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

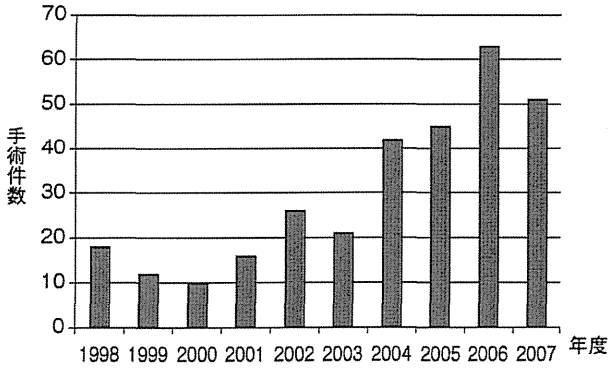

図 1 羊膜移植手術件数の年次推移.

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

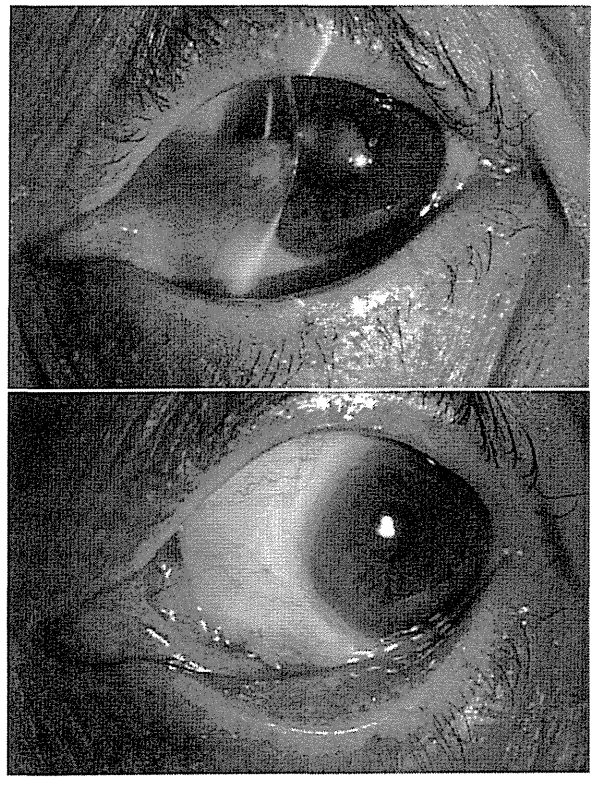

図 2 翼状片に対する羊膜移植併用翼状片切除術.
上：術前．再発翼状片であり，強い瞼球癒着を伴う．
下：術後．再発を認めず，眼表面は良好である．

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．

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) 島崎 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.

PLOS ONE

HLA-A*0206 with TLR3 Polymorphisms Exerts More than Additive Effects in Stevens-Johnson Syndrome with Severe Ocular Surface Complications

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Abstract

Background: Stevens-Johnson syndrome (SJS) is an acute inflammatory vesiculobullous reaction of the skin and mucosa, often including the ocular surface, and toxic epidermal necrolysis (TEN) occurs with its progression. Although SJS/TEN is thought to be initiated by certain types of medication coupled with possible infection. In the present study we examined the multiplicative interaction(s) between HLA-A*0206 and 7 Toll-like receptor 3 (TLR3) Single-nucleotide polymorphisms (SNPs) in patients with SJS/TEN.

Principal Findings: We analyzed the genotypes for HLA-A and 7 TLR3 SNPs in 110 Japanese SJS/TEN patients with severe ocular complications and 206 healthy volunteers to examine the interactions between the two loci. We found that HLA-A*0206 exhibited a high odds ratio for SJS/TEN (carrier frequency: OR = 5.1; gene frequency: OR = 4.0) and that there was a strong association with TLR3 rs.5743312T/T SNP (OR = 7.4), TLR3 rs.3775296T/T SNP (OR = 5.8), TLR3 rs.6822014G/G SNP (OR = 4.8), TLR3 rs.3775290A/A SNP (OR = 2.9), TLR3 rs.7668666A/A SNP (OR = 2.7), TLR3 rs.4861699G/G SNP (OR = 2.3), and TLR3 rs.11732384G/G SNP (OR = 1.9). There was strong linkage disequilibrium (LD) between rs.3775296 and rs.5743312 and between rs.7668666 and rs.3775290. The results of interaction analysis showed that the pair, HLA-A*0206 and TLR3 SNP rs3775296T/T, which exhibited strong LD with TLR3 rs.5743312, exerted more than additive effects (OR = 47.7). The other pairs, HLA-A*0206 and TLR3 rs.3775290A/A SNP (OR = 11.4) which was in strong LD with TLR3 rs7668666A/A SNP, and TLR3 rs4861699G/G SNP (OR = 7.6) revealed additive effects. Moreover, the combination HLA-A*0206 and TLR3 rs3775296T/T was stronger than the TLR3 rs6822014G/G and TLR3 rs3775290A/A pair, which reflected the interactions within the TLR3 gene alone.

Significance: By interaction analysis, HLA-A*0206 and TLR3 SNP rs3775296T/T, which were in strong LD with TLR3 SNP rs5743312T/T, manifested more than additive effects that were stronger than the interactions within the TLR3 gene alone. Therefore, multiplicative interactions of HLA-A and TLR3 gene might be required for the onset of SJS/TEN with ocular complications.

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Introduction

Stevens-Johnson syndrome (SJS) is an acute inflammatory vesiculobullous reaction of the skin and mucous membranes. It was first described in 1922 by Stevens and Johnson, [1] both pediatricians, who encountered 2 boys aged 8 and 7 who manifested an extraordinary, generalized skin eruption, persistent fever, inflamed buccal mucosa, and severe purulent conjunctivitis resulting in marked visual disturbance. Subsequently, other pediatricians reported that SJS was associated with infectious agents such as *Mycoplasma pneumoniae*, [2] and a viral etiology involving herpes simplex virus, Epstein-Barr virus, cytomegalovirus, and varicella zoster virus [3]. On the other hand, dermatologists claimed that more than 100 different drugs were implicated in eliciting SJS and its severe form, toxic epidermal necrolysis (TEN) [4,5]. The annual incidence of SJS and TEN has been estimated to be 0.4–1 and 1–6 cases per million persons, respectively; [6,7] the reported mortality rate is 3% and 27%, respectively [8]. Although rare, these reactions have high morbidity and mortality rates, and often result in severe and definitive sequelae such as vision loss. SJS/TEN is one of the most devastating ocular surface diseases leading to corneal damage and

loss of vision. The reported incidence of ocular complications in SJS/TEN is 50–68% [7,8].

In the acute stage, patients manifest vesiculobullous lesions of the skin and mucosa, especially that of the eyes and mouth, and severe conjunctivitis. The loss of finger nails in the acute or subacute stage due to paronychia was observed, has been observed in almost all SJS/TEN patients with severe ocular surface complications [9,10,11,12].

In the chronic stage, despite healing of the skin lesions, ocular surface complications such as conjunctival invasion into the cornea [10,11,12,13,14,15,16,17,18]. It is also reported that lid margin keratinization and tarsal scarring, together with lipid tear deficiency, contributes to corneal complications because of blink-related microtrauma [19].

Elsewhere we reported that the frequency of carriers of the HLA-A*0206 antigen is significantly higher among Japanese patients with severe ocular surface complications than in other populations [18,20]. Our single nucleotide polymorphism (SNP) association analysis of candidate genes documented the associated polymorphisms of several immune-related genes including *TLR3*, [12,17] *IL4R*, [14,16] *IL13*, [16] and *FasL* [15]in Japanese SJS/TEN patients with severe ocular surface complications. To elucidate the detailed pathophysiology of SJS/TEN we performed a genome-wide association study of SJS/TEN patients and found associations between 6 SNPs in the prostaglandin E receptor 3 (EP3) gene (*PTGER3*) and SJS/TEN accompanied by severe ocular surface complications [11]. Moreover, gene-gene interaction analysis in SJS/TEN patients with severe ocular surface complications revealed that the interaction between *TLR3* and *PTGER3* exerted SJS/TEN susceptibility effects, and there was a functional interaction between TLR3 and EP3 in a murine experimental allergic conjunctivitis model. [12].

