらの多くは末期緑内障であり，実際に適応となる症例は少ない．当科でも，11眼全例で良好な濾過胞形成を得たが，その後は適応となる症例が減少した．その理由として，重症の瘢痕性角結膜症を伴う緑内障症例が減少したこと，また抗緑内障治療薬の選択肢が増えて保存的に眼圧コントロールできる症例が増えたことが考えられた．

羊膜は，上皮化促進，癒着防止などのさまざまな効果があり，眼表面再建術において必要不可欠な存在となった．今回実施した304例において，羊膜に対する拒絶反応を生じた症例がないことは特筆すべき事実である．当教室の上田らは，羊膜そのものがアロ移植に伴う免疫反応を抑制することを報告しており[22]，今回の結果はそれを裏付けるものと考えられた．今回，良好な術後成績を示した翼状片，重症の瘢痕性角結膜上皮症，腫瘍性疾患には，今後も手術が継続して行われると予想される．また羊膜にはepidermal growth factor，keratinocyte growth factor，hepatocyte growth factorといったサイトカインが多く含まれ[23]，今後新たな用途が開発される可能性もある．

現在，国内13施設において，再発翼状片，角膜上皮欠損，角膜穿孔，角膜化学腐食，角膜瘢痕，瞼球癒着，結膜上皮内過形成，結膜腫瘍その他の眼表面疾患に対する先進医療として羊膜移植術が行われている．羊膜移植の術後成績が安定し，有用性が明らかになったことにより，今後，羊膜移植の症例数はさらに増加することが予想される．現在，日本角膜学会が定めたガイドラインに基づいて各病院で羊膜入手と処理を行っている．しかしすべての病院がガイドラインに準拠して羊膜を入手および使用するには，費用，その他の面で困難を伴う．安全性を確保した羊膜入手のため，組織バンクなどの整備が

今後の社会的課題である.

利益相反：利益相反公表基準に該当なし

文 献

1) 平野耕治：【特集 眼表面の再生医学と羊膜移植】羊膜の細胞生物学的な特殊性. 眼科手術 15：17-23, 2002.
2) 小泉範子, 木下 茂：【特集 羊膜移植】 組織移植としての問題. 眼科 42：245-250, 2000.
3) **Tseng SC, Espana EM, Kawakita T, Di Pascuale MA, Li W, He H,** et al：How does amniotic membrane work? Ocul Surf 2：177-187, 2004.
4) **de Rötth A**：Plastic repair of conjunctival defects with fetal membranes. Arch Ophthalmol 23：522-525, 1940.
5) **Kim JC, Tseng SC**：Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea 14：473-484, 1995.
6) **Tsubota K, Satake Y, Ohyama M, Toda I, Takano Y, Ono M,** et al：Surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens-Johnson syndrome. Am J Ophthalmol 122：38-52, 1996.
7) **Hori Y, Inoue R, Ikuno Y, Inoue T, Maeda N, Tano Y**：Severe methicillin-resistant *Staphylococcus aureus* infection after multilayer amniotic membrane transplantation. Jpn J Ophthalmol 53：61-62, 2009.
8) 稲富 勉, 小泉範子：【特集 羊膜移植】 羊膜の採取と保存. 眼科 42：251-256, 2000.
9) 片上千加子：【特集 羊膜移植】 翼状片への応用. 眼科 42：271-277, 2000.
10) **Shimazaki J, Kosaka K, Shimmura S, Tsubota K**：Amniotic membrane transplantation with conjunctival autograft for recurrent pterygium. Ophthalmology 110：119-124, 2003.
11) 福岡秀記, 稲富 勉, 中村隆宏, 外園千恵, 木下 茂：羊膜移植による再発翼状片手術の術後成績. あたらしい眼科 24：381-385, 2007.
12) 島﨑 潤：【特集 羊膜移植】 瘢痕性角結膜症への応用. 眼科 42：285-290, 2000.
13) **Tseng SC, Prabhasawat P, Lee SH**：Amniotic membrane transplantation for conjunctival surface reconstruction. Am J Ophthalmol 124：765-774, 1997.
14) **Paridaens D, Beekhuis H, van Den Bosch W, Remeyer L, Melles G**：Amniotic membrane transplantation in the management of conjunctival malignant melanoma and primary acquired melanosis with atypia. Br J Ophthalmol 85：658-661, 2001.
15) **Kobayashi A, Takahira M, Yamada A, Segawa Y, Tanahashi T, Shirao Y,** et al：Fornix and conjunctiva reconstruction by amniotic membrane in a patient with conjunctival mucosa-associated lymphoid tissue lymphoma. Jpn J Ophthalmol 46：346-348, 2002.
16) **Tomita M, Goto H, Muramatsu R, Usui M**：Treatment of large conjunctival nevus by resection and reconstruction using amniotic membrane. Graefes Arch Clin Exp Ophthalmol 244：761-764, 2006.
17) **Saw VP, Minassian D, Dart JK, Ramsay A, Henderson H, Poniatowski S,** et al：Amniotic membrane transplantation for ocular disease：a review of the first 233 cases from the UK user group. Br J Ophthalmol 91：1042-1047, 2007.
18) **Letko E, Stechschulte SU, Kenyon KR, Sadeq N, Romero TR, Samson CM,** et al：Amniotic membrane inlay and overlay grafting for corneal epithelial defects and stromal ulcers. Arch Ophthalmol 119：659-663, 2001.
19) **Fujishima H, Shimazaki J, Shinozaki N, Tsubota K**：Trabeculectomy with the use of amniotic membrane for uncontrollable glaucoma. Ophthalmic Surg Lasers 29：428-431, 1998.
20) 久保真人, 鈴木悦子, 森 秀樹, 村松隆次, 臼井正彦：難治性緑内障に対する羊膜移植併用トラベクレクトミー. あたらしい眼科 18：1201-1205, 2001.
21) 樋野景子, 森 和彦, 外園千恵, 池田陽子, 成瀬繁太, 石橋 健, 他：羊膜移植併用線維柱帯切除術を施行した薬剤性偽眼類天疱瘡の1例. 日眼会誌 110：312-317, 2006.
22) **Ueta M, Kweon MN, Sano Y, Sotozono C, Yamada J, Koizumi N,** et al：Immunosuppressive properties of human amniotic membrane for mixed lymphocyte reaction. Clin Exp Immunol 129：464-470, 2002.
23) **Koizumi N, Inatomi T, Sotozono C, Fullwood NJ, Quantock AJ, Kinoshita S**：Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res 20：173-177, 2000.

Interaction Between Conjunctival Epithelial Cells and Mast Cells Induces CCL2 Expression and Piecemeal Degranulation in Mast Cells

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Purpose. Intraepithelial mast cells are observed in giant papillae tissue samples obtained from patients with atopic keratoconjunctivitis (AKC)/vernal keratoconjunctivitis (VKC). We examined the roles of interaction between the conjunctival epithelial cells and mast cells.

Methods. The interaction between human mast cells and conjunctival epithelial cells (HCjE) was investigated using a coculture model. Protein array analysis, ELISA, and real-time PCR were performed to test the interaction. Tissue samples ($n = 6$) from giant papillae were resected for therapeutic purposes, and subjected to immunohistological analysis of CCL2 expression. Recombinant CCL2 (10 ng/mL) was reacted with the cultured human mast cells and ultrastructural analysis was performed. A ragweed (RW)-induced mouse experimental allergic conjunctivitis model was used to examine *ccl2* mRNA expression and mast cell morphology.

Results. Protein array and real-time PCR analyses showed that CCL2 protein/mRNA expression was induced by mast cell-HCjE coculture. Upregulation of *CCL2* mRNA was observed in mast cells, whereas in situ CCL2 expression was observed at the conjunctival epithelium of the giant papillae by immunohistochemistry. Ultrastructural analysis showed that recombinant CCL2 treatment induced piecemeal degranulation (PMD) in the mast cells. Ultrastructural analysis of tissues from the giant papillae showed PMD of mast cells within the conjunctival epithelial cells. The RW-induced experimental allergic conjunctivitis model showed increased *ccl2* mRNA expression and PMD morphology in the conjunctivae.

Conclusions. Mast cell–conjunctival epithelial cell interaction induces CCL2 expression and subsequent PMD.

Keywords: allergy, conjunctivitis, mast cell, degranulation

Mast cell activation and migration within and around the conjunctival epithelium is one of the histopathologic features of severe chronic allergic conjunctivitis, atopic keratoconjunctivitis (AKC),[1] and vernal keratoconjunctivitis (VKC).[2] In our study, we investigated possible interactions of mast cells and conjunctival epithelial cells using in vitro coculture models, and we found that CCL2 expression in mast cells was upregulated by coculture. A previous report showed that CCL2 could induce piecemeal degranulation (PMD) in basophils.[3] PMD and anaphylactic degranulation are known as two distinct types of mast cell degranulation.[4,5] Anaphylactic degranulation is a degranulation style with antecedent granule-to-granule and/or granule-to-plasma membrane fusions. On the other hand, gradual emptying of cytoplasmic secretory granules in the absence of granule-to-granule or granule-to-plasma membrane fusion events is observed in PMD. Previously, we reported that 34 of 168 mast cells observed in the giant papillae of eight eyes obtained from VKC patients showed PMD, whereas only 28 of the 168 mast cells had the morphology of anaphylactic degranulation.[6] These results suggested that not only anaphylactic degranulation, but also PMD might have some roles in the pathophysiology of AKC and VKC. In our study, we investigated the roles of mast cells and epithelial cells interactions, as well as the roles of PMD and CCL2 expression in mast cells, in the pathophysiology of AKC/VKC.

