あたらしい眼科 25(臨増):187～189, 2008

1. 緑内障の前眼部検査研究の最新情報は？

池田陽子* 森 和彦*

1. 種々の前眼部形状解析装置が開発され，非接触短時間で精密な画像撮影ができる．
2. Fourierドメイン式OCT(光干渉断層計)では濾過胞形状も非接触短時間で経過観察可能になる．
3. 角膜ヒステレーシスが緑内障進行のリスクファクターとなる．
4. Dynamic contour tonometerで測定される脈波は視神経乳頭血流と眼球剛性に影響する．
5. ワイヤレスコンタクトレンズ型眼圧測定装置が開発中．いずれ日内変動連続測定が可能となる．

I 種々の前眼部形状解析装置

近年の前眼部形状解析装置の進歩は目覚ましく，SPAC (scanning peripheral anterior chamber depth analyzer；Takagi, Japan)，Pentacam (Oculus Inc., Lynnwood, WA, USA)，Visante anterior segment optical coherence analyzer (Carl Zeiss Meditec, Jena, Germany)，SL-OCT (Slit-lamp optical coherence tomography；Heidelberg Engineering GmbH, Heidelberg, Germany)など数多くの新しい機器が登場している．SPACはスリット光で走査することにより周辺部隅角形状を解析することができるスクリーニング装置として開発され，PentacamはScheimpflugカメラ理論を応用した装置で非接触・短時間で角膜曲率，角膜厚，前房深度，隅角角度などの情報を得ることができる．しかしこれらの装置は直接隅角底を解析できるわけではなく，隅角検査として隅角鏡を用いた定性的な検査か，アイカップを装着した接触式のUBM (ultrasound biomicroscope)しか存在しなかった．前眼部OCTの出現により毛様体の情報に関しては劣るものの非接触式でUBMに匹敵する情報が得られるようになった(図1)．Visanteで強膜岬の描出が可能であることが報告[1]され，さらに新しいFourierドメイン式OCT (RTVue-100, Optovue社)では前眼部アタッチメントにより従来のタイムドメイン式OCTより格段に高速で前房隅角像が得られる．Asraniら[2]は従来型OCTでは不可能であったSchlemm管や線維柱帯がFourierドメイン式OCTによりきれいに描出できたと報告している．学会レベルでは隅角解析のみならず濾過胞形状解析にも応用されはじめており，今後が期待される．

II 角膜ヒステレーシス

角膜の生化学的特性を容易に測定できる機器が登場した．Ocular Response Analyzer (ORA；Reichart Ophthalmic Instruments, Buffalo, NY, USA；図2a)で測定できる角膜ヒステレーシス(CH)は角膜viscoelasticityを表すパラメータの一つで，空気圧による角膜の凹みはじめと戻りの圧差から規定している(図2b)．健常人のCHは8～15 mmHgであり，角膜実質コラーゲンの性状や脱水状態に影響される．Kirwanら[3]によれば，CHは角膜厚やGoldmann圧平眼圧(GAT)値とは相関を認めないが，円錐角膜やLASIK後，Fuchs角膜変性症やHaab's striaeのある先天緑内障では低い値をとる．健常者と原発開放隅角緑内障(POAG)患者および緑内障疑い患者を比較すると健常者＞緑内障疑い＞緑内障の順にCH値が低くなった．これらの事実よりCHは眼圧変動に対する緩衝能力に関与していると考えられ，CHが低いと眼圧ストレスが視神経に直接影響しやすいことか

* Yoko Ikeda & Kazuhiko Mori：京都府立医科大学大学院視覚機能再生外科学
〔別刷請求先〕池田陽子：〒602-0841 京都市上京区河原町広小路上ル梶井町465 京都府立医科大学大学院視覚機能再生外科学

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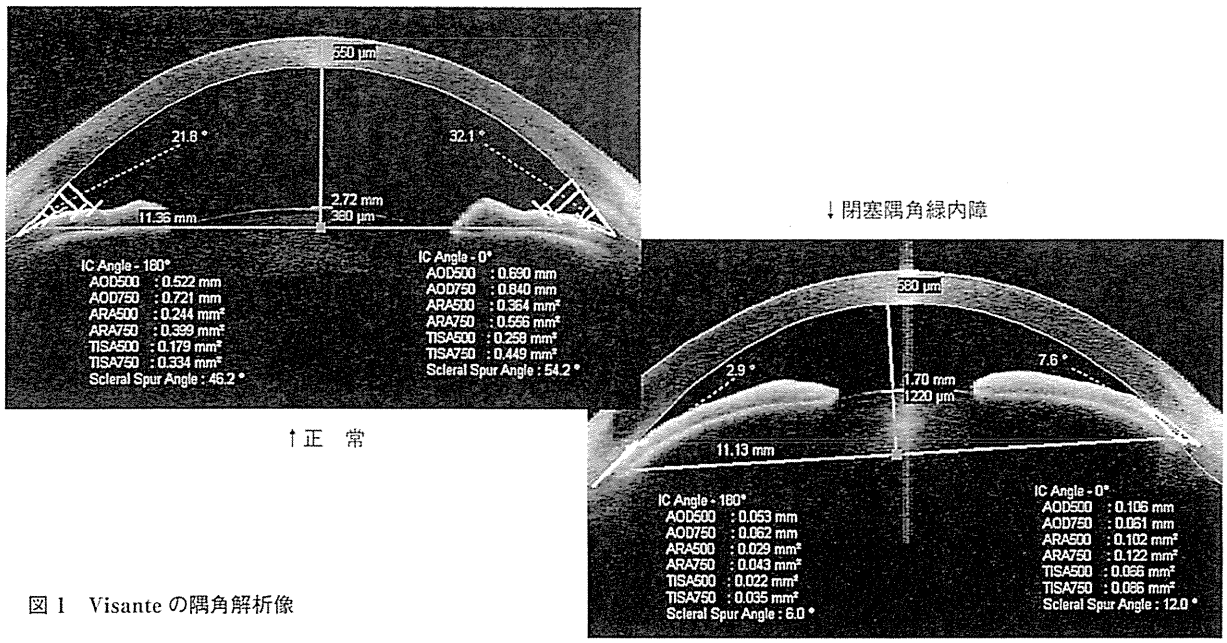

図 1　Visante の隅角解析像

ら視野進行のリスクファクターになりうると考えられている．

III　眼圧関連のトピックス

DCT (Pascal dynamic contour tonometer；Swiss Microtechnology AG, Port, Switzerland) は角膜厚の影響を受けにくい眼圧計として開発され，GAT よりも 1～4 mmHg 高く測定される．POAG 患者では視野異常の強い患者ほど平均 DCT 眼圧は高く，緑内障性視神経障害と相関することが示唆[4]されている．また DCT では脈波が測定できるのも特徴の一つである．眼圧は呼吸や心拍の変化に伴って変動し，その変動幅が眼圧脈波幅 (ocular pulse amplitude：OPA) である．OPA は眼血流の約 9 割を占める脈絡膜循環を反映しているとされ，健常者では 3 mmHg 前後である．OPA 値が大きいと緑内障の重症度が下がるという報告[5]があり，OPA は視神経乳頭血流と眼球剛性に影響する可能性が示唆されている．眼軸長と脈波の関係に関しては，短眼軸ほど脈波が大きくなる[6]という報告と相反する報告[7]もあり結論が出ていない．

2008 年の Association for Research in Vision and Ophthalmology (ARVO) では Pitchon ら[8]によってワイヤレスで眼圧と脈波がモニタリングできるシリコーンソフトコンタクトレンズ (図 3) が発表された．アンテナやマイクロチップが埋め込まれており，10 分間の装