In the present study we examined the multiplicative interaction(s) between HLA-A*0206 and 7 TLR3 SNPs (rs3775296 (uSNP), rs5743312 (iSNP), rs6822014 (gSNP), rs3775290 (sSNP), rs7668666 (iSNP), rs11732384 (iSNP), and rs4861699 (gSNP)) associated with the SJS/TEN patients [12,17] as the onset of SJS/TEN was associated not only with the administration of drugs but also with putative viral syndromes [10,11,12,17]. HLA-A is a component of HLA class I, which resides on the surface of all nucleated cells and alerts the immune system that the cell may be infected by a virus, thereby targeting the cell for destruction. TLR3 recognises viral double-stranded RNA [21].

Results

We analyzed the genotypes for HLA-A and 7 TLR3 SNPs in 110 Japanese SJS/TEN patients with severe ocular complications and 206 healthy volunteers to examine the interactions between the two loci.

We found that HLA-A*0206 exhibited a high odds ratio for SJS/TEN (carrier frequency: $p = 6.9 \times 10^{-10}$, OR = 5.1; gene frequency: $p = 2.5 \times 10^{-9}$, OR = 4.0) (Table 1).

We also found that there was a strong association with TLR3 rs.5743312T/T SNP (T/T vs T/C+C/C: $p = 2.5 \times 10^{-6}$, OR = 7.4), TLR3 rs.3775296T/T SNP (T/T vs T/G+G/G: $p = 8.2 \times 10^{-6}$, OR = 5.8), TLR3 rs.6822014G/G SNP (G/G vs G/A+A/A: $p = 1.2 \times 10^{-4}$, OR = 4.8), TLR3 rs.3775290A/A SNP (A/A vs A/G+G/G: $p = 7.1 \times 10^{-4}$, OR = 2.9), TLR3 rs.7668666A/A SNP (A/A vs A/G+G/G: $p = 1.2 \times 10^{-3}$, OR = 2.7), TLR3 rs.4861699G/G SNP (G/G vs G/A+A/A:

Table 1. Association between HLA-A*0206 and SJS/TEN with ocular complications.

HLA-A	Carrier frequency				Gene frequency			
	SJS (n = 110)	Normal (n = 206)	p-value (χ^2)	Odds Ratio	SJS (n = 220)	Normal (n = 412)	p-value (χ^2)	Odds Ratio
*0206	46.4% (51/110)	14.6% (30/206)	6.9×10^{-10}	5.07	24.1% (53/220)	7.3% (30/412)	2.5×10^{-9}	4.04
*0101	0% (0/110)	1.4% (3/206)	0.2	–	0% (0/220)	0.7% (3/412)	0.2	–
*0201	26.4% (29/110)	21.4% (44/206)	0.3	–	14.5% (32/220)	11.4% (47/412)	0.3	–
*0207	9.1% (10/110)	7.8% (16/206)	0.7	–	4.5% (10/220)	3.9% (16/412)	0.7	–
*0210	0% (0/110)	1.0% (2/206)	0.3	–	0% (0/220)	0.5% (2/412)	0.3	–
*0301	2.7% (3/110)	1.4% (3/206)	0.4	–	1.4% (3/220)	0.7% (3/412)	0.4	–
*0302	0% (0/110)	0.5% (1/206)	0.5	–	0% (0/220)	0.2% (1/412)	0.5	–
*1101	7.3% (8/110)	18.4% (38/206)	7.3×10^{-3}	0.35	3.6% (8/220)	9.2% (38/412)	1.0×10^{-2}	0.37
*1102	0% (0/110)	0.5% (1/206)	0.5	–	0% (0/220)	0.2% (1/412)	0.5	–
*2402	45.5% (50/110)	60.7% (125/206)	9.5×10^{-3}	0.54	25.0% (55/220)	36.7% (151/412)	2.9×10^{-3}	0.58
*2420	0% (0/110)	0.5% (1/206)	0.5	–	0% (0/220)	0.2% (1/412)	0.5	–
*2601	9.1% (10/110)	12.6% (26/206)	0.3	–	4.5% (10/220)	6.6% (27/412)	0.3	–
*2602	5.5% (6/110)	2.9% (6/206)	0.3	–	2.7% (6/220)	1.7% (7/412)	0.4	–
*2603	1.8% (2/110)	7.8% (16/206)	3.0×10^{-2}	0.2	0.9% (2/220)	3.9% (16/412)	3.2×10^{-2}	0.2
*2605	0% (0/110)	0.5% (1/206)	0.5	–	0% (0/220)	0.2% (1/412)	0.5	–
*2901	0% (0/110)	1.9% (4/206)	0.1	–	0% (0/220)	1.0% (4/412)	0.1	–
*3001	0.9% (1/110)	0% (0/206)	0.2	–	0.5% (1/220)	0% (0/412)	0.2	–
*3101	13.6% (15/110)	16.5% (34/206)	0.5	–	6.8% (15/220)	8.3% (34/412)	0.5	–
*3201	0% (0/110)	0.5% (1/206)	0.5	–	0% (0/220)	0.2% (1/412)	0.5	–
*3303	22.7% (25/110)	14.1% (29/206)	0.05	–	11.4% (25/220)	7.0% (29/412)	0.06	–

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Table 2. Association between TLR3 SNPs and SJS/TEN with ocular complications.

rs number of SNP	Genotypes		Case (N = 110)	Control (N = 206)	Allele 1 vs. Allele 2	Genotype 11 vs. 12+22	Genotype 11+12 vs. 22
					P-value[a]	P-value[a]	P-value[a]
					OR[b]	OR[b]	OR[b]
					(95%CI[c])	(95%CI[c])	(95%CI[c])
rs4861699	11	G/G	65/110 (59.1%)	79/206 (38.3%)	0.0016	4.2×10^{-4}	0.28
	12	G/A	36/110 (32.7%)	102/206 (49.5%)	1.80	2.32	1.55
	22	A/A	9/110 (8.2%)	25/206 (12.1%)	(1.25–2.59)	(1.45–3.72)	(0.70–3.45)
rs6822014	11	A/A	55/110 (50.0%)	127/206 (61.7%)	8.9×10^{-4}	0.046	1.2×10^{-4}
	12	A/G	37/110 (33.6%)	71/206 (34.5%)	0.54	0.62	0.21
	22	G/G	18/110 (16.4%)	8/206 (3.9%)	(0.37–0.78)	(0.39–0.99)	(0.09–0.49)
rs11732384	11	G/G	72/110 (65.5%)	103/206 (50.0%)	0.029	0.0085	0.88
	12	G/A	31/110 (28.2%)	89/206 (43.2%)	1.54	1.89	1.07
	22	A/A	7/110 (6.4%)	14/206 (6.8%)	(1.04–2.28)	(1.17–3.06)	(0.42–2.74)
rs3775296	11	G/G	49/110 (44.5%)	109/206 (52.9%)	0.0020	0.16	8.2×10^{-6}
	12	G/T	40/110 (36.4%)	89/206 (43.2%)	0.58	0.71	0.17
	22	T/T	21/110 (19.1%)	8/206 (3.9%)	(0.40–0.82)	(0.45–1.14)	(0.07–0.40)
rs5743312	11	C/C	52/110 (47.3%)	115/206 (55.8%)	0.0014	0.15	2.5×10^{-6}
	12	C/T	38/110 (34.5%)	85/206 (41.3%)	0.56	0.71	0.14
	22	T/T	20/110 (18.2%)	6/206 (2.9%)	(0.39–0.80)	(0.45–1.13)	(0.05–0.35)
rs7668666	11	C/C	36/110 (32.7%)	83/206 (40.3%)	0.0085	0.19	0.0012
	12	C/A	47/110 (42.7%)	101/206 (49.0%)	0.64	0.72	0.37
	22	A/A	27/110 (24.5%)	22/206 (10.7%)	(0.46–0.89)	(0.44–1.17)	(0.20–0.68)
rs3775290	11	G/G	38/110 (34.5%)	82/206 (39.8%)	0.016	0.36	7.1×10^{-4}
	12	G/A	45/110 (40.9%)	103/206 (50.0%)	0.66	0.80	0.35
	22	A/A	27/110 (24.5%)	21/206 (10.2%)	(0.48–0.93)	(0.50–1.29)	(0.18–0.65)

[a]*P*-value for allele or genotype frequency comparisons between cases and controls using the chi-square test.
[b]OR, odds ratio.
[c]CI, confidence interval.
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$p = 4.2\times10^{-4}$, OR = 2.3), and TLR3 rs.11732384G/G SNP (G/G vs G/A+A/A: $p = 8.5\times10^{-3}$, OR = 1.9) (Table 2). All SNPs were in Hardy-Weinberg equilibrium ($p>0.01$) in the samples from patients and the controls. Based on the squared correlation coefficient r^2, we investigated the linkage disequilibrium (LD) among the *TLR3* SNPs. We found strong LD between rs.3775296 and rs.5743312 (D' = 1, $r^2 = 0.911$), and between rs.7668666 and rs.3775290 (D' = 0.973, $r^2 = 0.934$) (Fig. 1).