Materials and Methods

Coculture Model of Mast Cells and Conjunctival Epithelial Cells

Human mast cell line LAD2 was provided by Dr Arnold Kirchenbaum (NIH) and maintained as described previously.[7] Human peripheral blood derived mast cells (p-mast) were raised and maintained as described previously.[8] The human conjunctival epithelial cell line (HCjE) was provided by Prof Ilene Gipson (Schepens Eye Research Inst., Boston, MA) and

maintained as described previously.[9] Coculture models of these cells were made using Costar Transwell permeable supports (for 12-well culture dishes).

Antibody Array Analysis and ELISA Analysis Using Culture Supernatant

For antibody array analysis, the culture supernatant was incubated with Human Inflammation Array No. 3 (Ray Biotech, Inc., Norcross, GA) according to the manufacturer's protocol. The results were visualized and quantified using an LAS 3000 image ware (Fuji Film, Tokyo, Japan). Human CCL2 ELISA was performed using a Quantikine CCL2 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Giant Papillae and Control Conjunctivae Samples

Giant papillae were resected for therapeutic purposes from 5 patients, 3 with AKC and 2 with VKC, and control conjunctival tissue was biopsied from 8 conjunctivochalasis patients during resection surgery after obtaining written informed consent as described previously.[10] Additional giant papillae were obtained from 2 AKC and 4 VKC patients for immunohistochemical analysis. All procedures were approved by the ethics committees of the Juntendo University School of Medicine and Kyoto Prefectural University of Medicine, and the study was conducted in accordance with the tenets of the Declaration of Helsinki. AKC was defined as a bilateral chronic inflammation of the conjunctiva and eyelids associated with atopic dermatitis, and VKC was defined as a chronic, bilateral, conjunctival inflammatory condition found in individuals as described previously.[11]

Reverse Transcription (RT) and Real-Time PCR

Total RNA was extracted from the cultured cells and tissues of the giant papillae using a NucleoSpin II RNA isolation kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany), and cDNAs were prepared from 1 μg of total RNA using random primers and the RevaTra-Ace reverse transcriptase (both from Toyobo, Tokyo, Japan) according to the manufacturer's protocol. We used TaqMann real-time PCR probes and primers specific for human *CCL2* (Hs00234140_ml) and 18SrRNA, obtained from Applied Biosystems (Assay-on-Demand gene expression products; Applied Biosystems, Inc., Foster City, CA). Real-time PCR analysis was performed on a 7500 Real-Time PCR system (Applied Biosystems). For *CCL2* mRNA expression, the comparative Ct method, which uses the 18SrRNA expression in the same cDNA as a control, was used. *CCL4* and *ccl2* mRNA were quantified using Fast SYBR green master mix (Applied Biosystems), and the following pairs of the primers: Forward 5′-CTGTGCTGATCCCAGTGAATC-3′, reverse 5′-TCAGTTCAGTTC CAGGTCATACA-3′ (*CCL4*) and forward 5′-AGCAGCAGGTGTC CCAAAGAAG-3′, reverse 5′-GCACAGACCTCTCTCTTGAG CTTG-3′ (*ccl2*). For *CCL4* and *ccl2* mRNA expression, the comparative Ct method, which uses the GAPDH/*gapdh* expression in the same cDNA as controls, was used.

Immunohistochemical Analysis

The specimens from the giant papillae were fixed immediately with 4% paraformaldehyde (PFA) in PBS for 3 hours. After washing with 30% sucrose in PBS, the tissues were frozen in Optimal Cutting Temperature (OCT; Sakura Finetek, Tokyo, Japan) compound using liquid nitrogen. Then, 5 μm frozen sections were made and air-dried. Immunohistochemical staining was performed according to the described previously methods.[11] A mouse anti-human CCL2 monoclonal antibody (R&D Systems) was used as a primary antibody (10 μg/mL). For mast cell staining, a rabbit anti-FcεRIβ antibody was prepared and used as described previously.[11] Alexa 488-, and 594-conjugated donkey anti-mouse IgG, and anti-rabbit IgG (all from Invitrogen Corporation, Carlsbad, CA) were used as secondary antibodies. Negative control staining was performed using isotype-matched IgG (normal mouse IgG1; BioLegend, San Diego, CA, and normal rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA) as substitutes for the primary antibodies. A confocal laser scanning microscope (FV-1000; Olympus, Tokyo, Japan) was used for imaging.

Ultrastructural Analysis of Human Cultured Mast Cells

P-mast cells were stimulated with 20 ng/mL recombinant human CCL2 (Peprotech, London, UK) for 3 minutes,[3] and fixed with 2.5% glutaraldehyde and postfixed with 2% osmic acid. HCjE cocultured p-mast cells also were prepared for ultrastructural analysis after 24-hour coculture experiments. For negative control, recombinant human CXCL8 (20 ng/mL for 3 minutes; Peprotech) stimulated p-mast cells, and cross-linked p-mast cells using human IgE (1 μg/mL; Chemicon/Millipore, Billerica, MA) and rabbit anti-IgE (1μg/mL; Dako Japan, Kyoto, Japan) were prepared. The samples were embedded in epoxy resin and ultrathin sections (60–80 nm) were made. The ultrathin sections then were examined using a transmission electron microscope (7000-100; Hitachi High-Technologies, Tokyo, Japan).

Alum Ragweed (RW)–Induced Experimental Allergic Conjunctivitis

RW-induced experimental allergic conjunctivitis models were prepared as described previously using male BALB/C mice at the age of 10 to 12 weeks (SLC, Hamamatsu, Japan).[12] The expression of *ccl2* mRNA was quantified for mouse conjunctival tissue 24 hours after final RW challenge. For comparison, a single-challenge RW eye drop model and 4-challenge RW eye drop model were used. Ultrastructural analysis was performed for conjunctival and eyelid samples after final RW challenge (4-challenge model). All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Results

Mast Cell HCjE Interaction Induces CCL2 Expression in Mast Cells

The coculture experiment showed synergistic increases of CCL2 and CCL4 protein in the supernatant of the mast cell (LAD2) HCjE coculture model (Figs. 1A–C). These results also were confirmed by ELISA analysis (Fig. 1D). The LAD2 HCjE coculture model induced 2-fold higher *CCL2* mRNA expression in LAD2 cells than in LAD2 cells cultured by themselves. The p-mast HCjE coculture model using also showed a significant increase of *CCL2* mRNA (Fig. 2A). For *CCL4* mRNA expression, only HCjE-cocultured LAD2 showed a *CCL4* mRNA increase; no change was observed in HCjE-cocultured p-mast cells (Fig. 2B).

Epithelial Cells and Infiltrating Cells in VKC/AKC Tissue Express CCL2 Protein In Situ

Immunohistologic analysis of the tissue samples from the giant papillae showed positive CCL2 immunostaining in epithelial

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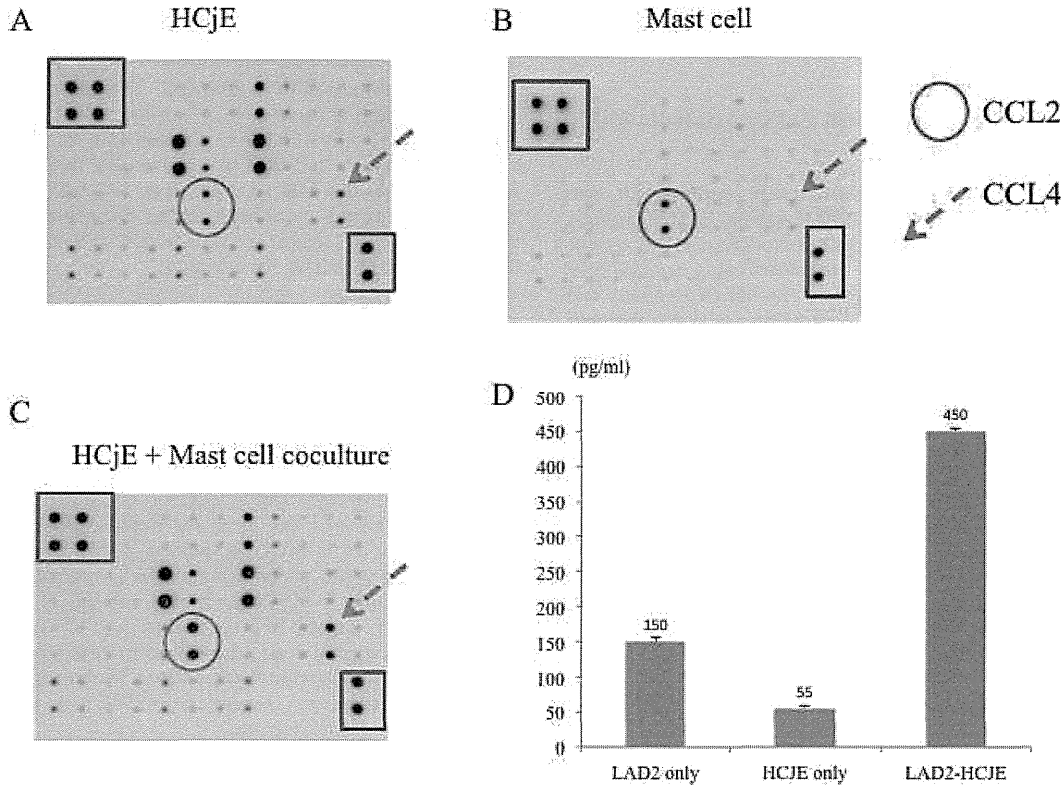