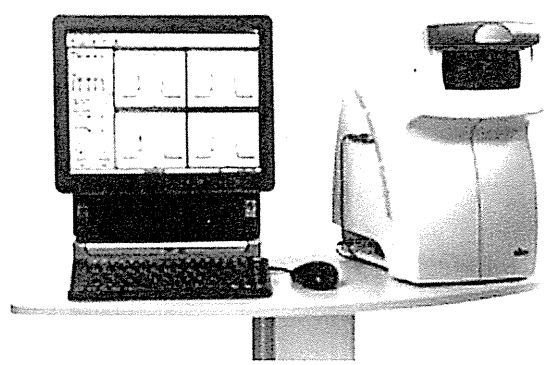

図 2a　Ocular Response Analyzer

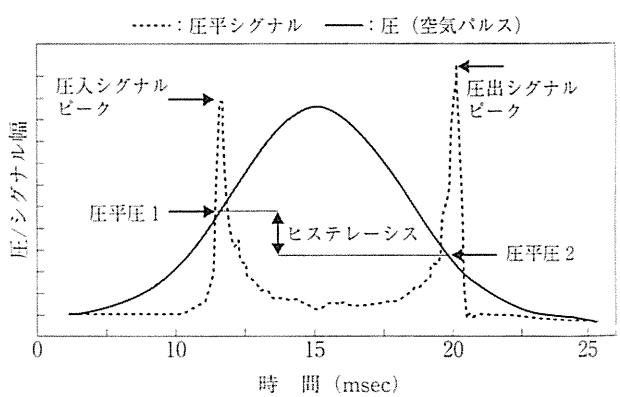

図 2b　波　形 (文献 3 より)

用データでは特に眼表面に問題はなく，眼圧は GAT とよく相関し脈波は DCT に匹敵するものであった．近い将来にはこれまで夢であった 24 時間眼圧測定が現実に

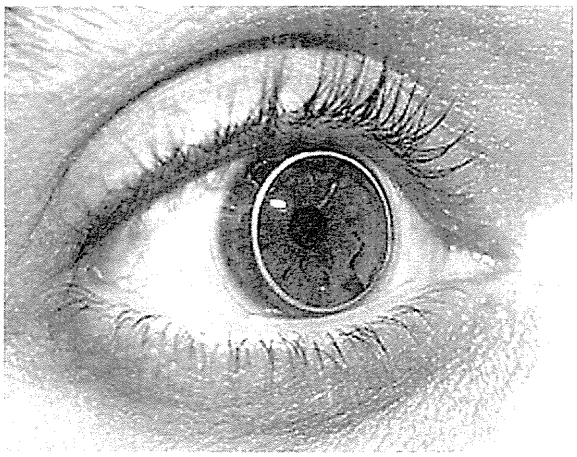

図3　ワイヤレスシリコーンレンズ

なりそうである.

文　献

1) Sakata LM, Lavanya R, Friedman DS et al : Assessment of the scleral spur in anterior segment optical coherence tomography images. *Arch Ophthalmol* **126** : 181-185, 2008
2) Asrani S, Sarunic M, Santiago C : Detailed visualization of the anterior segment using fourier-domain optical coherence tomography. *Arch Ophthalmol* **126** : 765-771, 2008
3) Kirwan C, O'Keefe M, Lanigan B : Corneal hysteresis and intraocular pressure measurement in children using the reichert ocular response analyzer. *Am J Ophthalmol* **142** : 990-992, 2006
4) Sullivan-Mee M, Halverson KD, Qualls C : Clinical comparison of pascal dynamic contour tonometry and goldmann applanation tonometry in asymmetric open-angle glaucoma. *J Glaucoma* **16** : 694-699, 2007
5) Weizer JS, Asrani S, Stinnett SS et al : The clinical utility of dynamic contour tonometry and ocular pulse amplitude. *J Glaucoma* **16** : 700-703, 2007
6) James CB, Trew DR, Clark K et al : Factors influencing the ocular pulse — axial length. *Graefes Arch Clin Exp Ophthalmol* **229** : 341-344, 1991
7) Kaufmann C, Bachmann LM, Robert YC et al : Ocular pulse amplitude in healthy subjects as measured by dynamic contour tonometry. *Arch Ophthalmol* **124** : 1104-1108, 2006
8) Pitchon EM, Leonardi M, Renaud PA et al : First in vivo Human Measure of the Intraocular Pressure Fluctuation and Ocular Pulsation by a Wireless Soft Contact Lens Sensor. ARVO abstract #687, 2008

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トランスレーショナルリサーチを支援する
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DNAチップ/マイクロアレイ臨床応用の実際

基礎、最新技術、臨床・創薬研究応用への実際から今後の展開・問題点まで

別　刷

株式会社 メディカルドゥ

1．疾病の解析 -発現解析各論-
7） 眼科領域におけるアレイ解析の動向

中野正和・米田一仁・木下　茂・田代　啓

ここ数ヵ月の間に，ヒト全ゲノムにわたる数十万ヵ所の一塩基多型（single nucleotide polymorphism：SNP）をタイピングし，数千人規模のアソシエーション解析を実施した研究成果が次々に報告され，geneticsの分野では疾患関連遺伝子の同定に沸いている。奇しくも，その先駆けの1つとなった研究例は眼科領域からのものであった。それは，2005年に加齢黄斑変性の原因遺伝子として補体系の調節因子の1つ，H因子遺伝子が同定された研究である。本稿では，DNAチップを用いたSNPをマーカーとするアソシエーション解析がもたらしたブレイクスルーについてこの研究を題材に概説するとともに，もう1つの代表的な眼疾患である緑内障のアソシエーション解析の現状やチップを用いた遺伝子発現解析が眼科領域に与える可能性について考察する。

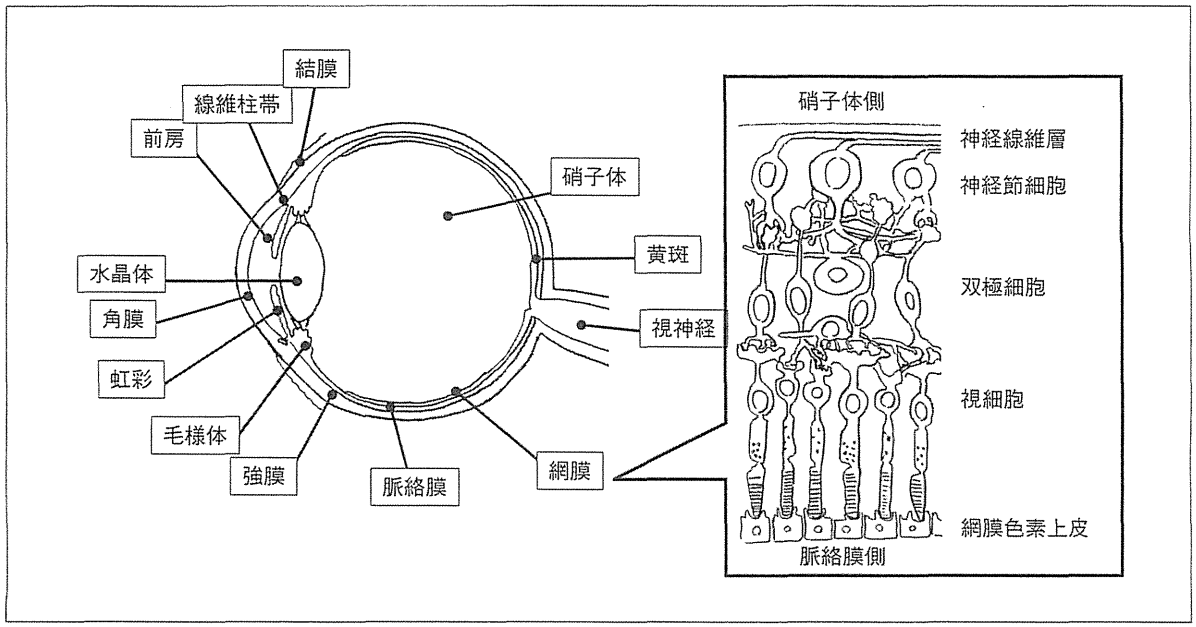
はじめに

近年，数万種類の既知遺伝子の発現解析やヒト全ゲノムにわたる百万ヵ所にも及ぶSNPsのタイピングだけでなく，タイリングアレイを用いたトランスクリプトーム解析や染色体構造変化をコピー数として捉えるcomparative genomic hybridization（CGH），さらにはリシークエンスまでもがチップベースの実験で行えるようになってきた。眼科領域では，歴史の浅いリシークエンスチップの報告例は現在のところゼロで，CGHの報告はretinoblastoma[1]とintraocular uveal melanoma[2]の2例のみであるが，それ以外のアプリケーションは活発に実用されている。これらのチップの技術革新の背景として，①国際HapMapプロジェクト[3]の進展やデータベース上のアノテーション情報の更新によるプローブの質の向上，②チップに搭載できるプローブの高密度化，③チップあたりの解析コストの劇的な低下が挙げられる。個々のチップにはそれぞれ実験上難易度が高い問題点があるものの，今後も質の高いデータをより簡便に得るためのチップ自体の技術開発は着実に進展していくことが予想される。