Results of interaction analysis showed that the pair, HLA-A*0206 and TLR3 SNP rs3775296T/T, which exhibited strong LD with TLR3 rs.5743312, exerted more than additive effects. We found that while 11 of the 110 patients (10%) manifested both HLA-A*0206 and TLR3 rs3775296T/T SNP, none of the 206 controls did ($p = 6.5\times10^{-6}$, OR = 47.7, Woolf's correction). The other pairs, HLA-A*0206 and TLR3 rs.3775290A/A SNP, which was in strong LD with TLR3 rs.7668666, or TLR3 rs4861699G/G SNP revealed additive effects: 16 of the 110 patients (14.5%) but only 3 of the 206 controls (1.5%) had both HLA-A*0206 and TLR3 rs.3775290A/A SNP ($p = 7.4\times10^{-6}$, OR = 11.4). In addition, 33 of the 110 patients (30%), compared to 11 of the 206 controls (5.3%), had both HLA-A*0206 and TLR3 rs.4861699G/G SNP ($p = 1.6\times10^{-9}$, OR = 7.6) (Table 3).

Moreover, to examine the interactions within the TLR3 gene alone we analyzed interactions between 2 each of 5 TLR3 SNPs (rs3775296, rs6822014, rs3775290, rs11732384, rs4861699). Combinations of high risk genotypes, on which the observed numbers in cases were greater than of the controls and greater than five, were analyzed. One of the 9 combinations, TLR3 rs6822014G/G and TLR3 rs3775290A/A, exerted more than additive effects (OR 16.1, $p = 2.0\times10^{-6}$) (Table 4). However, the combination HLA-A*0206 and TLR3 rs3775296T/T produced a stronger additive effect than it. In addition, we performed haplotype association analysis with the 7 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs5743312, rs7668666, rs3775290) and the 5 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs3775290), and found that no haplotype showed strong association ($p<0.001$) (Table S1). Thus, the haplotype associations appear to contribute little to the observed interactions.

Discussion

To our knowledge, ours is the first report documenting the additive effects of HLA-A*0206 and TLR3 polymorphisms. Our interaction analysis showed that the pair HLA-A*0206 and TLR3 SNP rs3775296T/T, which was in strong LD with TLR3 rs.5743312, exerted more than additive effects, and that other pairs, HLA-A*0206 and TLR3 rs.3775290A/A SNP in strong LD

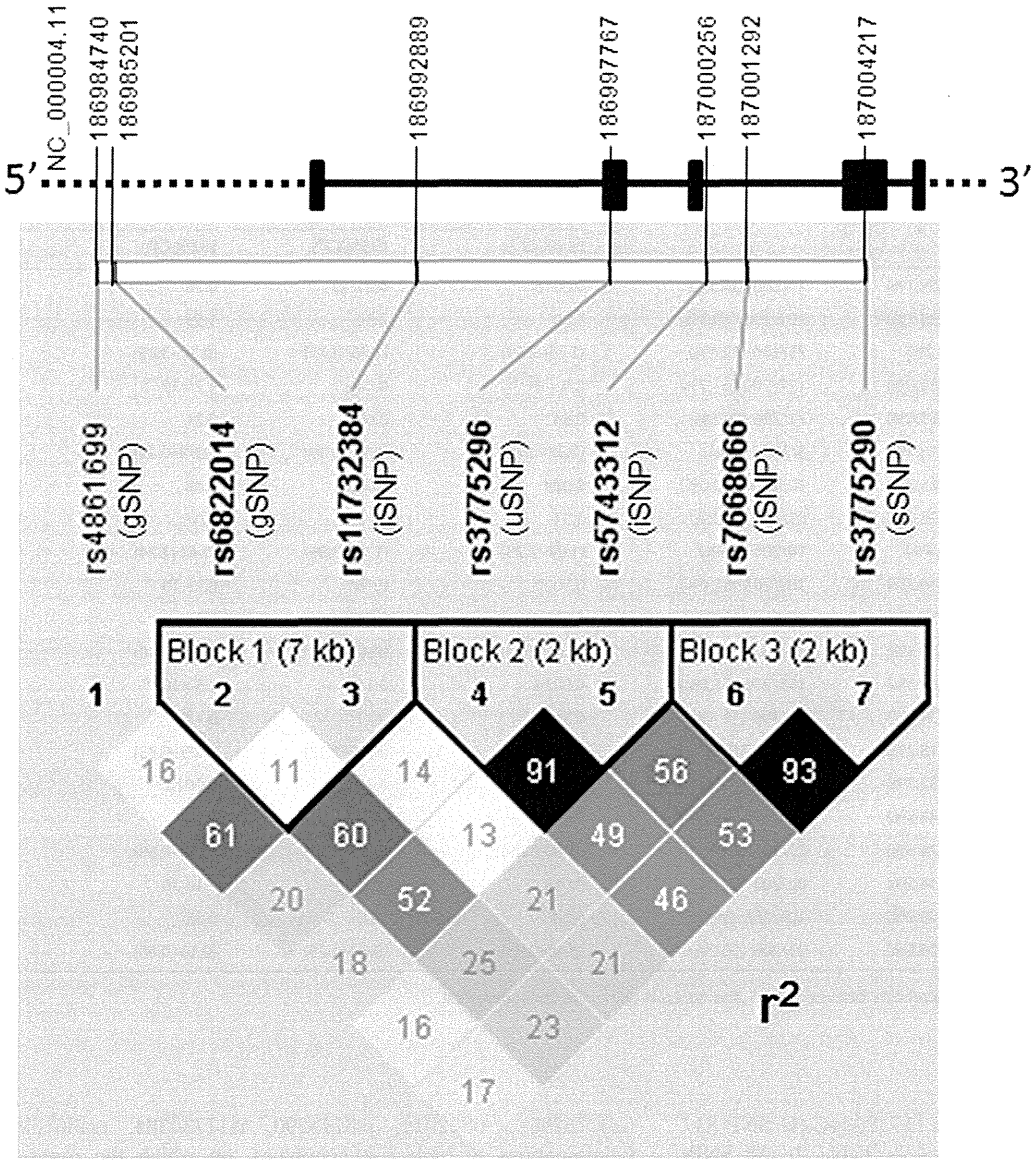

Figure 1. Linkage disequilibria among the 7 *TLR3* SNPs. Strong linkage disequilibrium was observed between rs.3775296 and rs.5743312, and between rs.7668666 and rs.3775290.
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with TLR3 rs.7668666, and TLR3 rs4861699G/G SNP exerted additive effects. Moreover, the combination HLA-A*0206 and TLR3 rs3775296T/T was stronger than the combination with TLR3 rs6822014G/G or TLR3 rs3775290A/A, the interactions within the TLR3 gene alone.

HLA-A, a component of HLA class I, alerts the immune system that the cell may be infected with a virus; TLR3 recognizes viral double-stranded RNA [21]. It is worth noting that about 80% of our SJS patients developed SJS after receiving treatment for the common cold with antibiotics, cold remedies, and/or NSAIDs; only about 5% of our SJS patient progressed to SJS after drug treatment to prevent the occurrence of convulsions [11,12]. Moreover, our review of medical records revealed that 9 of the 11 patients with both HLA-A*0206 and TLR3 SNP rs3775296T/T (and rs.5743312T/T) developed SJS after receiving cold medicine, leading us to suspect that they already had a viral infection before taking the cold medicine. Particulars on the other 2 patients are unknown because they developed SJS during childhood.