FIGURE 1. Mast cell conjunctival epithelial cell interaction induces CCL2 expression in mast cells. Antibody arrays were used to analyze HCjE (**A**), LAD2 (mast cell, [**B**]), and LAD2 HCjE coculture (**C**) supernatants. Synergistic increases of CCL2 (*red circles*) and CCL4 protein (*red arrows*) in the coculture supernatant (**C**). *Blue squares* are positive control samples for array reactions. The synergistic increase of CCL2 in the supernatant of a coculture sample also was confirmed by ELISA analysis (**D**).

cells (Fig. 3). FcεRIβ-immunopositive mast cells also were observed within and around CCL2-positive epithelial cells (Figs. 3B, 3C). We confirmed the specificity of the CCL2 immunostaining by using isotype-matched normal mouse IgG1 instead of the CCL2 antibody (Fig. 3F). The specificity of FcεRIβ immunostaining already was demonstrated in our previous report[11] and we always run negative controls specimens in our experiments (data not shown). CCL2-positive infiltrating cells also were observed in the substantia propria of the tissue (Fig. 4A). Higher magnification of the Figure revealed the CCL2/ FcεRIβ double-positive mast cells (arrows in Figs. 4B, 4C), as well as CCL2+/FcεRIβ– infiltrating cells (arrow-

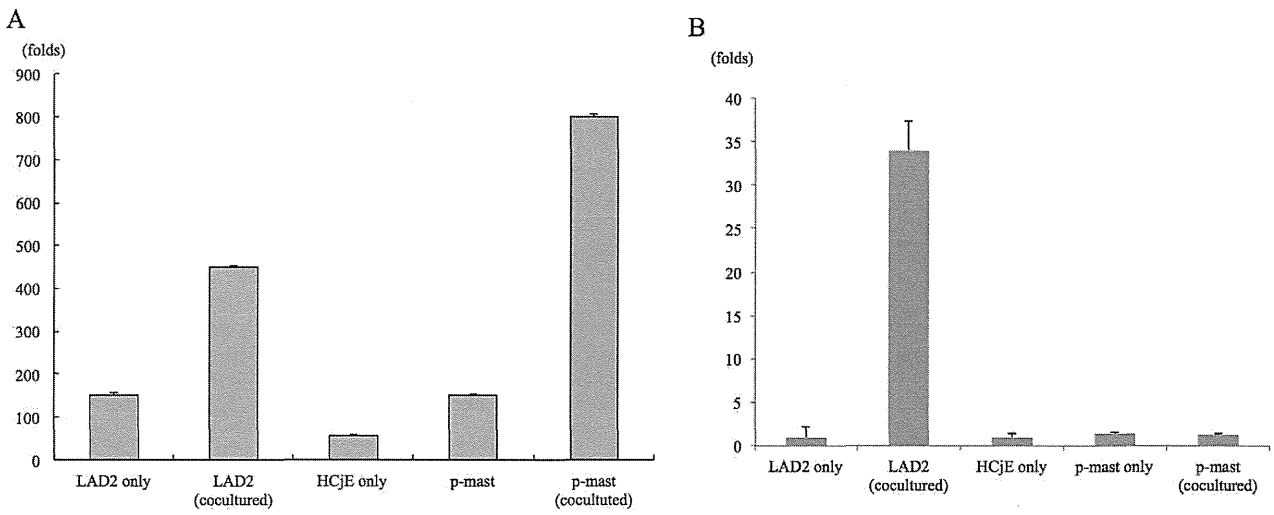

FIGURE 2. Mast cell HCjE coculture induces CCL2 mRNA expression. The mast cell HCjE coculture model induced significantly increased CCL2 mRNA expression in LAD2 and p-mast cells compared to that in mast cells cultured alone (**A**). On the other hand, CCL4 mRNA induction was observed only with HCjE-cocultured LAD2, and no change was observed in HCjE-cocultured p-mast cells (**B**).

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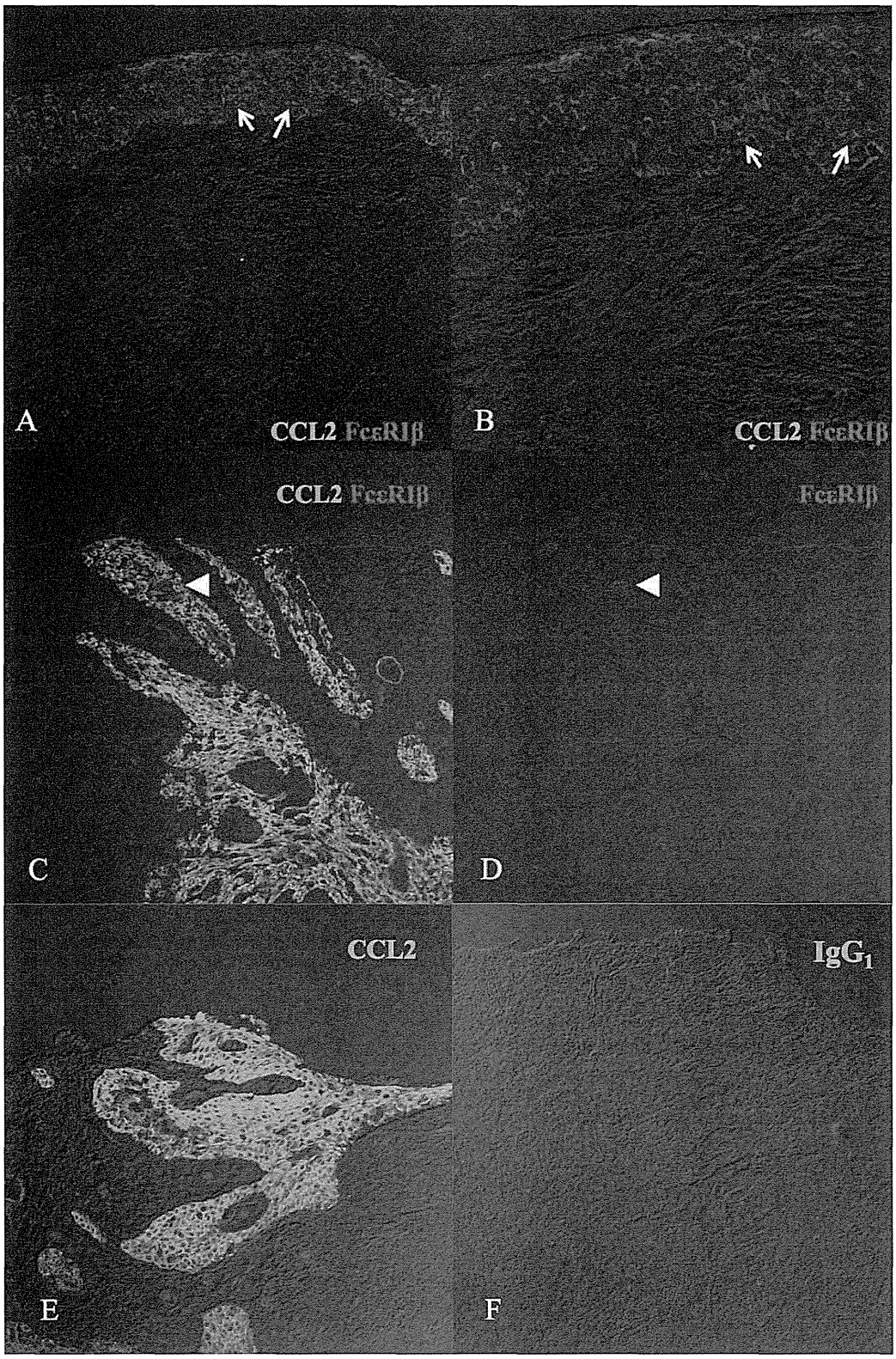

FIGURE 3. CCL2 expression in the conjunctival epithelial cells of giant papillae. Perpendicular (sagittal, [A] and [B]) and horizontal (coronal, [C] and [D]) sections of tissues from giant papillae showed positive CCL2 immunostaining (*green*) in epithelial cells. FcεRIβ-positive mast cells (*red*) also were observed within and around CCL2-positive epithelial cells (*arrows* in [A] and [B], *arrowhead* in [C]). The FcεRIβ single staining image of (C) also is shown in (D). Specificity of CCL2 immunostaining was shown by anti-CCL2 staining (E) and control mouse IgG_1 antibody staining using two adjacent sections (F). Original magnification (A), (C), and (D) ×200; (B), (E), and (F) ×400.