一方，チップ実験では，定性・定量データにかかわらず，抽出されるデータは膨大かつ無味乾燥である。したがって，解析対象の選択や分類などの事前計画はもちろん，データの処理方法とその解釈，ウェットなサポートデータも含めた研究全般にわたる実験設計に精度の高さが要求される。特に，疾患の発症や進行を制御するメカニズムを解明するためには，圧倒的な情報量により生命現象を包括的に捉える「力づく」だけで達成できるものではなく，ある程度フォーカスした設計を工夫する必要がある。本稿では，個々のチップ研究の設計方法にも着目しながら，眼科領域を取り巻くSNPs解析と遺伝子発現解析の現状を把握し，そ

key words
SNP，疾患関連遺伝子，アソシエーション解析，検出力，眼疾患，加齢黄斑変性，緑内障

図❶　眼球の断面図と各組織の名称および網膜の層構造（拡大）



の将来性について考察を加える。

Ⅰ．SNPをマーカーとするアソシエーション解析

疾患との関連性が示唆されている候補遺伝子上のSNPsをダイレクトシークエンス法やタックマン法で解析する従来のアプローチは眼科領域でも行われている。例えば近年，神経変性疾患としても捉えはじめられている緑内障におけるアルツハイマー病に関与するアポE[4]遺伝子をはじめ，網膜疾患で約50報，Stevens-Johnson症候群とToll-like receptor[5]やIL4R[6]，アトピー性結膜炎とIFNγレセプター[7]といった角結膜疾患でも約10報の興味深い解析例がある。今後，チップを用いた「全ゲノムアソシエーション解析」がこれらの研究をサポートするうえでも重要なアプローチになることは確実である。現在全世界で盛んに実施されはじめている全ゲノムアソシエーション解析は，原因遺伝子群を先入観のバイアスなしに同定できる可能性をおおいに秘めている。

2007年6月に英国のグループから全身性の多因子疾患7疾患についての結果が報告され[8]，各症例2000人と対照3000人のSNPデータを用いたアソシエーション解析のスケールの大きさに震撼させられた。同号のNature誌上に，SNPをマーカーとするアソシエーション解析が満たすべき明確な指針も提示された[9]。これは，これまでのいくつかの解析結果について再現性がとれないとの報告が相次いだためである[10]。この指針では，今後同様の解析を実施する場合，統計学的なパワー（検出力）が十分に高い実験設計に基づいていることと，同程度かそれ以上の規模の別集団で再現性を得ることが推奨されている。改めて大型プロジェクトの強みが浮き彫りになった形である。しかし興味深いことに，全ゲノムアソシエーション解析の先例となった以下に概説する眼疾患研究例では，この指針を必ずしも十分には満たしていなかった。

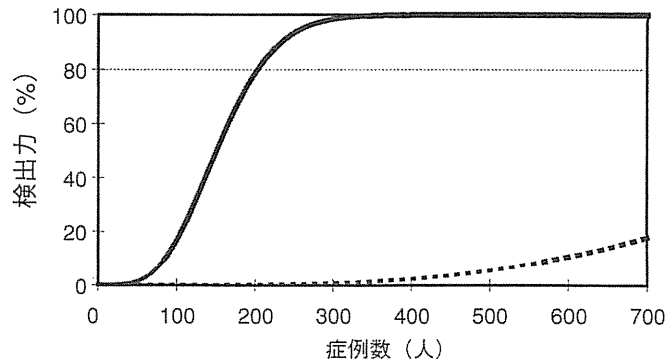
1．加齢黄斑変性

折しも2005年4月は，Affimetrix社やIllumina社が100Kチップを上市してはいたものの，同年8月に更新された国際HapMapプロジェクト[3]の第2相の成果はプローブにほとんど反映されておらず，ごく一部の施設がAffimetrix社500Kチップのベータ版を試用していた過渡期の頃であった。そのような状況下で，Affimetrix社100Kチップを用いた研究成果として，補体系のH因子（complement factor H：*CFH*）遺伝子が加齢黄斑変性[用語1]の原因遺伝子として同定された[11]。

加齢黄斑変性は，光を感じる神経の膜（網膜）の中央に位置し，物を見るために最も重要な視力

や色の判別を司る黄斑が加齢とともに変性する疾患である（図❶）。日本人の罹患率は50歳以上でも1％未満と推計されているが，米国では失明原因の第1位であり，現在は進行を遅延させるための対処療法しかない。本研究では，白人の症例96人と対照50人の全ゲノムアソシエーション解析を実施した結果，「過剰補正」とさえ言われている厳しいBonferroniの補正を突破した有意水準の高いマーカーSNPを2つ見出した。その後の連鎖不平衡ブロックによる領域の規定，領域内のダイレクトシークエンス，候補SNPsのハプロタイプ解析を経て，チロシンからヒスチジンへのアミノ酸変異をもたらす*CFH*遺伝子上のnonsynonymous SNPを同定することに成功した。この研究は，全ゲノム解析によるマーカーSNPから，これが真の生化学的疾患原因SNPであるという完全な証明には至っていないものの，追試されているという意味でかなり本当らしい疾患原因SNPの同定に結びついた最初の典型的な成功例である。

図❷　統計学的検出力のシミュレーション



加齢黄斑変性のマーカーSNPとして同定された1SNP（rs1329428）を題材に，本アソシエーション解析の検出力をフリーウェアソフト“Power and Sample Size Calculations”（Department of Biostatistics, Vanderbilt University）を用いて概算した。アレル頻度比較に基づき算出するため，オッズ比3.0（実線）とリスクアレル頻度0.52は文献12の情報から仮定した。検体数は，症例96人・対照50人（対照/疾患：0.52），有意水準はBonferroniの補正の閾値に近い$p < 1 \times 10^{-7}$に設定した。オッズ比1.5（点線）の場合についても同様の条件で検討した。

ところが，驚くべきことに本研究の理論上の検出力はかなり低い。症例96人・対照50人のアソシーション解析で，有意水準が$p < 1 \times 10^{-7}$のマーカーSNPを取得するための検出力は約15％に過ぎず（図❷，実線），検出力が80％を超えるためには少なくとも200人規模の症例が必要である。今回マーカーとして取得できた*CFH*遺伝子上のSNPのオッズ比が約3.0と比較的高かった[12]ことが幸運であったと言える。これが仮に多因子疾患で検出されるSNPの平均的なオッズ比1.5程度であった場合（図❷，点線），症例と対照群を合わせて2000人を超える例数を準備する必要があり，理論上，本研究の検体数では同様の結果を得ることは絶望的であった。偶然にもオッズ比が高かったとはいえ，検出力が15％しかない研究設計で高い有意水準のマーカーSNPを取得できたことについて，本研究が幸運に恵まれたことによるという理解は，今後，同様の研究を設計する際には忘れてはならない。

本研究は，同施設からの別集団を用いた報告[12]と他施設からの追随報告で再現性が実証されている。さらに，その後の解析から新たな原因遺伝子として補体系のC3因子上のnonsynonymous SNPも同定されている[13]。危うい実験設計ではあったが，まちがいなく全ゲノムアソシエーション解析が疾患の発症分子メカニズムの一端を解明する糸口になった。しかし残念ながら，*CFH*遺伝子上のSNPは日本人では関連がないことが本邦から報告され，この結果の差異は人種差によると推測されている[14]。今後，欧米人の加齢黄斑変性の発症メカニズムの全容の解明とともに，日本人独自の原因遺伝子の同定に向けた全ゲノムアソシエーション解析の実現が期待される。

2．緑内障

緑内障[用語2]は全人類共通の失明の原因の上位の疾患であることから，根治療法の開発に向けて世界中で原因遺伝子の探索の試みが報告されている。緑内障は，神経線維層が障害され視野が狭くなる疾患で，前房中の液体成分（前房水）の排出障害による眼圧の上昇が最大のリスク因子とされているが，正常眼圧緑内障の概念の確立から神経細胞