Although the TLR3 SNPs exerting additive- or more than additive effects with HLA-A*0206 were u-, i-, or gSNPs and without amino acid changes, it is possible that TLR3 SNPs and HLA-A*0206 were involved in the onset of SJS with severe ocular surface complications. Moreover, their interaction might influence the host immune response against viral infection with drug treatments.

Earlier reports indicated regional differences in HLA associations. Although in Japanese SJS patients we were unable to detect the HLA-Bw44 antigen, a subgroup of HLA-B12 [19,23], it was significantly increased in Caucasian SJS patients with ocular involvement [22].

On the other hand, the HLA-A*0206 antigen, which is not found in Caucasians [18,19] was significantly increased in our

Table 3. Interaction analysis between HLA-A*0206 and various TLR3 SNPs.

HLA-A*0206	TLR3 SNP	SJS patients (N = 110)	Controls (N = 206)	OR	p-value	Standardized OR
HLA-A*0206 & TLR3 rs3775296 T/T						
+	+	11/110 (10%)	0/206 (0%)	47.7*	6.5×10^{-6}**	262.7
+	−	40/110 (36.4%)	30/206 (14.6%)	3.4	8.8×10^{-6}	18.5
−	+	10/110 (9.1%)	8/206 (3.9%)	2.5	0.057	13.6
−	−	49/110 (44.5%)	168/206 (81.6%)	0.18	1.4×10^{-11}	1
HLA-A*0206 & TLR3 rs6822014G/G						
+	+	8/110 (7.3%)	3/206 (1.5%)	5.3**	0.019**	32.3
+	−	43/110 (39.1%)	27/206 (13.1%)	4.3	1.2×10^{-7}	25.9
−	+	10/110 (9.1%)	5/206 (2.4%)	4.0**	0.012**	24.5
−	−	49/110 (44.5%)	171/206 (83.0%)	0.16	1.4×10^{-12}	1
HLA A*0206 & TLR3 rs3775290A/A						
+	+	16/110 (14.5%)	3/206 (1.5%)	11.4**	7.4×10^{-6}**	49.0
+	−	35/110 (31.8%)	27/206 (13.1%)	3.1	6.6×10^{-5}	13.2
−	+	11/110 (10%)	18/206 (8.7%)	1.2	0.71	4.9
−	−	48/110 (43.6%)	158/206 (76.7%)	0.24	4.2×10^{-9}	1
HLA A*0206 & TLR3 rs11732384G/G						
+	+	37/110 (33.6%)	16/206 (7.8%)	6.0	4.5×10^{-9}	16.4
+	−	14/110 (12.7%)	14/206 (6.8%)	2	0.077	5.5
−	+	35/110 (31.8%)	87/206 (42.2%)	0.64	0.070	1.7
−	−	24/110 (21.8%)	89/206 (43.2%)	0.37	1.5×10^{-4}	1
HLA A*0206 & TLR3 rs4861699 G/G						
+	+	33/110 (30%)	11/206 (5.3%)	7.6	1.6×10^{-9}	25.7
+	−	18/110 (16.4%)	19/206 (9.2%)	1.9	0.060	6.5
−	+	32/110 (29.1%)	68/206 (33.0%)	0.83	0.48	2.8
−	−	27/110 (24.5%)	108/206 (52.4%)	0.30	1.8×10^{-6}	1

*Woolf's correction,
**Fisher's exact test.
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Japanese SJS patients with ocular complications. While there might be ethnic differences in the association of SJS/TEN with HLA,[18,19] specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype. [10,11,12,18,19].

Elsewhere [12] we reported that the epistatic interaction between TLR3 and PTGER3 confers an increased risk for SJS with ocular complications. Since SJS/TEN is a rare condition that probably has a complex genetic background, it is reasonable to posit that multiplicative interactions of genes such as HLA-A & TLR3, and TLR3 & PTGER3, are required for the phenotypic manifestation.

In summary, we show that HLA-A*0206 with TLR3 polymorphisms exerts more than additive effects in SJS with severe

Table 4. Interaction analysis of two SNPs of the TLR3 SNPs (SJS > control and SJS >5).

Combination of 2 TLR3 SNPs		SJS (N = 110)	Controls (N = 206)	OR	p-value
rs3775296 T/T +	rs3775290 A/A +	19/110 (17.3%)	6/206 (2.9%)	7.0	6.6×10^{-6}
rs11732384 G/G +	rs3775290 A/A +	27/110 (24.5%)	21/206 (10.2%)	2.9	7.1×10^{-4}
rs6822014 G/G +	rs3775290 A/A +	15/110 (13.6%)	2/206 (1.0%)	16.1	2.0×10^{-6}
rs4861699 G/G +	rs3775290 A/A +	26/110 (23.6%)	16/206 (7.8%)	3.7	7.5×10^{-5}
rs11732384 G/G +	rs3775296 T/T +	21/110 (19.1%)	8/206 (3.9%)	5.8	8.2×10^{-6}
rs6822014 G/G +	rs3775296 T/T +	17/110 (15.5%)	4/206 (1.9%)	9.2	4.3×10^{-6}
rs4861699 G/G +	rs3775296 T/T +	21/110 (19.1%)	8/206 (3.9%)	5.8	8.2×10^{-6}
rs6822014 G/G +	rs11732384 G/G +	18/110 (16.4%)	8/206 (3.9%)	4.8	1.2×10^{-4}
rs4861699 G/G +	rs6822014 G/G +	18/110 (16.4%)	8/206 (3.9%)	4.8	1.2×10^{-4}

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ocular surface complications and we suggest that gene-gene interactions should be considered in addition to major single-locus effects.

Materials and Methods

Patients

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine and the University of Tokyo, Graduate School of Medicine. All experimental procedures were conducted in accordance with the principles of the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants, and their prior written informed consent was obtained.

Diagnosis of SJS/TEN was based on a confirmed history of acute onset of high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites including the ocular surface [9,11,12,17,18].

To investigate the gene-gene interaction between HLA-A*0206 and TLR3, we enrolled 110 SJS/TEN patients in the chronic or subacute phase; all presented with symptoms of ocular surface complications. None of the patients were relatives. The controls were 206 healthy volunteers. All participants and volunteers were Japanese residing in Japan. The average age of the 110 patients and 206 controls was 43.6±18.0 (SD) and 35.4±11.1 (SD) years, respectively. The male:female ratios in the patient and control groups were 42:68 and 82:124, respectively. Some of the SJS/TEN patients and controls in this study were subjects in our earlier reports [12,17,18,19].

TLR3 SNPs Genotyping

Genomic DNA was isolated from human peripheral blood at SRL Inc. (Tokyo, Japan). Genotyping for 2 SNPs of TLR3 (rs3775290, 3775296) was performed by PCR-direct sequencing as reported previously [17]. For direct sequencing, PCR amplification was conducted with AmpliTaq Gold DNA Polymerase (Applied Biosystems) for 35 cycles at 94°C for 1 min, annealing at 60°C for 1 min, and 72°C for 1 min on a commercial PCR machine (GeneAmp; Perkin-Elmer Applied Biosystems). The PCR products were reacted with BigDye Terminator v3.1 (Applied Biosystems) and sequence reactions were resolved on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Genotyping for 5 SNPs of TLR3 (rs4861699, rs6822014, rs11732384, rs5743312, rs7668666) as performed using DigiTag2 assay [12]. Multiplex PCR was performed in 10 μl of Multiplex PCR buffer containing 25 ng genomic DNA, 25 nM of each multiplex primer mix, 200 μM of each dNTP, 2.25 mM $MgCl_2$, and 0.4 U KAPA2G Fast HotStart DNA polymerase (Kapa Biosystems). Cycling was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 68°C for 2 min. The primers and probes used in this study previously were reported [12,17].

HLA-A Genotyping

For HLA-A genotyping, we performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial bead-based typing kits (WAK Flow, Wakunaga, Hiroshima, Japan), as described previously [18,19].