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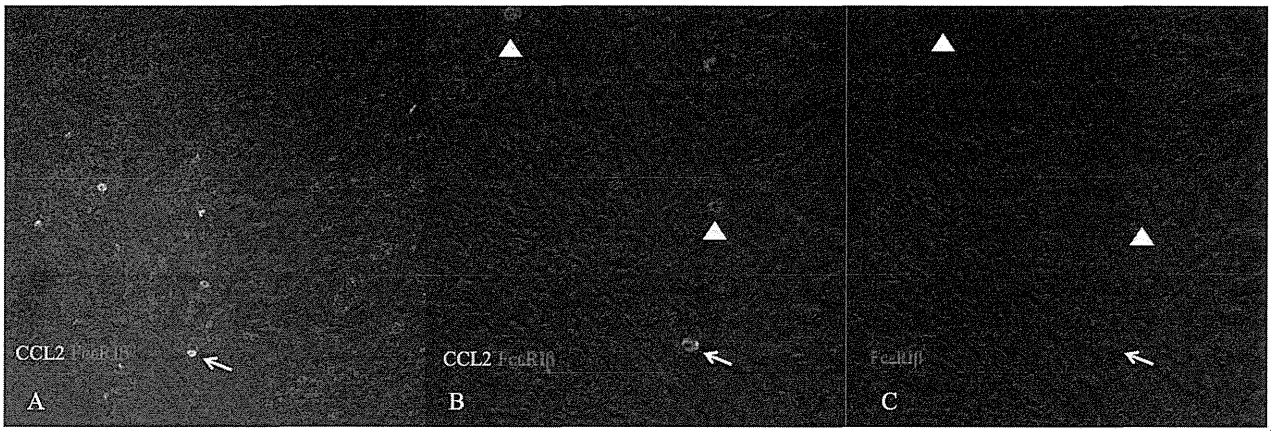

FIGURE 4. CCL2 expression in the substantia propria of giant papillae. CCL2 immunohistochemical staining of the substantia propria of giant papillae is shown. CCL2/ FcεRIβ double-positive mast cell (*arrow*) and CCL2+/ FcεRIβ- infiltrating cells (*arrowheads*) are observed in the substantia propria of giant papillae tissue. (**B**) is a high magnification image of (**A**), (**C**) is the FcεRIβ single staining image of (**B**). Original magnification (**A**) ×200, (**B**) and (**C**) ×400.

heads). The results of CCL2 immunohistochemical staining are summarized in Table 1. The existence of mast cells was also verified by anti-tryptase immunostaining of adjacent sections (Supplementary Fig. S1).

Significantly Higher *CCL2* mRNA Expression was Observed in Tissues From Giant Papillae Than in Conjunctivochalesis Tissue Samples

Five samples from giant papillae and eight conjunctivochalasis samples (Table 2) were collected, and cDNA was prepared. Real-time PCR analysis showed significantly increased CCL2 mRNA in the samples from giant papillae compared to the conjunctivochalasis samples (Fig. 5).

Mast Cells in the Giant Papillae Showed PMD and Recombinant CCL2 Could Induce PMD

Three-minute treatment of p-mast cells with recombinant CCL2 (20 ng/mL) could induce PMD (Figs. 6B, 6F) compared to naïve p-mast cells (Figs. 6A, 6E). P-mast HCjE coculture (for 24 hours) also induced PMD morphology (Figs. 6C, 6G). IgE/anti-IgE crosslinking treatment induced anaphylactic degranulation in p-mast cells (Fig. 6D). In contrast to CCL2 treatment, CXCL8 (IL-8) treatment did not induce PMD (Fig. 6H). Ultrastructural analysis of giant papillae showed that intraepithelial mast cells had PMD morphology (Fig. 7A) as well as anaphylactic degranulation morphology (Fig. 7B).

Ccl2 Expression in Alum-RW Induced Mouse Experimental Allergic Conjunctivitis

Increased *ccl2* mRNA expression (Fig. 8A) was observed in RW-induced allergic conjunctivitis (4-challenge RW eye drop model) compared to PBS-challenged conjunctivae. The single-challenge RW eye drop model did not show a significant *ccl2* mRNA increase compared to the PBS-challenged conjunctivae. PMD morphology was observed in the conjunctival mast cells of RW conjunctivitis (Fig. 8B).

DISCUSSION

In our study, we showed that mast cell-conjunctival epithelial cell interaction induced CCL2 expression at the mRNA and at protein levels. We used LAD2 cells (a cell line) and p-mast cells (primary-cultured cells) as the source of mast cells. Initial coculture experiments, including antibody array experiments, were performed using LAD2, and confirmatory experiments were performed with primary p-mast cells due to their limited availability. Increased concentrations of CCL2 and CCL4 (MIP-1β) protein were observed in LAD2 HCjE coculture supernatant (Fig. 1), and upregulation of *CCL2* mRNA and *CCL4* mRNA was confirmed in LAD2 cells in the coculture model. We then tried to replicate the results using p-mast cells. *CCL2* mRNA upregulation was observed in cocultured p-mast cells, but no CCL4 mRNA upregulation was observed (Figs. 2A, 2B), so we focused at the role of CCL2 for further studies.

TABLE 1. Summary of CCL2 Immunostaining

Case No.	Age	Sex	Total IgE	Specific IgE	Diagnosis	CCL2 Immunostaining		Treatment
						Epithelium	Substanita Propria	
1	16	F	509	Positive	VKC	+	+	Dex, CsA
2	22	M	89	Positive	VKC	+	−	Dex
3	13	M	2319	Positive	VKC	+	−	Dex
4	18	M	375	Positive	AKC	+	−	Dex, CsA
5	21	M	1904	Positive	AKC	+	+	Dex
6	29	M	56	Positive	VKC	+	+	Dex

Dex, 0.1% dexamethasone eyedrop; CsA, 0.1% cyclosporine eyedrop.

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TABLE 2. Clinical Information for CCL2 Expression Analysis

	Age	Sex	Diagnosis
Control 1	69	F	Conjunctivochalasis
Control 2	65	F	Conjunctivochalasis
Control 3	59	M	Conjunctivochalasis
Control 4	74	M	Conjunctivochalasis
Control 5	69	F	Conjunctivochalasis
Control 6	80	F	Conjunctivochalasis
Control 7	65	F	Conjunctivochalasis
Control 8	73	F	Conjunctivochalasis
Case 1	21	M	AKC
Case 2	18	M	AKC
Case 3	12	M	VKC
Case 4	32	M	AKC
Case 5	29	M	VKC

To examine the relevance to the pathophysiology of AKC/VKC, we next evaluated CCL2 expression in the tissues of giant papillae tissue obtained from patients.

Immunohistochemical analysis showed CCL2-positive staining of conjunctival epithelial cells (Fig. 3). Double immunohistochemical staining with a mast cell marker (FcεRIβ) showed mast cells within and beneath the CCL2-positive conjunctival epithelial cells (Figs. 3A–C). We also found positive CCL2 immunostaining at the substantia propria of the tissue from the giant papillae (Fig. 4). Abu El-Asrar et al. found increased number of CCL2-positive staining of cells infiltrating the substantia propria of the limbal tissue of VKC.[13] Although they reported negative CCL2 expression in the conjunctival epithelium in limbal VKC tissue, we clearly detected CCL2-positive immunostaining of the conjunctival epithelium tarsal form of giant papillae (Fig. 3). Giustizieri et al. demonstrated CCL2 mRNA expression in the epithelial cells of the lesional skin of atopic dermatitis patients by in situ hybridization.[14] Gordon reported increased CCL2 expression in epidermal cells and dermal cells in a dinitrophenyl serum albumin-induced mouse passive cutaneous anaphylaxis (PCA) model, using immunohistological analysis.[15] Mercer et al. reported positive CCL2 immunostaining at the epithelial cells of human idiopathic pulmonary fibrosis tissue but not at the epithelial cells of lung tumor tissue (control tissue).[16] These three reports on CCL2 expression are supportive for our results for positive CCL2 immunostaining of epithelial cells. Since we did not examine the expression of CCL2 in the limbal VKC tissue, the reason for the difference between our results and those of Abu El-Asrar et al.[13] is unknown. We speculate that there may be a difference between the limbal and tarsal forms of VKC for the epithelial expression of CCL2.