死に関わる他因子にも注目が集まっている。古くから緑内障は遺伝性疾患であると考えられており，緑内障家系を用いた連鎖解析によりゲノム上の候補領域が特定されている。それらの領域から，ミオシリン[15]，オプチニューリン[16]，*WDR36*[17]などが同定され，「緑内障遺伝子」と呼称されている。しかし，検出力が十分に高い解析で追試されていないので，これら3つの遺伝子が緑内障の発症メカニズムに関与する可能性はあると考えられるが，どの程度の寄与があるかは今後の研究を待つ必要がある。

日本人でも，ミオシリンなどの遺伝子についてのアソシエーション解析が報告されており[18]，新たな原因遺伝子の候補も挙がっている。しかし，一定水準以上の検出力を有する全ゲノムアソシエーション解析を実施した報告例は皆無である。症例・対照群の設計精度の高い，ある程度規模の大きな集団を準備することができれば，あるいは発症メカニズムの解明や治療法の開発に向けて突破口になるような「真の緑内障遺伝子群」を同定することができるかもしれない。

最近，続発緑内障の一病型である落屑緑内障の原因遺伝子が同定されたとの報告がなされた[19]。本報告を受けて一部では「緑内障の原因遺伝子を同定」とも報じられている。しかし，同定されたSNPは最も一般的な緑内障である開放隅角緑内障では関連が認められず，落屑緑内障のみで強い関連を示した。さらに，緑内障を伴わない落屑症候群に対しても関連が示されていることから，正確には「落屑症候群とそれに伴う続発緑内障の原因遺伝子が同定された」と言うべきであろう。

Ⅱ．遺伝子発現解析

遺伝子の発現の変動を定量的に解析する場合，異なる由来の同一組織（症例と対照など）を準備するか，もしくは特定の組織を異なる条件で処理するか（薬剤の処理の有無など），大別して2通りの検討方法が考えられる。当然のことながら，いずれの場合においても出発材料として解析対象となる組織や細胞のtotal RNAが必要になる。ヒトの組織を入手するには制限があるので，チップデータの意義づけには動物実験も含めて研究の設計が極めて重要である。

1．眼科領域におけるヒト由来の組織・細胞

研究計画が所属施設医学倫理委員会をクリアし，インフォームドコンセントが十分に得られるという条件つきではあるが，眼科領域で入手可能なヒト由来の組織を**表❶**に示した。日本でも米国でも健常者から採取できる組織は角膜と結膜の上皮の一部に限られる。その他の組織については，手術などの治療行為で摘出した組織に限って，当人の同意の下，研究目的での使用が可能である。一方，米国では本邦とは異なり，アイバンクへ提供された眼球であっても，適正な手続きを経ることで眼の各組織を研究目的で使用することができる。実質上この制度が研究社会へ多大な貢献をしている。

2．眼疾患の遺伝子発現変動解析

特定の眼組織での遺伝子発現プロファイルを解析することで，眼疾患の発症や進行との関連（**表❶**）を明らかにしようとする試みが多数報告されている。例えば，年齢差のあるヒト眼球由来の網膜を比較して発現が変動している遺伝子を列挙し，加齢に伴い発症する疾患との関連を考察している報告がある[20]。また，網膜色素上皮細胞を用いて，加齢とともに増加する種々の酸化ストレスの有無により発現が変動する遺伝子を解析した報告も散見される。加齢黄斑変性では，SNP解析から原因遺伝子として補体系の因子が同定されたことを契機に，今後免疫系との関わりが盛んに検討されることが予想される。しかし，眼組織はある種の「免疫寛容」の状態にあり，網膜に侵入した外来抗原は免疫反応を誘起しにくいことが知られている[21]。全身性の補体系が網膜における加齢黄斑変性の発症メカニズムにどのように関わっているのか，興味深い課題が残されている。

緑内障では，障害部位である神経線維層のもととなる神経節細胞と眼圧の維持に関与している線維柱帯細胞を用いた報告が圧倒的に多い。神経節細胞はヒト由来の細胞の入手が困難であることから，ラットから樹立した初代培養細胞で代用している。反面，線維柱帯細胞ではヒト眼球由来の初代培養系が確立されており[22]，眼圧の上昇や下降

表❶ 研究材料として入手可能な眼組織と関連する眼疾患

組織	日本			米国			関連疾患
	健常者	患者（手術）	アイバンク	健常者	患者（手術）	アイバンク	
角膜	○*	○	移植治療のみ	○*	○	○	Stevens-Johnson症候群，感染症など
結膜	○*	○	×	○*	○	○	Stevens-Johnson症候群，翼状片など
線維柱帯	×	○	×	×	○	○	緑内障
虹彩	×	○	×	×	○	○	虹彩炎
水晶体	×	○	×	×	○	○	白内障
網膜	×	○	×	×	○	○	緑内障，加齢黄斑変性など

*Epi-LASIKあるいはPRK屈折手術時における角膜上皮，impression cytologyによる結膜上皮に限る。

に関与しているTGF-βファミリーなどのサイトカインの影響を検討した例が多く見受けられる。筆者らは，前房水に含まれるTGF-β3濃度を定量できる微量測定系を構築し，続発緑内障の一病型である偽落屑緑内障の患者から採取した前房水中のTGF-β3濃度が他の病型の患者に比べて有意に高いことを見出した[23]。現在，TGF-βファミリーが相互に眼圧を制御する機構を統合的に理解するため，線維柱帯細胞にTGF-β1，-β2，-β3をそれぞれ添加し，発現が変動する遺伝子群を検討している。その際，二次的な作用の影響をできるだけ回避するために各サイトカインを添加してからごく早期にtotal RNAを抽出してチップ実験に供している。本研究がTGF-βファミリーによる眼圧の制御機構と緑内障の発症や進行のメカニズムとの関わりを解明する一助になることを期待している。

おわりに

10年以上の歴史があるチップを用いる遺伝子発現解析の結果は連続変数であり，グレーのグラデーションとして表現されるが，SNPs解析は白か黒かの結果を出さなくてはならない。白黒を判定できる実験の質を確保する努力が各研究者に求められる。結果が後世の大規模研究で覆されることがおおいにありうるからである。今後，チップの多様なアプリケーションがもたらす無数のデータを，従来の分子生物学的手法で整理・統合しながら生命現象を捉えていく機会がますます増えていくものと思われる。眼科領域では，日本眼科学会の専門別研究会の1つ「眼科DNAチップ研究会」などで先進的な研究の情報交換や議論をさらに活性化することが重要であると考えられる。

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用語解説

1．**加齢黄斑変性**：黄斑が加齢に伴い変性することによって発症する疾患。脈絡膜から発生する異常な血管（新生血管）の有無で滲出型と萎縮型に分類される。特に滲出型は急激な視力低下を生じ，失明原因となる難治性疾患である。日本人では，男性の発病率が女性の約3倍，50歳以上の罹患率は1％未満と推計されている。米国では失明原因の第1位。

2．**緑内障**：網膜の神経線維層が何らかの原因で障害され視野が狭くなる疾患。糖尿病網膜症とともに「目の成人病」と言われ，全人類共通の失明原因の上位の疾患である。前房水の排出障害による眼圧の上昇が最大のリスク因子とされているが，日本人患者の約6割が「正常眼圧」緑内障である。緑内障は，原発緑内障，先天緑内障，続発緑内障に大別され，原発緑内障はさらに開放隅角緑内障（このうち眼圧が正常範囲のものを正常眼圧緑内障と呼ぶ）と閉塞隅角緑内障に分類される。