Statistical Analysis

Statistical significance of the association with each SNP was assessed using Chi-square test or Fisher's exact test on two-by-two contingency tables. When the value obtained for the control was 0 the odds ratio was calculated using Woolf's correction.

Haploview software (ver. 4.2) was used to infer the linkage disequilibrium structure of the 7 TLR3 SNPs and to perform a haplotype analysis of TLR3 gene.

Supporting Information

Table S1 Haplotype analysis of TLR3 gene. Haplotype association analysis with the 7 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs5743312, rs7668666, rs3775290) and the 5 TLR3 SNPs (rs4861699, rs6822014, rs11732384, rs3775296, rs3775290)
(DOCX)

Author Contributions

Conceived and designed the experiments: MU. Performed the experiments: MU KT HS. Analyzed the data: MU KT HS GT. Contributed reagents/materials/analysis tools: MU CS TI SK. Wrote the paper: MU.

References

1. Stevens AM, Johnson FC (1922) A new eruptive fever associated with stomatitis and opthalmia: report of two cases in children. Am J Dis Child 24: 526–533.
2. Leaute-Labreze C, Lamireau T, Chawki D, Maleville J, Taieb A (2000) Diagnosis, classification, and management of erythema multiforme and Stevens-Johnson syndrome. Arch Dis Child 83: 347–352.
3. Forman R, Koren G, Shear NH (2002) Erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis in children: a review of 10 years' experience. Drug Saf 25: 965–972.
4. Roujeau JC, Kelly JP, Naldi L, Rzany B, Stern RS, et al. (1995) Medication use and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. N Engl J Med 333: 1600–1607.
5. Wolf R, Orion E, Marcos B, Matz H (2005) Life-threatening acute adverse cutaneous drug reactions. Clin Dermatol 23: 171–181.
6. Auquier-Dunant A, Mockenhaupt M, Naldi L, Correia O, Schroder W, et al. (2002) Correlations between clinical patterns and causes of erythema multiforme majus, Stevens-Johnson syndrome, and toxic epidermal necrolysis: results of an international prospective study. Arch Dermatol 138: 1019–1024.
7. Yetiv JZ, Bianchine JR, Owen JA Jr. (1980) Etiologic factors of the Stevens-Johnson syndrome. South Med J 73: 599–602.
8. Power WJ, Ghoraishi M, Merayo-Lloves J, Neves RA, Foster CS (1995) Analysis of the acute ophthalmic manifestations of the erythema multiforme/Stevens-Johnson syndrome/toxic epidermal necrolysis disease spectrum. Ophthalmology 102: 1669–1676.
9. Sotozono C, Ueta M, Koizumi N, Inatomi T, Shirakata Y, et al. (2009) Diagnosis and treatment of Stevens-Johnson syndrome and toxic epidermal necrolysis with ocular complications. Ophthalmology 116: 685–690.
10. Ueta M, Kinoshita S (2010) Innate immunity of the ocular surface. Brain Res Bull 81: 219–228.
11. Ueta M, Sotozono C, Nakano M, Taniguchi T, Yagi T, et al. (2010) Association between prostaglandin E receptor 3 polymorphisms and Stevens-Johnson syndrome identified by means of a genome-wide association study. J Allergy Clin Immunol 126: 1218–1225 e1210.
12. Ueta M, Tamiya G, Tokunaga K, Sotozono C, Ueki M, et al. (in press) Epistatic interaction between TLR3 and PTGER3 confers an increased risk for Stevens-Johnson syndrome with ocular complications. J Allergy Clin Immunol.
13. Sotozono C, Ang LP, Koizumi N, Higashihara H, Ueta M, et al. (2007) New grading system for the evaluation of chronic ocular manifestations in patients with Stevens-Johnson syndrome. Ophthalmology 114: 1294–1302.
14. Ueta M, Sotozono C, Inatomi T, Kojima K, Hamuro J, et al. (2007) Association of IL4R polymorphisms with Stevens-Johnson syndrome. J Allergy Clin Immunol 120: 1457–1459.
15. Ueta M, Sotozono C, Inatomi T, Kojima K, Hamuro J, et al. (2008) Association of Fas Ligand gene polymorphism with Stevens-Johnson syndrome. Br J Ophthalmol 92: 989–991.
16. Ueta M, Sotozono C, Inatomi T, Kojima K, Hamuro J, et al. (2008) Association of combined IL-13/IL-4R signaling pathway gene polymorphism with Stevens-Johnson syndrome accompanied by ocular surface complications. Invest Ophthalmol Vis Sci 49: 1809–1813.

17. Ueta M, Sotozono C, Inatomi T, Kojima K, Tashiro K, et al. (2007) Toll-like receptor 3 gene polymorphisms in Japanese patients with Stevens-Johnson syndrome. Br J Ophthalmol 91: 962–965.
18. Ueta M, Sotozono C, Tokunaga K, Yabe T, Kinoshita S (2007) Strong Association Between HLA-A*0206 and Stevens-Johnson Syndrome in the Japanese. Am J Ophthalmol 143: 367–368.
19. Ueta M, Tokunaga K, Sotozono C, Inatomi T, Yabe T, et al. (2008) HLA class I and II gene polymorphisms in Stevens-Johnson syndrome with ocular complications in Japanese. Mol Vis 14: 550–555.
20. Di Pascuale MA, Espana EM, Liu DT, Kawakita T, Li W, Gao YY, et al. (2005) Correlation of corneal complications with eyelid cicatricial pathologies in patients with Stevens-Johnson syndrome and toxic epidermal necrolysis syndrome. Ophthalmology. 112: 904–912.
21. Kawai T, Akira S (2007) TLR signaling. Semin Immunol 19: 24–32.
22. Mondino BJ, Brown SI, Biglan AW (1982) HLA antigens in Stevens-Johnson syndrome with ocular involvement. Arch Ophthalmol 100: 1453–1454.
23. Kaniwa N, Saito Y, Aihara M, Matsunaga K, Tohkin M, et al. (2010) HLA-B*1511 is a risk factor for carbamazepine-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese patients. Epilepsia 51: 2461–2465.

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Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: case-control study

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ABSTRACT

Objectives: To confirm the downregulation of PTGER4 mRNA in the conjunctiva of Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and ocular cicatricial pemphigoid (OCP) patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders.

Design: Case-control study.

Setting and participants: We performed quantitative reverse transcription-PCR (RT-PCR) analysis of *PTGER4* mRNA in conjunctival tissue sections from patients with SJS/TEN and OCP to confirm the downregulation of *PTGER4* mRNA expression. We also analysed EP4 immunohistologically in other ocular surface disorders. Conjunctival tissues were obtained from patients undergoing surgical reconstruction of the ocular surface due to chemical eye burns, subacute SJS/TEN or chronic SJS/TEN, chronic OCP, severe graft versus host disease (GVHD) and from patients with Mooren's ulcers treated by resection of the inflammatory conjunctiva.

Primary and secondary outcome measures: The expression of *PTGER4* mRNA and EP4 protein assessed by quantitative RT-PCR assay and immunohistological methods.

Results: *PTGER4* mRNA was significantly lower in conjunctival tissues from SJS and OCP patients than in the control conjunctivochalasis samples. EP4 protein was detected in conjunctival epithelium from patients with chemical eye burn and in control conjunctival epithelium from patients with conjunctivochalasis. Its expression varied in conjunctival epithelium from patients with Mooren's ulcer. We did not detect EP4 immunoreactivity in conjunctival epithelium from patients with subacute SJS/TEN, severe GVHD, chronic SJS/TEN or OCP.

Conclusions: The strong downregulation of EP4 expression in conjunctival epithelium from patients with OCP or SJS/TEN may be attributable to ocular surface inflammation.

ARTICLE SUMMARY

Article focus

- We previously reported that EP4 protein was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP). Article focus of this study are to confirm the downregulation of *PTGER4* mRNA, which protein is EP4, in the conjunctiva of SJS/TEN and OCP patients and to examine the expression of its EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders in addition chronic SJS/TEN and OCP.

Key messages

- EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the contrary, it is strongly downregulated in conjunctival epithelium from patients with OCP and chronic SJS/TEN and subacute SJS/TEN.