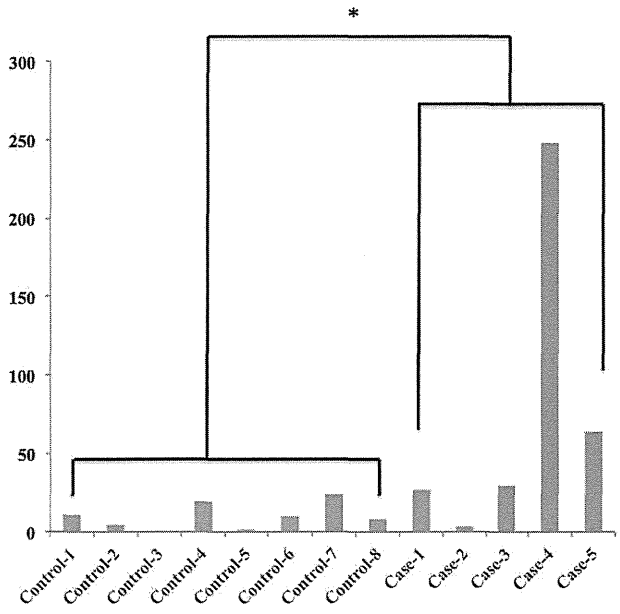

FIGURE 5. Increased CCL2 mRNA expression in the tissues from giant papillae. Five samples from giant papillae and eight control (conjunctivochalasis) samples were analyzed. Real-time PCR analysis showed significantly higher CCL2 mRNA expression in the samples from giant papillae than in the control samples. *$P < 0.05$, Mann-Whitney U test.

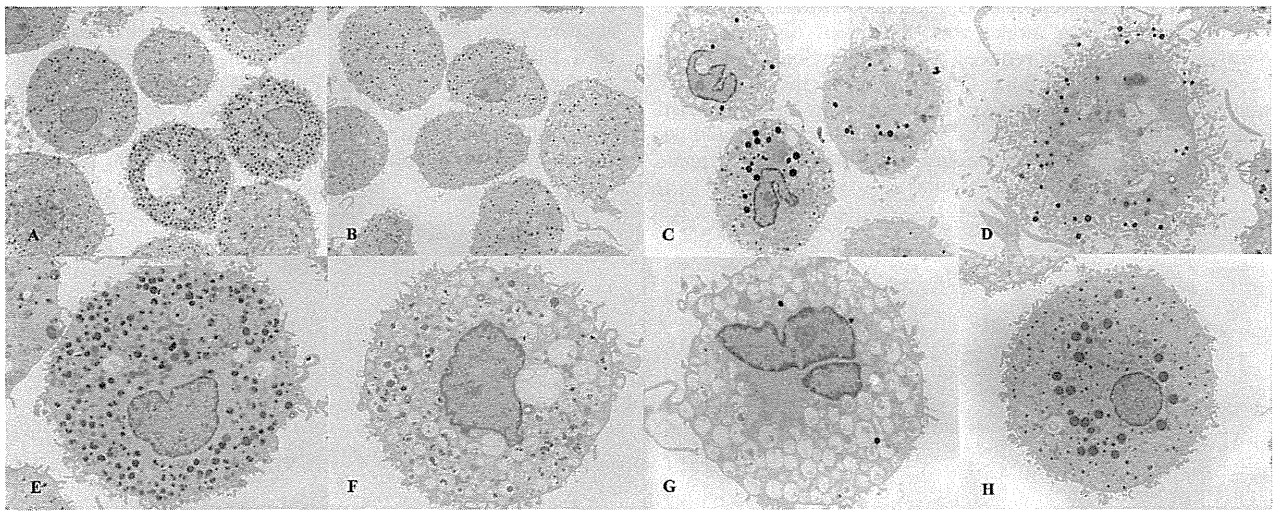

FIGURE 6. Recombinant CCL2 stimulation could induce PMD morphology. CCL2-(20 ng/mL, for 3 minutes) stimulated p-mast cells (**B**, **F**) show PMD morphology compared to naïve p-mast cells (**A**, **E**). P-mast HCjE coculture also induced PMD morphology (**C**, **G**). Anaphylactic degranulation morphology of a p-mast cell (**D**) induced by IgE/anti-IgE crosslinking is shown (**D**). A CXCL8-stimulated (20 ng/mL, for 3 minutes) p-mast cell (**H**) is shown as a negative control.

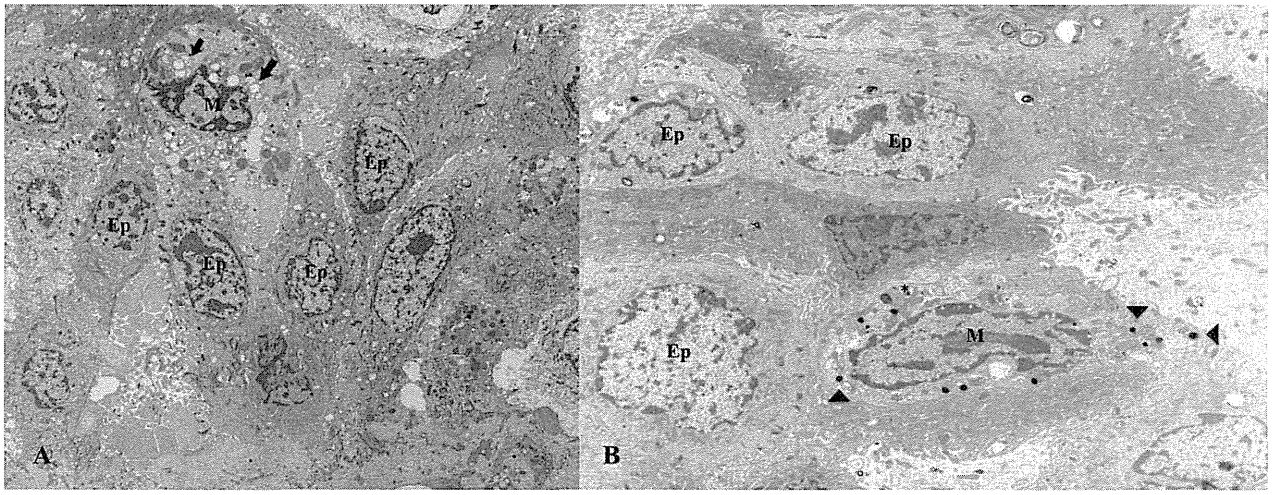

FIGURE 7. Intraepithelial mast cells show PMD morphology in situ. Ultrastructural analysis of a giant papilla obtained from a VKC patient shows intraepithelial mast cells (*M*) with PMD morphology (**A**) and with anaphylactic degranulation morphology (**B**). Empty granule chambers (*arrows*) in a mast cell with PMD morphology, and released granules (*arrowheads*) and labyrinth formation (*asterisk*) in a mast cell with anaphylactic degranulation morphology are shown. Ep, conjunctival epithelial cells.

We found few CCL2/FcεRIβ double-positive mast cells (Fig. 4B) by immunohistochemical analysis. Although the main CCL2 mRNA-producing cells were mast cells (Fig. 2A), CCL2 protein also was secreted from mast cells as we found in culture supernatant samples (Fig. 1D). Therefore, we hypothesized that continuous CCL2 secretion from mast cells was the reason we found few CCL2/FcεRIβ double-positive mast cells. This discrepancy between abundant CCL2 mRNA expression and poor CCL2 retention in mast cells has been reported previously.[15,17]

We obtained tarsal giant papilla tissues from refractory AKC/VKC patients, all of whom were treated with topical dexamethasone eye drops for at least 4 weeks (Table 1), so treatment may have downregulated the CCL2 expression as reported previously.[18] Nonetheless, significantly higher CCL2 mRNA expression in samples from giant papillae than in conjunctival tissues obtained from conjunctivochalasis patients was observed by real-time PCR analysis (Fig. 5).

Interestingly, the report of Gordon also showed that CCL2 expression in a PCA model was dependent on mast cells because of significantly reduced CCL2 expression in the skin of mast cell-deficient mice (*W/Wv*) during the PCA reaction.[15] Their results suggesting that interaction between mast cells and other components of conjunctival cells (including conjunctival epithelial cells) could upregulate CCL2 expression during allergic reactions agreed with our results in this study.