参考文献

1) Zielinski B, Gratias S, et al : Genes Chromosomes Cancer 43, 294-301, 2005.
2) Hughes S, Damato BE, et al : Br J Cancer 93, 1191-1196, 2005.
3) The International HapMap Consortium : Nature 437, 1299-1320, 2005.
4) Copin B, Brézin AP, et al : Am J Hum Genet 70, 1575-1581, 2002.
5) Ueta M, Sotozono C, et al : Br J Ophthalmol 91, 962-965, 2007.
6) Ueta M, Sotozono C, et al : J Allergy Clin Immun 120, 1457-1459, 2007.
7) Matsuda A, Ebihara N, et al : Invest Ophthalmol Vis Sci 48, 583-589, 2007.
8) The Wellcome Trust Case Control Consortium : Nature 447, 661-678, 2007.
9) Chanock SJ, Manolio T, et al : Nature 447, 655-660, 2007.
10) Morgan TM, Krumholz HM, et al : JAMA 297, 1551-1561, 12007.
11) Klein RJ, Zeiss C, et al : Science 308, 385-389, 2005.
12) Zareparsi S, Branham KE : Am J Hum Genet 77, 149-153, 2005.
13) Yates JR, Sepp T, et al : N Engl J Med 357, 19-27, 2007.
14) Gotoh N, Yamada R, et al : Hum Genet 120, 139-143, 2006.
15) Stone EM, Fingert JH, et al : Science 275, 668-670, 1997.
16) Rezaie T, Child A, et al : Science 295, 1077-1079, 2002.
17) Monemi S, Spaeth G, et al : Hum Mol Genet 14, 725-733, 2005.
18) Funayama T, Mashima Y, et al : Invest Ophthalmol Vis Sci 47, 5368-5375, 2006.
19) Thorleifsson G, Magnusson KP, et al : Science 317, 1397-1400, 2007.
20) Yoshida S, Yashar BM, et al : Invest Ophthalmol Vis Sci 43, 2554-2560, 2002.
21) Streilein JW : Prog Retin Eye Res 18, 357-370, 1999.
22) Yue BY, Higginbotham E, et al : Exp Cell Res 187, 65-68, 1990.
23) Yoneda K, Nakano M, et al : Growth Factors 25, 160-167, 2007.

参考ホームページ

- Power and Sample Size Estimation Software
  http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize
- 眼科DNAチップ研究会
  http://eye.ophth.kpu-m.ac.jp/dna_chip/index.html

中野正和

1995年　東京理科大学基礎工学部生物工学科卒業
1997年　同大学院修士課程修了
　　東レ株式会社医薬事業部
2002年　東京理科大学大学院博士後期課程修了（工学博士）
　　日本学術振興会PD特別研究員（東京大学医科学研究所遺伝子解析施設）
2004年　京都府立医科大学ゲノム医科学部門助手
2007年　同助教

Prostaglandin E receptor subtype EP3 in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis

Mayumi Ueta, MD, PhD,[a,b] Toshiyuki Matsuoka, MD, PhD,[c] Shuh Narumiya, MD, PhD,[c] and Shigeru Kinoshita, MD, PhD[a] *Kyoto, Japan*

Background: We previously demonstrated that the prostaglandin E_2 (PGE_2)–EP3 pathway negatively regulates allergic reactions in a murine allergic asthma model.
Objectives: We investigated whether the PGE_2-EP3 pathway also regulates the development of murine experimental allergic conjunctivitis (EAC).
Methods: The expression of EP3 was examined by means of RT-PCR and immunohistochemistry in wild-type mice, as well as by means of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining in mice deficient in *EP3* (*Ptger3*$^{-/-}$ mice) carrying the β-galactosidase gene at the *EP3* gene locus. EAC was induced by immunization of mice with short ragweed pollen (RW), followed by challenge with eye drops of RW, and eosinophil infiltration and *eotaxin-1* mRNA expression in the conjunctiva were examined. Mice were also treated with a topical application of an EP3-selective agonist during the elicitation phase. Quantitative RT-PCR was used to detect expression of *COXs* and *prostaglandin E synthases*, and ELISA was used to measure PGE_2 production in the eyelid.
Results: EP3 was constitutively expressed in conjunctival epithelium on the ocular surface. *Ptger3*$^{-/-}$ mice demonstrated significantly increased eosinophil infiltration in conjunctiva after RW challenge compared with wild-type mice. Consistently, significantly higher expression of *eotaxin-1* mRNA was observed in *Ptger3*$^{-/-}$ mice. Conversely, treatment of wild-type mice with an EP3-selective agonist resulted in a significant decrease in eosinophil infiltration, which was blunted in *Ptger3*$^{-/-}$ mice. Expression of *COX-2* and *prostaglandin E synthases* was upregulated and PGE_2 content was increased in the eyelids after RW challenge.
Conclusion: These data suggest that PGE_2 acts on EP3 in conjunctival epithelium and downregulates the progression of EAC. (J Allergy Clin Immunol 2009;123:466-71.)

Key words: *Conjunctival epithelium, prostaglandin E receptor subtype EP3, allergic conjunctivitis, eosinophilic conjunctival inflammation, EP3 agonist*

Allergy, especially type I allergy, such as asthma, allergic rhinitis, allergic conjunctivitis, allergic dermatitis, food allergy, and severe anaphylactic response, has increased enormously in prevalence during the past 2 decades[1] and has become one of the major health problems in our society. The course of allergic diseases can be divided into 2 phases: the early-phase reaction and the late-phase reaction. The early-phase reaction occurs within 1 hour after allergen exposure and is driven by the cross-linking of allergen-specific IgE bound to the high-affinity IgE receptor FcεRI on the surface of resident mast cells, the key effector cell in the early-phase reaction. The late-phase reaction occurs 12 to 24 hours after allergen challenge and is characterized by eosinophil-dominant inflammatory cell infiltration in which chemokines such as eotaxin play a key role.

Allergic conjunctivitis is ocular-surface inflammation associated with type I hypersensitivity reactions accompanied by the characteristic symptoms (itching, tearing, conjunctival edema, redness, and photophobia) during the early phase, and eosinophil infiltration occurs in conjunctivas during the late phase. The signs and symptoms of allergic conjunctivitis have a significant effect on patients' comfort, health, and quality of life. Patients with allergic conjunctivitis have a poor quality of life associated with their symptoms, and current treatments for allergic conjunctivitis are inadequate to cure them completely and sometimes lead to side effects, such as an increased risk for the development of cataracts and glaucoma caused by corticosteroids.[2] Therefore safer and more effective treatments are being sought.

One candidate for such treatment is the manipulation of prostanoids and their receptor-signaling pathways because they were reported to regulate allergic reactions in a mouse allergic asthma model. Although prostaglandin D_2 acts on its receptor, DP, and functions as a mediator of allergic asthma,[3] prostaglandin E_2 (PGE_2) acts on one of its 4 receptor subtypes, the prostaglandin E receptor subtype EP3, and negatively regulates allergic

From [a]the Department of Ophthalmology, Kyoto Prefectural University of Medicine; [b]the Research Center for Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto; and [c]the Department of Pharmacology and Faculty of Medicine, Kyoto University.

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Reprint requests: Mayumi Ueta, MD, PhD, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kajiicho, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan. E-mail: mueta@koto.kpu-m.ac.jp.

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Abbreviations used
alum: Aluminum hydroxide
cPGES: Cytosolic prostaglandin E synthase
EAC: Experimental allergic conjunctivitis
mPGES: Membrane-bound prostaglandin E synthase
PGE_2: Prostaglandin E_2
PGES: Prostaglandin E synthase
RT: Room temperature
RW: Short ragweed pollen
TARC: Thymus and activation-regulated chemokine
X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

reactions.[4] In the latest analysis significantly more pronounced allergic inflammation developed in EP3-deficient *(Ptger3*$^{-/-}$*)* mice than in wild-type mice, and the EP3-selective agonist suppressed the inflammation. Intriguingly, EP3 is expressed in airway epithelial cells but not infiltrating cells.[4] On the other hand, nonsteroidal anti-inflammatory drugs, which reduce the increase in both prostaglandin D_2 and PGE_2 levels, are used not only for prevention of posterior capsular opacification[5] but also at times for the treatment of allergic conjunctivitis.[6]

In this study we tested a hypothesis that ocular-surface epithelial cells express EP3 and regulate the inflammation of allergic conjunctivitis through the PGE_2–EP3 pathway, and we addressed this issue by examining ocular-surface EP3 expression and analyzing its role in murine experimental allergic conjunctivitis (EAC) by using *Ptger3*$^{-/-}$ mice and a selective EP3 agonist, ONO-AE-248.

METHODS

Mice, compounds, and reagents

BALB/c mice were purchased from CLEA (Tokyo, Japan) and used at 6 to 12 weeks of age for sensitization. *Ptger3*$^{-/-}$ mice were generated as previously described,[7] back-crossed more than 10 generations to BALB/c mice, and subjected to EAC at 9 to 15 weeks of age with age-matched, wild-type BALB/c mice as control animals. Mice were maintained on a 12-hour/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology "Statement for the use of animals in ophthalmic and vision research."