Strengths and limitations of this study

- The function of EP4 in conjunctival epithelial cells is not elucidated.

INTRODUCTION

The prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are lipid mediators that form in response to various stimuli. They are released extracellularly immediately after their synthesis and they act by binding to a G protein-coupled rhodopsin-type receptor on the surface of target cells.[1] PGE_2 is produced during inflammatory responses and it suppresses the production of cytokines and chemokines induced by lipopolysaccharide-stimulated macrophages[2 3] and dendritic cells.[4] Elsewhere we reported that PGE_2 modulates the expression of polyI:C-induced proinflammatory genes in human conjunctival epithelial cells.[5]

There are four PGE receptor subtypes, EP1, EP2, EP3 and EP4. The intestinal epithelium has been reported to express EP4 mRNA,[6] and intestinal homeostasis was

maintained and the immune response downregulated by EP4.[7] The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused on the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.

We documented that while normal human conjunctival epithelium expressed EP4 protein, it was down-regulated in devastating ocular surface inflammatory disorders such as chronic Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) and chronic ocular cicatricial pemphigoid (OCP).[8] Here we examined the mRNA expression of *PTGER4*, which is the gene of EP4 protein, in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that *PTGER4* mRNA EP4 is down-regulated in their conjunctiva. We also examined the expression of *PTGER4* mRNA protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD) and of patients in the subacute stage of SJS/TEN.

MATERIALS AND METHODS

Human conjunctival tissues

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experiments were conducted in accordance with the principles set forth in the Helsinki Declaration.

For quantitative reverse transcription-PCR (RT-PCR) the controls were nearly normal conjunctival tissues obtained at surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in four patients in the chronic stage of SJS/TEN and four patients in the chronic stage of OCP.

The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in three patients with chemical (alkali) eye burn (two in the chronic stage and one in the subacute stage), two patients with subacute SJS/TEN, one patient with severe GVHD and from four patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva. SJS/TEN, OCP, Mooren's ulcer, chemical burn and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute stage but also in the chronic stage.

Quantitative RT-PCR

Total RNA was isolated from conjunctival tissue sections using the RNeasy mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The RT reaction was with the SuperScript preamplification kit (Invitrogen, Carlsbad, California, USA). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, California, USA). The probes for human PTGER4 and human GAPDH were from Applied Biosystems. For cDNA amplification we performed PCR in a 25 µl total volume that contained a 1 µl cDNA template in 2×TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The results were analysed with sequence detection software (Applied Biosystems). The quantification data were normalised to the expression of the housekeeping gene GAPDH.

Immunohistochemistry

For EP4 staining we used rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan, USA). The secondary antibody (Biotin-SP-conjugated AffiniPure $F(ab')_2$ fragment donkey antirabbit IgG (H+L), 1:500 dilution; Jackson Immuno Research, Baltimore, Maryland, USA) was applied for 30 min. The VECTASTAIN ABC reagent (Vector Laboratories, Inc, Burlingame, California, USA) was used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector) as a chromogenic substrate.

Data analysis

Data were expressed as the mean±SEM and evaluated by the Student's t test using the Microsoft Excel software program.

RESULTS

We previously documented that EP4 protein expression was down-regulated in conjunctival epithelium of devastating ocular surface inflammatory disorders such as chronic SJS/TEN and chronic OCP.[8] In this study, to confirm the down-regulation of EP4 in the ocular surface of SJS/TEN and OCP patients we examined the expression of *PTGER4* mRNA in control conjunctival tissues from six conjunctival chalasis patients and in conjunctival tissues from four SJS/TEN patients and four OCP patients. Representative findings of EP4 immunoreactivity in each of these groups are shown in figure 1A. Although EP4 protein was detected in the control tissues, conjunctival epithelium from SJS patients and OCP patients did not manifest EP4 immunoreactivity. *PTGER4* mRNA was significantly lower in conjunctival tissues from SJS/TEN and OCP patients than in the control conjunctivochalasis samples (figure 1B).

Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders. EP4 protein was detected in nearly normal conjunctival epithelium from patients with conjunctivochalasis (figure 2A) and in conjunctival tissues from three patients with chemical eye burn (figure 2B). Its expression varied in conjunctival epithelium from four patients with Mooren's ulcer (figure 2C): in one patient is was similar to the control,

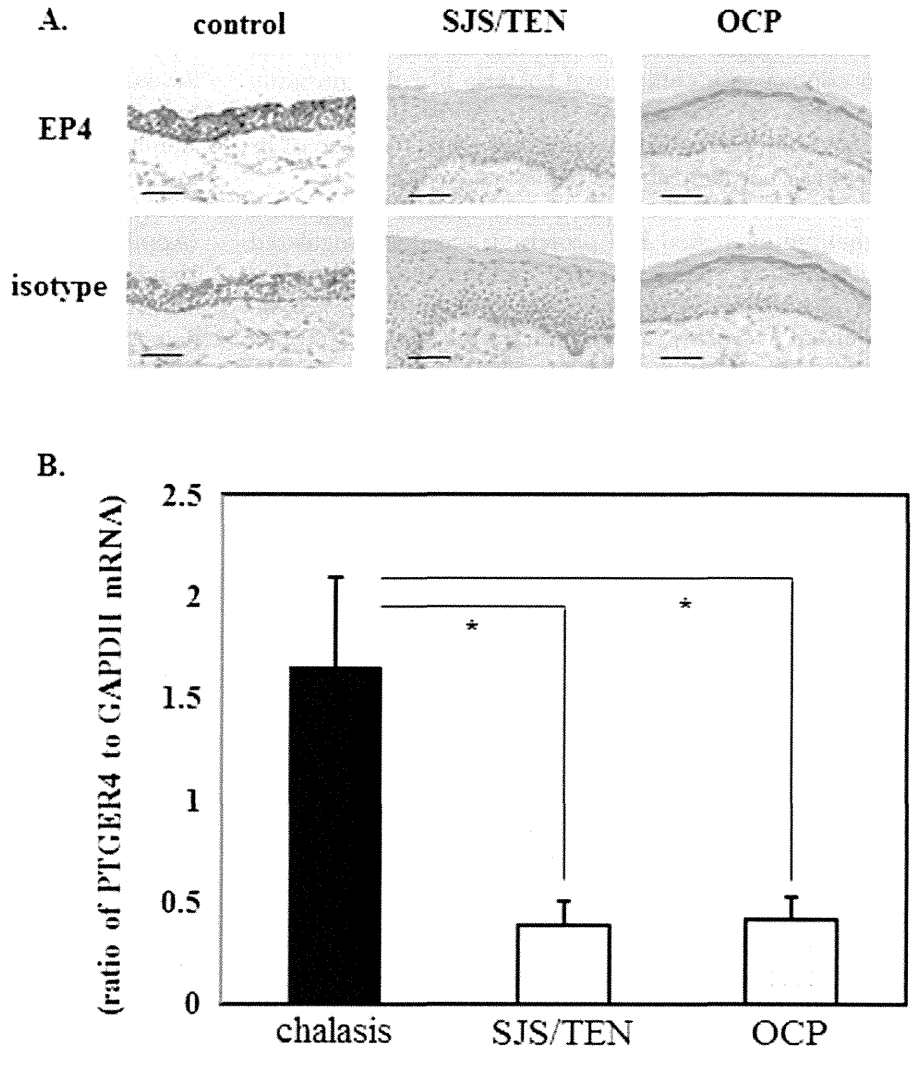

Figure 1 The expression of *PTGER4* mRNA in conjunctival tissues from patients with Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN), ocular cicatricial pemphigoid (OCP) and the controls. (A) Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP). (B) Expression of *PTGER4* mRNA in human conjunctival tissues (*p<0.05).

in two it was slightly lower than in the control and in the remaining patient it was not detected. There was no EP4 immunoreactivity in conjunctival epithelium from two patients with subacute SJS/TEN (figure 2D), a patient with severe GVHD (figure 2E) as same as patients with chronic SJS/TEN or OCP.[8]

We found that, as in normal human conjunctival epithelium, EP4 is expressed in conjunctival epithelium from patients with chemical eye burn. On the other hand, EP4 immunoreactivity was not detected in conjunctival epithelium from patients with SJS/TEN, OCP or severe GVHD. We did not detect EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined.