To elucidate further the role of CCL2 protein in the pathophysiology of AKC/VKC, we examined the activation pattern of mast cells with special reference to PMD. We found that recombinant CCL2 stimulation (Figs. 6B, 6F) as well as HCjE coculture procedures (Figs. 6C, 6G) could induce PMD in cultured mast cells in vitro. We also tried to inhibit the effect of CCL2 by adding a CCR2 inhibitor (RS504393 from TOCRIS Bioscience) to the coculture model, and found partial inhibition of the PMD phenomenon (data not shown). Consistent with the results of a previous report,[19] p-mast cells stimulated with another chemokine (CXCL8) did not show PMD morphology (Fig. 6H). This result also supported the specificity of the CCL2-induced PMD phenomenon. Although we could not deny the possibility of other conjunctival epithelial cell-derived mast cell activators, CCL2 in the coculture medium had some roles in PMD. Continuous studies are ongoing in our laboratory to elucidate possible additional activators. We also found PMD in the intraepithelial mast cells of a VKC patient, showing the relevance of PMD to the pathophysiology of VKC (Fig. 7). In our previous study, 20% of the mast cells in the giant papillae samples showed the PMD morphology and 17% of the mast cells in the giant papillae samples showed anaphylactic degranulation in VKC patients.[6]

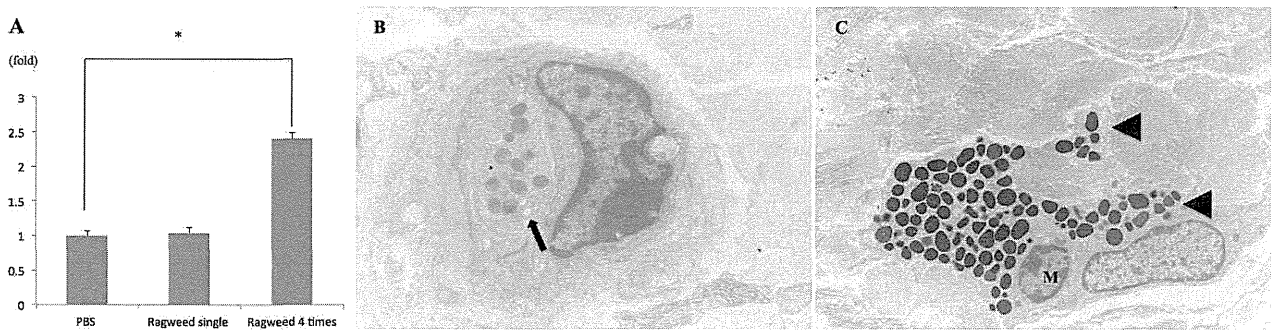

FIGURE 8. *Ccl2* expression in mouse experimental allergic conjunctivitis. Alum-RW-induced mouse experimental conjunctivitis shows increased ccl2 mRNA. (**A**) PMD morphology (*arrow*) and anaphylactic degranulation (*arrowheads*) are observed in the mast cells of conjunctival tissue (**B**, **C**). $^*P < 0.05$ by Student's *t*-test. The nucleus of a degranulated mast cell is shown (*M*).

These results suggested the importance of PMD and subsequent slow/persistent mediator release[20] during chronic allergic keratoconjunctivitis. The magnitude of inflammation with PMD seems to be smaller than with anaphylactic degranulation; however, the PMD reaction lasts longer without IgE crosslinking by the antigen.[5] On the other hand, the mediator release from mast cells is not long lasting in the case of anaphylactic degranulation because it needs some time to regain the cytoplasmic granules.[4]

We also confirmed *Ccl2* mRNA upregulation and PMD of mast cells in the RW-induced mouse experimental allergic conjunctivitis model. After 4 RW eye drop challenges, but not after a single RW eye drop challenge, increased *ccl2* mRNA expression compared to PBS-challenged control conjunctival tissue and PMD morphology were observed in the RW-challenged conjunctival tissue (Fig. 8A). This result suggested that chronic antigen stimuli were *ccl2* mRNA-inducing factors. Although there are no appropriate mouse models for AKC/VKC, and RW-induced mouse experimental allergic conjunctivitis is considered to be an animal model of seasonal allergic conjunctivitis,[21] RW-induced allergic conjunctivitis can be used as a model of chronic allergic inflammation induced by repeated antigen stimuli in which eosinophil infiltration and T cell activation[22,23] are observed.

A study by Miyazaki et al. showed that CCL2 protein was expressed in the conjunctival epithelium of mouse experimental allergic conjunctivitis and CCL2 subconjunctival injection induced mast cell degranulation.[24] Our results are consistent with their findings. They also reported that blocking the CCL2-CCR2 signaling cascade could attenuate signs and symptoms of the acute phase of experimental allergic conjunctivitis.[24] Further experiments analyzing RW-induced experimental allergic conjunctivitis using mast cell–deficient mice to clarify mast cell–conjunctival epithelial cell interactions are now ongoing.

In conclusion, we showed that mast cell–conjunctival epithelial cell interaction could induce higher CCL2 expression and PMD in cultured human mast cells, which also was observed in situ samples of chronic allergic conjunctivitis. These results suggested that suppression of CCL2-CCR2 signaling cascades might be useful for alternative therapy for severe chronic allergic conjunctivitis.

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References

1. Tuft SJ, Kemeny DM, Dart JK, Buckley RJ. Clinical features of atopic keratoconjunctivitis. *Ophthalmology.* 1991;98:150–158.
2. Bonini S, Lambiase A, Marchi S, et al. Vernal keratoconjunctivitis revisited: a case series of 195 patients with long-term followup. *Ophthalmology.* 2000;107:1157–1163.
3. Dvorak AM, Schroeder JT, MacGlashan DW Jr, et al. Comparative ultrastructural morphology of human basophils stimulated to release histamine by anti-IgE, recombinant IgE-dependent histamine-releasing factor, or monocyte chemotactic protein-1. *J Allergy Clin Immunol.* 1996;98:355–370.
4. Dvorak AM, Kissell S. Granule changes of human skin mast cells characteristic of piecemeal degranulation and associated with recovery during wound healing in situ. *J Leukoc Biol.* 1991;49:197–210.
5. Crivellato E, Nico B, Mallardi F, Beltrami CA, Ribatti D. Piecemeal degranulation as a general secretory mechanism? *Anat Rec A Discov Mol Cell Evol Biol.* 2003;274:778–784.
6. Ebihara N, Watanabe Y, Murakami A. The ultramicrostructure of secretory granules of mast cells in giant papillae of vernal keratoconjunctivitis patients [in Japanese]. *Nippon Ganka Gakkai Zasshi.* 2008;112:581–589.
7. Kirshenbaum AS, Akin C, Wu Y, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk Res.* 2003;27:677–682.
8. Saito H, Kato A, Matsumoto K, Okayama Y. Culture of human mast cells from peripheral blood progenitors. *Nat Protoc.* 2006;1:2178–2183.
9. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci.* 2003;44:2496–2506.
10. Matsuda A, Ebihara N, Yokoi N, et al. Functional roles of thymic stromal lymphopoietin for chronic allergic keratoconjunctivitis. *Invest Ophthalmol Vis Sci.* 2010;51:151–155.
11. Matsuda A, Okayama Y, Ebihara N, et al. Hyperexpression of the high-affinity IgE receptor-beta chain in chronic allergic keratoconjunctivitis. *Invest Ophthalmol Vis Sci.* 2009;50:2871–2877.
12. Ishida W, Fukuda K, Sumi T, et al. Adjuvants determine the contribution of basophils to antigen sensitization in vivo. *Immunol Lett.* 2011;136:49–54.
13. Abu El-Asrar AM, Struyf S, Al-Kharashi SA, Missotten L, Van Damme J, Geboes K. Chemokines in the limbal form of vernal keratoconjunctivitis. *Br J Ophthalmol.* 2000;84:1360–1366.
14. Giustizieri ML, Mascia F, Frezzolini A, et al. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. *J Allergy Clin Immunol.* 2001;107:871–877.
15. Gordon JR. Monocyte chemoattractant peptide-1 expression during cutaneous allergic reactions in mice is mast cell dependent and largely mediates the monocyte recruitment response. *J Allergy Clin Immunol.* 2000;106:110–116.
16. Mercer PF, Johns RH, Scotton CJ, et al. Pulmonary epithelium is a prominent source of proteinase-activated receptor-1-inducible CCL2 in pulmonary fibrosis. *Am J Respir Crit Care Med.* 2009;179:414–425.
17. Burd PR, Rogers HW, Gordon JR, et al. Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J Exp Med.* 1989;170:245–257.
18. Kato A, Chustz RT, Ogasawara T, et al. Dexamethasone and FK506 inhibit expression of distinct subsets of chemokines in human mast cells. *J Immunol.* 2009;182:7233–7243.
19. Swensson O, Schubert C, Christophers E, Schroder JM. Inflammatory properties of neutrophil-activating protein-1/interleukin 8 (NAP-1/IL-8) in human skin: a light- and electronmicroscopic study. *J Invest Dermatol.* 1991;96:682–689.

20. Dvorak AM, MacGlashan DW Jr, Morgan ES, Lichtenstein LM. Vesicular transport of histamine in stimulated human basophils. *Blood*. 1996;88:4090–4101.
21. Merayo-Lloves J, Zhao TZ, Dutt JE, Foster CS. A new murine model of allergic conjunctivitis and effectiveness of nedocromil sodium. *J Allergy Clin Immunol*. 1996;97:1129–1140.
22. Fukushima A. Roles of T-cells in the development of allergic conjunctival diseases. *Cornea*. 2007;26:S36–S40.
23. Li DQ, Zhang L, Pflugfelder SC, et al. Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. *J Allergy Clin Immunol*. 2011;128:1318–1325.
24. Tominaga T, Miyazaki D, Sasaki S, et al. Blocking mast cell-mediated type I hypersensitivity in experimental allergic conjunctivitis by monocyte chemoattractant protein-1/CCR2. *Invest Ophthalmol Vis Sci*. 2009;50:5181–5188.