ONO-AE-248, a selective EP3 agonist, was supplied by ONO Pharmaceutical Co, Ltd (Osaka, Japan); the ligand-binding specificities of the compounds for each prostaglandin E receptor subtype have previously been described.[8] Short ragweed pollen (RW), RW extract, and aluminum hydroxide (alum) were purchased from Polysciences, Inc (Warrington, Pa); LSL Co, Ltd (Tokyo, Japan); and Sigma (St Louis, Mo), respectively.

RT-PCR analysis

By using TRIzol (Invitrogen, Carlsbad, Calif), total RNA was isolated from mouse kidney, corneal, and conjunctival tissues, according to the manufacturer's instructions. For the RT-PCR, we used the SuperScript preamplification system (Invitrogen). Amplification was performed with DNA polymerase (Takara, Shiga, Japan) for 40 cycles at 94°C for 1 minute, 66°C for 1 minute, and 72°C for 1 minute for mouse *EP3* (GeneAmp; PE Applied Biosystems, Foster City, Calif). The primers for mouse *EP3* were as follows: forward 5′-ATCCTCGTGTACCTGTCACAGCGACGCTGG-3′ and reverse 5′-TGCT CAACCTACATCTGATTGAAGATCATT-3′. For mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT), they were as follows: forward 5′-GTTGGATACAGGCCAGACTTTGTT-3′ and reverse 5′-GAGGGTAG GCTGGCCTATAGGCT-3′. A kidney, conjunctiva, or cornea was isolated from wild-type mice.

Histochemistry and histologic analysis

For staining for β-galactosidase activity, freshly isolated eye tissue with the eyelids and conjunctiva was embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and then flash-frozen in liquid nitrogen and cut into 8-μm-thick sections with a cryostat. The sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS; washed with PBS with 1 mmol/L $MgCl_2$; incubated for 24 hours at 37°C with a solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 1 mg/mL), 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, and 1 mmol/L $MgCl_2$; and then counterstained with hematoxylin and eosin.

For staining of eosinophils, the whole eyeball, together with the eyelids and conjunctiva, were dissected and fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Vertical 6-μm-thick sections were affixed to microscope slides and deparaffinized. The slides were then stained with the Luna method (stain in a working 1% Biebrich scarlet solution, dip in lithium carbonate solution, wash in running water, counterstain in a working hematoxylin solution, wash in running water, and dehydrate), and eosinophils were examined with a light microscope. The number of infiltrating eosinophils in the lamina propria mucosae of the tarsal of the conjunctivas in the entire section was counted. The sections used were those from the central portion of the eye, which included the pupil and optic nerve head. Because the cell numbers vary depending on the counting area, the cell-count data are expressed as infiltrating eosinophil numbers divided by the area (in square millimeters), as measured with Scion Image (Scion Corp, Frederick, Md). The data are presented as means ± SEMs of all the mice examined.

Immunohistochemistry

The whole eyeball, together with the eyelids and conjunctiva, was embedded in OCT compound (Sakura Finetek) and then flash-frozen in liquid nitrogen. Sections 6 μm in thickness were cut and fixed with 100% acetone at 4°C for 10 minutes and blocked for 30 minutes with 10% normal donkey serum in PBS. Rabbit polyclonal antibody was to EP3 or to membrane-bound prostaglandin E synthase (mPGES) 1 (Cayman Chemical Co, Ann Arbor, Mich). Nonspecific rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was used as the negative control. For EP3 staining, *Ptger3*$^{-/-}$ mice also were used as the negative control. The secondary antibody (Biotin-SP–conjugated AffiniPure F[ab′]2 Fragment Donkey Anti-Rabbit IgG[H+L], 1:500 dilution; Jackson Immuno Research, Baltimore, Ms) was applied for 30 minutes. VECTASTAIN ABC Reagents (Vector Laboratories, Inc, Burlingame, Calif) were used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories, Inc) as a chromogenic substrate.

Sensitization and challenge

Mice were immunized by means of subcutaneous injection into their left hind footpads of RW adsorbed on alum (200 μg of RW and 2.6 mg of alum in a total volume of 200 μL) on day 0, followed by intraperitoneal injection of RW adsorbed on alum on day 7. On day 18, the eyes of the immunized mice were challenged with RW in PBS (500 μg in 5 μL per eye) or with PBS alone (5 μL per eye). For histologic analysis, the eyes were collected 24 hours after challenge.[9] For RT-PCR analysis of *eotaxin-1*–specific mRNA, the upper and lower eyelids were isolated 6 hours after challenge. For measurement of *COX* values, *prostaglandin E synthase (PGES)*–specific mRNA, and PGE_2 content, the upper and lower eyelids were harvested with a time course of 0, 1, 3, 6, and 12 hours after challenge.

Quantitative RT-PCR of eotaxin-1, COXs, and PGES-specific mRNA in eyelids

Quantitative RT-PCRs of *eotaxin-1, COXs,* and *PGES*-specific mRNA in the eyelids were performed as previously reported.[9] Briefly, the upper and lower lids were collected with the previously specified time course after RW challenge and homogenized in liquid nitrogen. Total RNA was extracted with the RNeasy mini kit (Qiagen, Tokyo, Japan). ReverTra Ace (TOYOBO, Otsu, Japan) was used for reverse transcription. The primers and probes for mouse *eotaxin-1, COX-1, COX-2, mPGES-1, mPGES-2, cytosolic PGES (cPGES),*

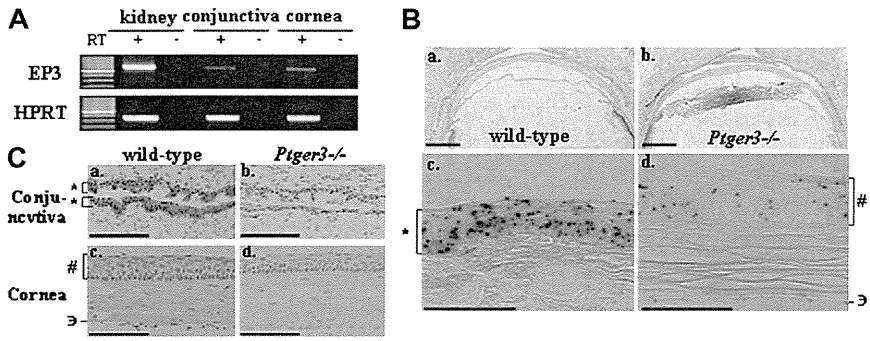

FIG 1. Expression and localization of EP3 in the conjunctiva and cornea. **A**, RT-PCR analyses of the expression of *EP3*-specific mRNA in the cornea and conjunctiva of wild-type mice. **B**, Histochemical staining for EP3 (X-gal): wild-type mice *(a)* and *Ptger3*$^{-/-}$ mice *(b-d)*. Data are representative of 3 experiments. **C**, Immunohistologic analysis for EP3. Each *bar* represents a length of 500 (*B, a, b*), 50 (*B, c, d*), or 100 *(C)* μm. Data are representative of 3 experiments. *Conjunctival epithelium, #corneal epithelium, ɜCorneal endothelium.

and *glyceraldehyde-3-phosphate dehydrogenase (GADPH)* were from Applied Biosystems. The results were analyzed with sequence-detection software (Applied Biosystems); the expression level of *eotaxin-1*, *COXs*, and *PGES*-specific mRNA was normalized to the expression of the mouse housekeeping gene *GADPH*. The time after RW challenge, 6 hours, was optimal for maximum induction of *eotaxin-1* mRNA expression.

Measurement of PGE_2

PGE_2 measurement in eyelids were assessed by means of ELISA with the Prostaglandin E2 Biotrak Enzyme-immunoassay System (Amersham Biosciences, Buckinghamshire, United Kingdom), according to the manufacturer's recommendation.

Data analysis

Data were expressed as the mean ± SEM, and statistical analyses were performed by means of ANOVA and the Student *t* test.