DISCUSSION

Elsewhere we reported the expression of EP4 in normal human conjunctival epithelium and its down-regulation in conjunctival epithelium from patients with SJS/TEN and OCP.[8] Here we confirmed that in conjunctival tissues from SJS/TEN and OCP patients its mRNA expression was significantly down-regulated, and we also document that EP4 is expressed normally in conjunctival epithelium from patients with severe chemical eye burn which, like SJS/TEN and OCP, is a devastating ocular surface disorder.

On the ocular surface of patients with severe chemical eye burn, conjunctival invasion into the cornea may occur due to the stem cell deficiency of corneal epithelial cells. This results in devastating ocular surface disorders similar to OCP and SJS/TEN. However, in the conjunctiva of patients with severe chemical eye burns, EP4 expression was not down-regulated.

In patients with Mooren's ulcer, an ocular surface inflammatory disease, the expression of EP4 protein varied; in some patients it was down-regulated. In patients in the subacute stage of SJS/TEN with ocular surface inflammation, the expression of EP4 protein was remarkably down-regulated.

Our results suggest that it is possible that EP4 in conjunctival epithelium might contribute the ocular surface homeostasis, while the EP4 may not necessarily be down-regulated in all devastating ocular surface disorders.

Kabashima *et al*[7] reported that in mice, EP4 deficiency impaired mucosal barrier function and induced

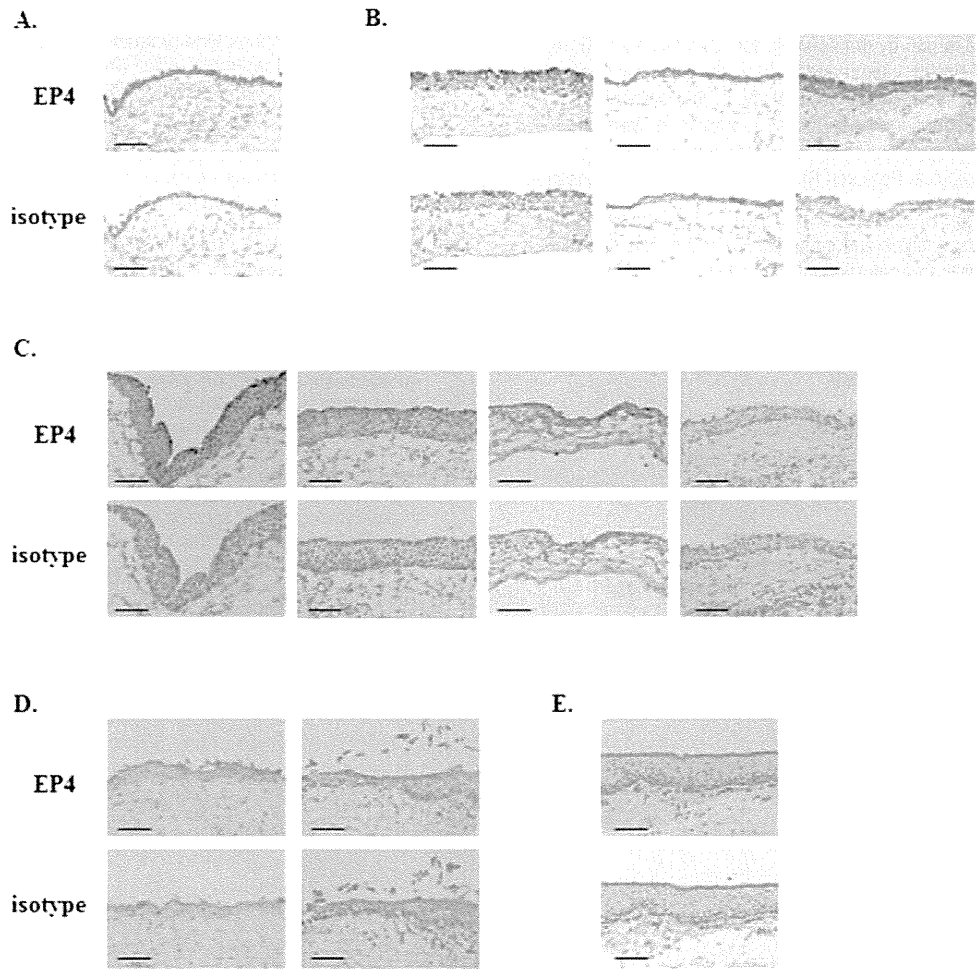

Figure 2 Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases. (A) Nearly normal conjunctival tissues from patients with conjunctivochalasis. (B) Conjunctival tissues from patients with chemical eye burn requiring ocular surface reconstruction. (C) Inflammatory conjunctival tissues from patients with active Mooren's ulcer requiring resection of the inflammatory conjunctiva. (D) Conjunctival tissues from Stevens-Johnson syndrome/toxic epidermal necrolysis patients in the subacute stage. (E) Conjunctival tissues from a patient with severe graft versus host disease. Each scale bar represents 100 μm.

the aggregation of lymphocytes and neutrophils in the colon, and that the administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis. In mice treated with an EP4-selective antagonist the recovery from colitis was suppressed, leading them to conclude that EP4 maintains intestinal homeostasis by preserving mucosal integrity and down-regulating the immune response. On the other hand, Yao *et al*[9] found that PGE_2 acting on its receptor EP4 on T cells and dendritic cells not only facilitated T helper 1 (T_H1) cell differentiation but also amplified interleukin-23-mediated T_H17-cell expansion in vitro. The administration of an EP4-selective antagonist to mice with experimental autoimmune encephalomyelitis or contact hypersensitivity decreased the accumulation of both T_H1 and T_H17 cells in regional lymph nodes and suppressed disease progression. Based on these observations they concluded that PGE_2-EP4 signalling promotes immune inflammation.

In human conjunctival tissues EP4 protein was expressed in epithelial cells but not in cells infiltrating subconjunctival tissues. We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN and Mooren's ulcer.

On the other hand, elsewhere we reported that although EP3 and EP2 agonists suppressed the production of CCL5, CXCL11 and CCL20 in response to polyI:C stimulation, these chemokines were not suppressed by the EP4 agonist in human conjunctival epithelial cells.[5] Studies are underway in our laboratory to elucidate the function of EP4 in conjunctival epithelial cells.

In summary, EP4 is expressed not only in normal conjunctival epithelium but also in conjunctival epithelium from patients with chemical eye burns and some patients with Mooren's ulcer. On the other hand, it is strongly down-regulated in conjunctival epithelium from patients with OCP and chronic SJS/TEN and subacute SJS/TEN.

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REFERENCES

1. Matsuoka T, Narumiya S. Prostaglandin receptor signaling in disease. *ScientificWorldJournal* 2007;7:1329–47.
2. Takayama K, Garcia-Cardena G, Sukhova GK, *et al.* Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem* 2002;277:44147–54.
3. Xu XJ, Reichner JS, Mastrofrancesco B, *et al.* Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production. *J Immunol* 2008;180:2125–31.
4. Shiraishi H, Yoshida H, Saeki K, *et al.* Prostaglandin E2 is a major soluble factor produced by stromal cells for preventing inflammatory cytokine production from dendritic cells. *Int Immunol* 2008;20:1219–29.
5. Ueta M, Matsuoka T, Yokoi N, *et al.* Prostaglandin E2 suppresses polyinosine-polycytidylic acid (polyI:C)-stimulated cytokine production via prostaglandin E2 receptor (EP) 2 and 3 in human conjunctival epithelial cells. *Br J Ophthalmol* 2011;95:859–63.
6. Morimoto K, Sugimoto Y, Katsuyama M, *et al.* Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract. *Am J Physiol* 1997;272:G681–7.
7. Kabashima K, Saji T, Murata T, *et al.* The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J Clin Invest* 2002;109:883–93.
8. Ueta M, Sotozono C, Yokoi N, *et al.* Prostaglandin E receptor 4 expression in human conjunctival epithelium and its downregulation in devastating ocular surface inflammatory disorders. *Arch Ophthalmol* 2010;128:1369–71.
9. Yao C, Sakata D, Esaki Y, *et al.* Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med* 2009;15:633–40.

Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: case-control study

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Prostaglandin E2 Suppresses Poly I:C-Stimulated Cytokine Production Via EP2 and EP3 in Immortalized Human Corneal Epithelial Cells

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Purpose: We previously reported that prostaglandin (PG) E_2 acts as a ligand for prostaglandin E receptor 3 (EP3) in conjunctival epithelial cells, that it downregulates the progression of experimental murine allergic conjunctivitis, and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via prostaglandin E receptor 2 (EP2) and EP3, suggesting that PGE_2 might have important roles in ocular surface inflammation such as allergic conjunctivitis. Here, we investigated whether PGE_2 also downregulates polyI:C-induced cytokine production in human corneal epithelial cells.

Methods: We used enzyme-linked immunosorbent assay and quantitative reverse transcription–polymerase chain reaction to examine the effects of PGE_2 on polyI:C-induced cytokine expression by immortalized human corneal-limbal epithelial cells (HCLE). Using reverse transcription–polymerase chain reaction, we examined the messenger RNA (mRNA) expression of the PGE_2 receptor, EP1–4.

Results: PGE_2 significantly attenuated the expression of CC chemokine ligand (CCL)5 ($P < 0.0005$), CCL20 ($P < 0.0005$), C-X-C chemokine (CXCL)10 ($P < 0.0005$), CXCL11 ($P < 0.05$), and interleukin (IL)-6 ($P < 0.005$) in human corneal-limbal epithelial cells. Human corneal epithelial cells manifested the mRNA expression of EP2, EP3, and EP4, but not EP1. The EP2 agonist significantly suppressed the polyI:C-induced expression of CCL5 ($P < 0.005$), CXCL10 ($P < 0.0005$), and CXCL11 ($P < 0.05$) but not of CCL20 and IL-6. The EP3 agonist significantly suppressed the expression of CCL5 ($P < 0.05$), CCL20 ($P < 0.005$), CXCL10 ($P < 0.0005$), CXCL11 ($P < 0.0005$), and IL-6 ($P < 0.005$). The EP4 agonist failed to suppress cytokine production induced by polyI:C stimulation.

Conclusions: Our results show that in human corneal epithelial cells, PGE_2 attenuated the mRNA expression and production of CCL5, CXCL10, and CXCL11 via both EP2 and EP3, and that the mRNA expression and production of CCL20 and IL-6 was attenuated only by EP3.

Key Words: prostaglandin E_2 (PGE_2), human corneal epithelial cells, prostaglandin E receptor 3, prostaglandin E receptor 2

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Prostanoids are a group of lipid mediators that form in response to various stimuli. They include prostaglandin (PG)D_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane (TX)A_2. They are released extracellularly immediately after their synthesis, and they act by binding to a G protein–coupled rhodopsin-type receptor on the surface of target cells. There are 8 types of prostanoid receptors: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP).[1]

PolyI:C, a synthetic double-stranded (ds)RNA, which mimics viral dsRNA, is the well-known ligand of Toll-like receptor 3.[2] We have reported that polyI:C stimulation induces the secretion of inflammatory cytokines such as interleukin (IL)-6, IL-8, type I interferon (IFN) such as IFN-β, IFN-inducible proteins such as C-X-C chemokine (CXCL)10 and CXCL11, and allergy-related proteins such as CC chemokine ligand (CCL)5 and thymic stromal lymphopoietin in human ocular surface epithelium, both corneal and conjunctival.[3–5] Moreover, we also reported that not only Toll-like receptor 3, but also cytoplasmic helicase proteins, RIG-I (retinoic acid–inducible protein I) and MDA5 (melanoma differentiation–associated gene 5) contribute to polyI:C-inducible responses in conjunctival epithelium.[6]

We previously reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells, that it downregulates the

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progression of experimental murine allergic conjunctivitis,[7] and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via not only EP3 but also EP2,[8] suggesting that PGE_2 might have important roles in the ocular surface inflammation such as allergic conjunctivitis.

PGE_2 was reported to be produced during inflammatory responses and to suppress the production of cytokines and chemokines induced by lipopolysaccharide (LPS) stimulation in macrophages[9,10] and dendritic cells.[11] Elsewhere, we documented that human corneal and conjunctival epithelial cells produce cytokines such as IL-6, IL-8, and IFN-β in response to stimulation with polyI:C but not LPS.[3,12,13] In this study, we examined the expression of the PGE_2 receptors, EP1, EP2, EP3, and EP4, in human corneal epithelial cells and investigated whether polyI:C-induced cytokine production is downregulated by PGE_2 in these cells.

MATERIALS AND METHODS

Human Corneal Epithelial Cells

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the tenets set forth in the Declaration of Helsinki.

For reverse transcription–polymerase chain reaction (RT-PCR) assay, we obtained human corneal epithelial cells from corneal grafts of patients who had undergone corneal transplantation for bullous keratopathy. Immortalized human corneal-limbal epithelial cells (HCLE), a gift from Dr Irene K. Gipson, were cultured in low calcium–defined keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) with defined growth-promoting additives that included insulin, epidermal and fibroblast growth factors, and 1% antibiotic–antimycotic solution. The cells were used after reaching 80% confluence.[7]

Reverse Transcription–Polymerase Chain Reaction

RT-PCR assay was as previously described.[7] Briefly, total RNA was isolated from HCLE and human corneal epithelium using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript Preamplification kit (Invitrogen). Amplification was with DNA polymerase (Takara, Shiga, Japan) for 38 cycles at 94°C for 1 minute, annealing for 1 minute, and 72°C for 1 minute on a commercial PCR machine (GeneAmp; PE Applied Biosystems). The primers were as previously reported.[7] RNA integrity was assessed by electrophoresis in ethidium bromide–stained 1.5% agarose gels. We performed 2 separate experiments.

Enzyme-Linked Immunosorbent Assay

Protein production was confirmed by enzyme-linked immunosorbent assay (ELISA). The amount of IL-6, CCL5, CCL20, CXCL11, and CXCL10 released into the culture supernatant was determined by ELISA using the human CCL5, CCL20, CXCL11, CXCL10 DuoSet (R&D Systems Inc, Minneapolis, MN) or the OptEIA IL-6 set (BD Pharmingen, San Diego, CA).[4,7,14]

We performed 3 separate experiments, each being carried out in 6 wells per group.

Quantitative RT-PCR

Total RNA was isolated from HCLE using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RT reaction was with the SuperScript Preamplification kit (Invitrogen). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, CA) using a previously described protocol.[4,7,14] The primers and probes were from Applied Biosystems [assay ID: CCL5 (Hs00174575), CCL20 (Hs01011368), CXCL10 (Hs00171042), CXCL11 (Hs00171138), IL-6 (Hs00174131), and human GAPDH (Hs 4326317E)]. For complementary DNA (cDNA) amplification, we performed PCR in a 25 μl total volume that contained a 1-μl cDNA template in 2× TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. We performed 3 separate experiments, each being carried out in 6 wells per group.

Data Analysis

Data are expressed as the mean ± SEM and were evaluated by Student t test using the Microsoft Excel software program.

RESULTS

PGE2 Downregulated the Production of Cytokines Induced by Poly I:C Stimulation

Using HCLE and ELISA, we examined whether PGE_2 downregulated the production of IL-6, IL-8, CCL5, CCL20, CXCL10, and CXCL11 induced by polyI:C stimulation in human corneal epithelial cells. HCLE were exposed to 10 μg/mL polyI:C and 100 μg/mL PGE_2 for 24 hours (ELISA) or 6 hours (quantitative RT-PCR). We found that PGE_2 significantly attenuated the production of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (all, $P < 0.0005$) (Fig. 1A). Quantitative RT-PCR assay confirmed that the messenger RNA (mRNA) expression of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (respectively, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$, $P < 0.05$ and $P < 0.005$) was significantly downregulated by PGE_2 (Fig. 1B).

Human Corneal Epithelial Cells Expressed EP2-, EP3-, and EP4-Specific mRNA

We then performed RT-PCR to assay the mRNA expression of the PGE_2 receptors, EP1, EP2, EP3, and EP4, in human corneal epithelial cells. PCR products of expected