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A novel mutation (p.Glu1389AspfsX16) of the phosphoinositide kinase, FYVE finger containing gene found in a Japanese patient with fleck corneal dystrophy

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Purpose: The phosphoinositide kinase, FYVE finger containing (*PIKFYVE*) gene has been identified as a gene responsible for fleck corneal dystrophy (FCD). The purpose of this study is to report a novel mutation of the *PIKFYVE* gene in a Japanese patient with fleck corneal dystrophy.

Methods: Slit-lamp microscopy, corneal topography, and optical coherence tomography were performed for the clinical examination of the patient's eye. For genetic analysis, peripheral blood was obtained from the patient and her sister. DNA was extracted from the blood and subjected to mutation analysis by sequencing of the *PIKFYVE* gene. The sequencing results were validated with a PCR-fragment length polymorphism analysis.

Results: A 63-year-old woman presented at our clinic with complaints of decreased vision and metamorphopsia in her right eye occurring 1 month before presentation. Both eyes exhibited small, dot-like, white flecks scattered throughout all layers of the corneal stroma, which corresponds to the typical FCD phenotype. The opacities were relatively dominant at the peripheral region of the cornea, yet were found throughout the entire cornea. Sequence analysis revealed that the patient has a heterozygous c.4166_4169delAAGT mutation located at exon 24 of the *PIKFYVE* gene that may cause p.Glu1389AspfsX16 flame-shift mutation, which has never before been reported for FCD.

Conclusions: To the best of our knowledge, this is the first study to show that a novel mutation (p.Glu1389AspfsX16) causing the truncation of the *PIKFYVE* protein causes fleck corneal dystrophy in the Japanese population.

The cornea is one of the most transparent and non-vascularized tissues in the human body, and several active genes [1,2] are thought to be involved in maintaining the homeostasis of the cornea. Recent advances in molecular biology techniques have allowed the genes responsible in most hereditary corneal dystrophies to be identified, including transforming growth factor, beta-induced (TGFBI)-related corneal dystrophies (i.e., granular corneal dystrophy, lattice corneal dystrophy type I, granular corneal dystrophy type 2 (Avellino corneal dystrophy), Reis-Bücklers corneal dystrophy, and Thiel-Behnke corneal dystrophy) [3], Meesmann corneal dystrophy [4,5], macular corneal dystrophy [6], gelatinous drop-like corneal dystrophy [7], and Fuchs' endothelial dystrophy [8].

Fleck corneal dystrophy (FCD, Online Mendelian Inheritance in Man (OMIM) #121850) was first reported in 1957 by Francois and Neetens [9], and is one of the hereditary corneal dystrophies in which the causative genes have already been identified. This corneal dystrophy is a rare autosomal dominant disease characterized by numerous tiny, dot-like white flecks scattered in all layers of the corneal stroma. Typically, the stroma located in between the flecks is clear, and the endothelium, the epithelium, Bowman's layer, and Descemet's membrane are normal. Patients are usually asymptomatic with normal vision, yet a small number of patients report the sensation of a minor photophobia. The flecks in FCD can appear as early as at 2 years of age, or sometimes even at birth, and appear not to progress significantly throughout life [10,11]. Histologically, the corneal flecks appear to correspond to abnormal keratocytes swollen with membrane-limited intracytoplasmic vesicles containing complex lipids and glycosaminoglycans [12]. It has been reported that there are no extracellular abnormalities [12].

In this study, we report a case of FCD bearing a heterozygous flame-shift mutation within the phosphoinositide kinase, FYVE finger containing (*PIKFYVE*) gene. The patient had no obvious vision loss or any complaints related to this corneal dystrophy, and the appropriateness of our identified mutation as a causative one for FCD is theoretically discussed.

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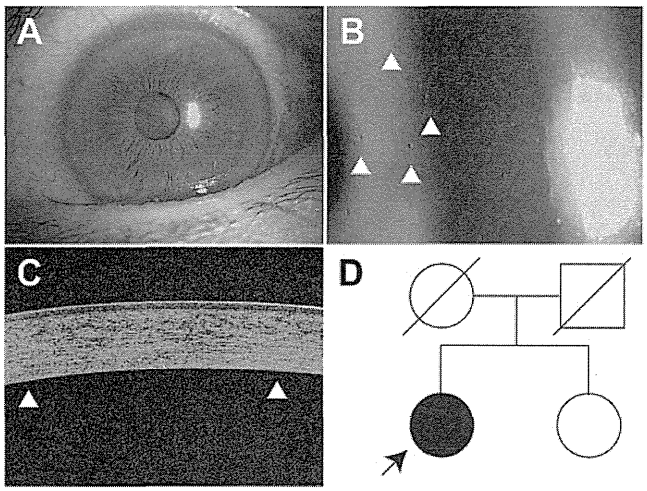

Figure 1. Images demonstrating the corneal phenotypes of a patient with fleck corneal dystrophy. **A**: Stromal flecks are not perceptible under diffuse illumination in both of the patient's eyes. **B**: Under iris retroillumination, stromal flecks (arrowheads) became evident. **C**: Optical coherence tomography analysis successfully detected the stromal flecks as slightly bright small areas (arrowhead). **D**: The pedigree of the patient with fleck corneal dystrophy is demonstrated.

METHODS

Measurement of corneal irregularity and higher-order aberration: Corneal irregularity and higher-order aberration in the patient were investigated using a commercially available corneal topography device (KR-1W; Topcon Corp., Tokyo, Japan).

Optical coherence tomography of cornea: An optical section of the patient's cornea was obtained using a commercially available optical coherence tomography (OCT) device (Cirrus HD-OCT; Carl Zeiss Meditec Co. Ltd., Tokyo, Japan).

Mutation analysis: A 63-year-old woman presented at our clinic with complaints of decreased vision and metamorphopsia in her right eye occurring 1 month before presentation. Her best-corrected visual acuity was 0.7 in her right eye and 1.2 in her left eye. The decreased visual acuity and metamorphopsia seemed to be due to a transient focal retinal detachment caused by the traction of the posterior vitreous membrane.

All experimental procedures were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine. This study was performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

Peripheral blood was obtained from the patient and her younger sister, the patient's only remaining living relative using a plastic syringe attached with a 23G needle. Prior informed consent was obtained from both subjects after a detailed explanation of the study protocols. Genomic DNA was extracted from the blood using a commercially available kit (DNeasy Blood & Tissue Kit; Qiagen GmbH, Hilden, Germany). Genomic DNA samples from 96 normal Japanese volunteers (48 men and 48 women) were obtained from a research-resource bank (Human Science Research Resource Bank, Osaka, Japan). Using 10 ng of genomic DNA, all exons of the *PIKFYVE* gene were amplified with polymerase chain reaction (PCR) in a 50 µl reaction buffer containing 1 x ExTaq buffer, 0.2 mM dNTP, 0.2 µM primer pair, and 1.25 U Taq polymerase (ExTaq Hot Start version; Takara Bio Inc., Otsu, Japan). All primer pairs were designed according to a previous study [13]. The PCR products were treated with a mixture of exonuclease and alkaline phosphatase (ExoSAP-IT; GE Healthcare UK, Ltd., Buckinghamshire, UK), heat-inactivated, and sequenced using a commercially available kit (BigDye 3.1; Applied Biosystems Inc., Foster City, CA). The sequencing products were purified with a commercially available kit (BigDye Xterminater Purification Kit; Applied Biosystems), electrophoresed on an automated sequencer (3130×l Genetic Analyzer; Applied Biosystems), and analyzed with sequence alignment software (Variant Reporter Version 1.0; Applied Biosystems). Thermal cycle conditions for all primer pairs were 30 cycles of three-temperature thermal cycles at 94 °C for 30 s for heat denaturation, at 55 °C for 30 s for annealing, and 72 °C for 30 s for extension.