RESULTS

Expression and localization of EP3 in the ocular surface

To examine the expression of *EP3* in the ocular surface, we performed RT-PCR analyses. As a positive control, we used mRNA isolated from the kidney. The expected length of PCR products (608 bp) was obtained from kidney, corneal, and conjunctival samples, suggesting that the ocular-surface tissues, both cornea and conjunctiva, express *EP3* mRNA (Fig 1, *A*). PCR products were isolated and sequenced to confirm the specificity of *EP3* mRNA detection. The sequences obtained from these PCR products were identical to the mouse EP3 cDNA sequence. We next examined EP3 localization using *Ptger3*$^{-/-}$ mice in which the β-galactosidase gene was "knocked in" at the *EP3* gene locus and with immunohistochemistry of wild-type mice. In *Ptger3*$^{-/-}$ mice X-gal staining of ocular-surface tissue revealed dense positive signals in conjunctival epithelia and scattered positive signals in corneal epithelia (Fig 1, *B*, *b-d*), suggesting a strong EP3 expression in conjunctival epithelial cells and a slight expression in corneal epithelial cells. Interestingly, a few positive signals were seen in corneal endothelia (Fig 1, *B*, *d*). In other eye compartments, such as the lens, retina, uvea, and sclera, of the *Ptger3*$^{-/-}$ mice, there were no positive signals (Fig 1, *B*, *b*). X-gal staining of ocular-surface tissue did not reveal positive signals in wild-type mice (Fig 1, *B*, *a*). Immunohistochemistry of conjunctival and corneal tissue revealed that conjunctival epithelial cells expressed EP3 protein (Fig 1, *C*, *a*), although we could not find the positive signal in corneal epithelial and endothelial cells, presumably because of decreased expression of EP3 protein (Fig 1, *C*, *c*).

Antibody responses to RW sensitization

We next examined whether immunization of RW induced immune responses specific to RW equally in wild-type and *Ptger3*$^{-/-}$ mice (see the Methods section of the Online Repository at www.jacionline.org). RW sensitization significantly increased the serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 in both wild-type and *Ptger3*$^{-/-}$ mice compared with levels seen in the unsensitized control mice. There were no significant differences in serum levels of total IgE, anti-RW IgE, and anti-RW IgG1 between the sensitized wild-type and *Ptger3*$^{-/-}$ mice (see Fig E1 in this article's Online Repository at www.jacionline.org). These results suggest that wild-type and *Ptger3*$^{-/-}$ mice had been equally sensitized to RW.

Eosinophil accumulation in the lamina propria mucosae of the conjunctivas

To investigate whether EP3 plays a role in the late-phase reaction of EAC, we examined conjunctival tissue from wild-type and *Ptger3*$^{-/-}$ mice. Histologic analyses of conjunctivas revealed that the RW challenge on RW-sensitized mice led to inflammatory cell infiltrations in the lamina propria mucosae of the conjunctivas at 24 hours after challenge in our EAC model (Fig 2). The infiltrated cells consisted predominantly of eosinophils; only a few lymphocytes and neutrophils were detected. Both wild-type and *Ptger3*$^{-/-}$ mice had eosinophil-dominant inflammatory cell infiltrations in the lamina propria mucosae at 24 hours after challenge; however, the numbers of eosinophils in *Ptger3*$^{-/-}$ mice were significantly greater than in wild-type mice (Table I). Either RW sensitization or RW challenge alone did not significantly increase the numbers of eosinophils in wild-type and *Ptger3*$^{-/-}$ mice (data not shown). These results suggest that the PGE_2–EP3 pathway negatively regulates EAC development in the late-phase reaction, which cause the pronounced allergic inflammation in *Ptger3*$^{-/-}$ mice.

Expression of *eotaxin-1*–specific mRNA in eyelids

Given the critical roles of eotaxin-1 in eosinophil recruitment,[10-12] we examined the *eotaxin-1*–specific mRNA expression

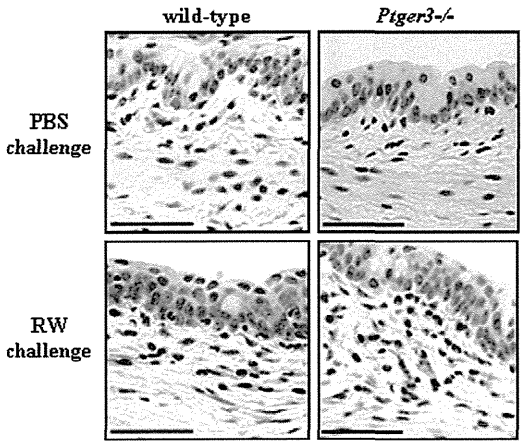

FIG 2. Histologic analyses of eosinophil inflammation in conjunctiva. Infiltration of eosinophils into the conjunctiva of wild-type and *Ptger3*$^{-/-}$ mice were detected by using the Luna method, which stained eosinophil granules with a distinctive red. *Scale bars* = 50 μm.

TABLE I. Number of eosinophils in the lamina propria mucosae of the tarsal conjunctiva

	Wild-type mice		*Ptger3*$^{-/-}$ mice	
Sensitization	+		+	
Challenge	−	+	−	+
No. of mice	19	19	19	19
No. of eosinophils, mean ± SEM/0.1 mm^2	4.6 ± 1.1	42.4 ± 3.1	4.2 ± 0.8	86.5 ± 5.4*

*P < .0005 (42.4 ± 3.1 vs 86.5 ± 5.4).

in the eyelids from RW-sensitized wild-type and *Ptger3*$^{-/-}$ mice at 6 hours after RW challenge.

Quantitative real-time RT-PCR analyses demonstrated that the RW challenge significantly increased the *eotaxin-1* mRNA expression in both genotypes compared with that in vehicle-treated control animals. The *eotaxin-1* mRNA expression increase in RW-challenged *Ptger3*$^{-/-}$ mice is significantly larger than that seen in wild-type mice (Fig 3), which is consistent with pronounced eosinophil inflammation in *Ptger3*$^{-/-}$ mice.

Upregulation of *COX-2, PGES*-specific mRNA expression, and PGE_2 contents in eyelids during EAC

Because COX and PGES action is necessary for PGE_2 synthesis,[13] we examined the expression of mRNA for *COX* isoforms and *PGES* isoforms in eyelids during EAC. After RW challenge, the relative expression levels of *COX-2* mRNA increased and peaked at 1 hour (about 9 times higher than the basal level at 0 hour) and then gradually decreased (Fig 4, *A*), whereas the expression of *COX-1* mRNA stayed at an almost basal level (data not shown). After RW challenge, the relative expression levels of *mPGES-1* mRNA also increased and peaked at 3 hours and then gradually decreased (Fig 4, *A*), and similar gene expression patterns of *mPGES-2* and *cPGES* were also observed (data not shown). We also examined the PGE_2 contents in eyelids during EAC. After RW challenge, the PGE_2 contents in eyelids increased time dependently until 12 hours (Fig 4, *B*). These results suggest that RW challenge increased PGE_2 synthesis in eyelids through upregulation of inducible enzymes, such as COX-2 and

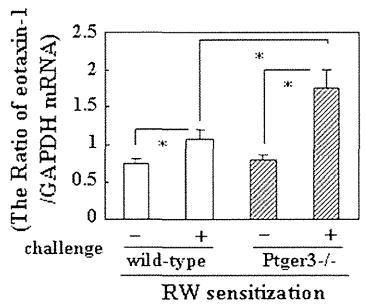

FIG 3. Real-time PCR analyses of the expression of *eotaxin-1* mRNA in the eyelids 6 hours after RW challenge. Relative mRNA expression levels for *eotaxin-1* normalized by *GAPDH* are shown. Data are shown as the mean ± SEM of 7 samples per group. *P < .05.

mPGES-1, during the elicitation phase of EAC. Furthermore, we examined the localization of PGE_2 synthesis by using immunohistochemistry of mPGES-1. Immunohistologic analysis of ocular-surface tissue, which was obtained from RW-sensitized wild-type mice at 6 hours after RW challenge, revealed that conjunctival epithelial cells expressed mPGES-1 protein (Fig 4, *C*), suggesting that PGE_2 synthesis through mPGES-1 might occur in conjunctival epithelium during the elicitation phase of EAC.