Polymerase chain reaction–fragment length polymorphism analysis: Sequencing data were validated with PCR–fragment length polymorphism (PCR–FLP). Briefly, a partial sequence of exon 24 of the *PIKFYVE* gene was amplified by PCR using a primer pair (PIKFYVE_FLP_F_Ex24; 5′-CTC AGT TAT TCT CCC ATT CGG CTT C-3′, PIKFYVE_FLP_R_Ex24; 5′-AAT GAA TAT TTT GGG GAG TGG AAC A-3′). The PCR product was electrophoresed on a 10% acrylamide gel. After the electrophoresis, the gel was stained with a DNA-staining fluorescent dye (SYBR® Green I; Takara Bio), observed on a UV transilluminator, and photographed in

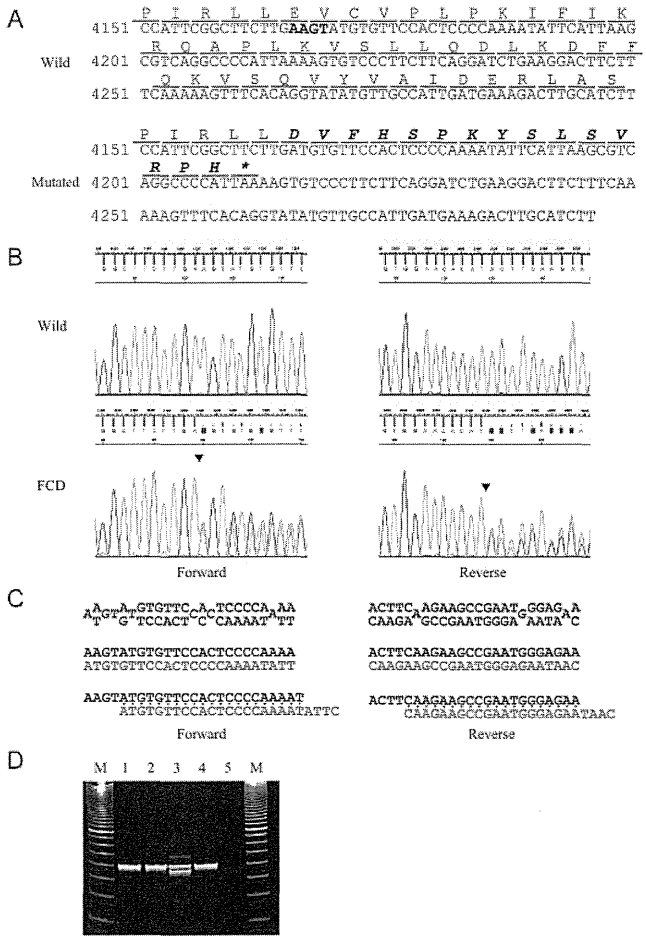

Figure 2. Results of sequencing and polymerase chain reaction (PCR)-fragment length polymorphism (PCR-FLP) analyses for the patient with FCD are demonstrated. **A**: Nucleotide and amino acid sequence of the wild (upper) and mutated (lower) *PIKFYVE* gene around the identified p.Glu1389AspfsX16 mutation are demonstrated. The deleted four bases of the c.4166_4169delAAGT mutation are indicated in bold type in the wild-type sequence. The altered amino acid sequence downstream of the deleted four bases is indicated in bold italics in the mutated sequence. Asterisk (*) means an ochre (TAA) stop codon. **B**: Results of sequencing analysis for exon 24 of the *PIKFYVE* gene in normal volunteer (upper) and the patient with FCD (lower) from forward (left) and reverse (right) directions are demonstrated. Arrowheads indicate the breakpoint of the c.4166_4169delAAGT mutation. **C**: The mixed base sequence (upper) downstream of the breakpoints was subtracted (middle) from the reference sequence (black type) to extract the mutated sequence (red type) in both directions (left: forward, right: reverse). Note that the mutated sequence is fully matched to the reference sequence from four bases downstream of the breakpoints (lower), indicating that the mutated sequence is deleted with four bases, AAGT sequence. **D**: Results of PCR-FLP analysis for exon 24 of the *PIKFYVE* gene in normal volunteers (lanes 1 and 2) and the patient with FCD (lane 3) and her sister (lane 4) are demonstrated. Lane 5 means negative control. Note that the shorter PCR band in the patient with FCD (lane 3) was amplified from the mutated allele while the longer PCR band was from the wild-type allele.

a dark box equipped with a charge-coupled device (CCD) camera (LAS-3000 UV mini; GE Healthcare UK).

RESULTS

Both eyes exhibited small, dot-like, white-fleck opacities scattered in all layers of the corneal stroma. The opacities were relatively dominant at the peripheral region of the cornea, yet were found throughout the entire cornea. The opacities were almost invisible under diffuse illumination (Figure 1A), but became more apparent under slit-lamp illumination or iris retroillumination (Figure 1B). It seems difficult to recognize the opacities in ordinary care, especially for ophthalmologists unfamiliar with such faint corneal opacity. OCT analysis successfully identified some of the small stromal flecks (Figure 1C). Higher-order aberration was within the normal limit in both corneas. Specular microscopy examination demonstrated that the endothelial cell density was 2,000 cells/mm^2 in her right cornea and 2,200 cells/mm^2 in her left cornea, which is sufficient for endothelial function but appears slightly decreased compared to the average cell density in persons of her same age. She had previously undergone clinical examination by an ophthalmologist several times in her life; however, it was never pointed out to her that she had such corneal abnormalities. Her sister did not exhibit any corneal manifestations in either eye (Figure 1D).

TABLE 1. LIST OF NUCLEOTIDE CHANGES IDENTIFIED IN OUR FCD PATIENT.

#	Region	Nucleotide change	Zygosity	Type of mutation	Effects on amino acid	SNP
1	Intron 9	g.32610C>T	Homozygous	Substitution	none (non-coding)	none
2	Intron 15	g.48601A>G	Homozygous	Substitution	none (non-coding)	none
3	Exon 16	c.2087G>A	Homozygous	Substitution	p.696S>N	rs10932258
4	Exon 16	c.2106C>T	Homozygous	Substitution	p.702p>P	rs10932259
5	Exon 19	c.2795T>C	Homozygous	Substitution	p.932L>S	rs2363468
6	Exon 19	c.2984A>T	Homozygous	Substitution	p.995Q>L	rs893254
7	Exon 19	c.2993C>G	Homozygous	Substitution	p.998T>S	rs893253
8	Exon 19	c.2984A>T	Homozygous	Substitution	p.995Q>L	rs893254
9	Exon 19	c.2993C>G	Homozygous	Substitution	p.998T>S	rs893253
10	Exon 19	c.3547C>A	Homozygous	Substitution	p.1183Q>K	rs1529979
11	Exon 19	c.3564T>C	Homozygous	Substitution	p.1188n>N	rs1529978
12	Exon 24	c.4166_4169delAAGT	Heterozygous	Insertion	p.Glu1389AspfsX16	none
13	Intron 27	g.65496T>C	Homozygous	Substitution	none (non-coding)	none
14	Intron 31	g.73584G>A	Homozygous	Substitution	none (non-coding)	none
15	Intron 32	g.73754C>T	Homozygous	Substitution	none (non-coding)	none
16	Exon 34	c.5334G>A	Homozygous	Substitution	p.1778T>T	rs2304545
17	Exon 35	c.5397A>G	Homozygous	Substitution	p.1799T>T	rs2118297
18	Intron 35	g.79205A>G	Homozygous	Substitution	none (non-coding)	none
19	Exon 36	c.5526A>G	Homozygous	Substitution	p.1842E>E	rs994697
20	Exon 38	c.5727G>T	Heterozygous	Substitution	p.1909A>A	none
21	Intron 39	g.82947A>G	Homozygous	Substitution	none (non-coding)	none

The notation convention of the nucleotide and protein changes follows the nomenclature guidelines of human genome variation society (HGVS).

No treatment was undergone by this patient, but her retinal problem spontaneously ameliorated as judged by OCT findings along with the improvement of visual acuity from 0.7 to 1.2 in 2 weeks, indicating that the decrease in the visual acuity of her right eye at her first visit was not due to the corneal opacities.

Mutation analysis: The sequence data revealed that the patient had a heterozygous 4-base-pair deletion mutation (c.4166_4169delAAGT or c.4167_4170delAGTA) within the *PIKFYVE* gene (Figure 2A-C). This mutation may produce a new reading flame starting from amino acid number 1389, leading to a premature termination at the 16th codon counted from the first affected amino acid (p.Glu1389AspfsX16), which has never been reported in patients with FCD. PCR-FLP analysis confirmed these sequencing data (Figure 2D). In addition to this mutation, 20 nucleotide changes were found within the *PIKFYVE* gene (Table 1), yet all were of known single-nucleotide polymorphisms, of synonymous amino acid alteration, or located at the non-coding region, and hence are considered non-pathological. The c.4166_4169delAAGT mutation was not found in any of the examined 96 normal Japanese volunteers (data not shown). The sister of the patient did not have the c.4166_4169delAAGT mutation.

DISCUSSION

In 2003, Jiao et al. [14] performed linkage analysis of four families with FCD and found that the critical region for FCD mapped to a 27.9 cM region of chromosome 2q35 flanked by the genomic markers D2S117 and D2S126. Subsequently, in 2005, Li et al. [15] further narrowed the linked region to a 24 cM interval containing 18M bases. Li et al. subsequently sequenced genes included within the narrowed region and found mutations in the *PIKFYVE* gene in patients with FCD. The *PIKFYVE* gene encodes a widely expressed, 2,089-amino-acid-long, phosphoinositide 3-kinase family member that functions in post-Golgi vesicular sorting [15].

In the present study, we found a heterozygous c.4166_4169delAAGT mutation within the *PIKFYVE* gene in our patient with FCD. As this mutation is of 4-base-pair deletion, the mutation may cause a flame-shift amino acid