Effect of an EP3-selective agonist

To investigate whether allergic inflammation can be suppressed by stimulating the PGE_2–EP3 pathway in EAC, we next examined the effects of an EP3-selective agonist, ONO-AE-248. We topically administered ONO-AE-248 to the eyes of RW-sensitized mice 3 times at 1 hour before and at 3 and 7 hours after RW challenge. First, to determine the optimal dose, ONO-AE-248 was administered at various doses (0.01, 0.1, 1, 10, and 100 ng) in 5 μL of PBS per eye to wild-type mice. ONO-AE-248 suppresses eosinophil infiltration dose dependently until 1 ng, and then at the higher doses of 10 ng and 100 ng, its suppression becomes weak (see Fig E2 in this article's Online Repository at www.jacionline.org). We therefore determined that the optimal dose of AE248 was 1 ng in 5 μL of PBS (0.02%). In wild-type mice topical administration of 1 ng of ONO-AE-248 significantly inhibited the antigen-induced infiltration of eosinophils compared with that seen in the vehicle-treated groups (Fig 5 and see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, this inhibitory effect of ONO-AE-248 was absent in *Ptger3*$^{-/-}$ mice, confirming that it was mediated by EP3 (Fig 5 and see Fig E3). Although vehicle treatment mildly decreased eosinophil infiltration, it is very likely that a 3-time vehicle administration washed out the challenged RW antigen and resulted in reduced allergic responses. These results suggest that allergic inflammation can be suppressed by stimulating EP3 with a selective agonist.

DISCUSSION

In this study we investigated the roles of the PGE_2–EP3 pathway in allergic conjunctivitis by using RW-induced EAC as a model in mice. First, we examined EP3 expression in ocular tissues and found that EP3 is expressed in the conjunctival epithelium on the ocular surface in mice. Because *Ptger3*$^{-/-}$ mice have allergic inflammation that was much more pronounced than that seen in wild-type mice, negative regulation of the

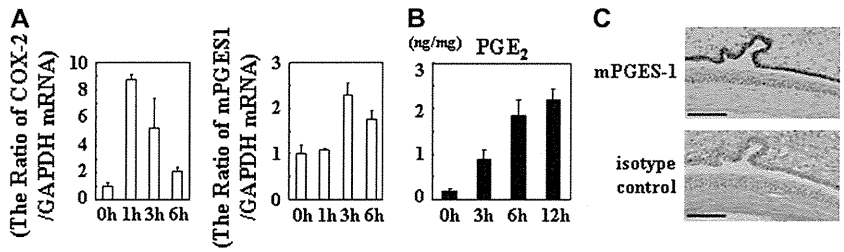

FIG 4. RW challenge increased PGE_2 synthesis in eyelids. **A**, Time course of *COX-2* and *mPGES-1* mRNA expression in eyelids during EAC. The *y-axis* shows the increase of specific mRNA over that seen in 0-hour samples. The *x-axis* shows the time after the RW challenge. Data are shown as the mean ± SEM of 3 samples. **B**, Time course of PGE_2 content in eyelids during EAC. The *x-axis* shows the time after the RW challenge. Data are shown as the mean ± SEM of 4 samples. **C**, Localization of mPGES-1 on the ocular surface. Each *bar* represents a length of 100 μm.

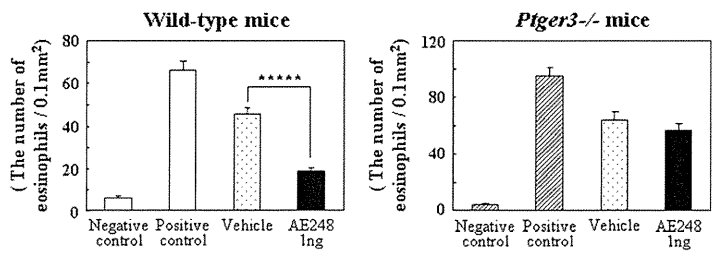

FIG 5. The effects of an EP3-selective agonist on eosinophil infiltration in conjunctiva. Data are shown as the mean ± SEM of samples from all the mice examined. Negative control (RW sensitization without challenge), positive control (RW sensitization with challenge) only vehicle, and AE248 are shown. (Wild-type mice: negative control, n = 20; positive control, n = 20; vehicle only, n = 25; AE248, n = 30; $Ptger3^{-/-}$ mice: negative control, n = 11; positive control, n = 11; vehicle only, n = 14; AE248, n = 16). *****$P < .0005$.

development of EAC through the PGE_2–EP3 pathway was suggested. Consistently, significantly greater *eotaxin-1* mRNA expression was observed in $Ptger3^{-/-}$ mice. Conversely, treatment with an EP3-selective agonist resulted in significantly decreased eosinophil infiltration in wild-type mice, which was blunted in $Ptger3^{-/-}$ mice. We examined the expression of mRNA of *COX* isoforms, *PGES* isoforms, and PGE_2 content in eyelids during EAC and found that *COX-2*, *PGES* mRNA, and PGE_2 content in eyelids increased after RW challenge. Interestingly, the expression of mPGES-1 was localized in conjunctival epithelium, suggesting that PGE_2 synthesis through mPGES-1 might occur in conjunctival epithelium. These results suggest not only the critical roles of the PGE_2–EP3 pathway in allergic conjunctivitis but also a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE_2–EP3 pathway.

The allergic response in conjunctivitis is typically elicited by ocular exposure to an allergen, such as grass or tree pollen, that causes cross-linkage of membrane-bound IgE, which triggers mast cell degranulation, releasing a cascade of allergic and inflammatory mediators. Thus mast cells play an important role in the early-phase reactions. Given the predominant role of mast cells in the early-phase reaction, most patients presenting with allergic conjunctivitis are treated with eye drops containing mast cell stabilizers or antihistamines. It is also suggested that mediators released by mast cells during the early-phase reactions contribute to the development of the late-phase reaction.[14] However, we previously demonstrated that mast cells do not play an essential role in the development of a late-phase reaction of eosinophilic conjunctival inflammation by showing that mast cell–deficient mice similarly had eosinophilic conjunctival inflammation compared with congenic littermates with EAC.[9] In this study we showed that conjunctival epithelial cells might be also implicated in the eosinophilic conjunctival inflammation seen in allergic conjunctivitis.

Late-phase reaction is characterized by eosinophil-dominant infiltrations in the local tissue.[9,12] The increase of eosinophil infiltration correlates well with both the severity and condition of this disease.[10-12] For eosinophil recruitment, eotaxin-1 is now accepted as the central mediator, and it was reported that its deficiency ablates eosinophilic responses in allergic conjunctivitis.[12] Hence we examined the *eotaxin-1*–specific mRNA expression in the eyelids from RW-sensitized wild-type and $Ptger3^{-/-}$ mice at 6 hours after RW challenge. Because we observed a large number of eosinophils in our EAC at 24 hours after RW challenge, this condition is thought to mimic the late-phase reaction in human allergic conjunctivitis. Recently, T cells[15] and fibroblasts[16] were also reported to contribute to the development of the late-phase reaction. Although various cell types can contribute to orchestrate the allergic response and various mechanisms are thought to develop the late-phase reaction, our findings in this study strongly suggest that conjunctival epithelium, which predominantly expresses EP3 among ocular tissues, substantially contributes to the late-phase reaction in our EAC.

In an allergic asthma model PGE_2 was reported to act at EP3 on airway epithelial cells and regulate the extent of the late-phase reaction by attenuating the expression of chemokine genes, such as *eotaxin-1* and *thymus and activation-regulated chemokine (TARC)*.[4] Given that RW challenge–induced *eotaxin-1* mRNA increase in eyelids was much more pronounced in $Ptger3^{-/-}$ mice, it was suggested that the PGE_2–EP3 pathway negatively regulates allergic reactions by suppressing chemokine gene expression in

EAC. With regard to the clinical relevance of eotaxin-1 in allergic conjunctivitis, it was reported that eotaxin-1 was expressed in the conjunctival epithelium of patients with vernal keratoconjunctivitis,[17] although we failed to show the eotaxin-1 or TARC production by cultured conjunctival epithelial cells stimulated with combinations of TNF-α and IL-4 (data not shown). The difference between *in vivo* and *in vitro* conditions might contribute to the different cell responses. On the other hand, cultured conjunctival fibroblasts could release eotaxin-1 or TARC in response to stimulation with combinations of TNF-α and IL-4 (data not shown). The mechanisms of eosinophilic conjunctival inflammation at the late-phase reaction are still elusive. Further investigations are required to identify the precise molecular mechanisms of allergic conjunctivitis.

It is well known that prostaglandins are produced in substantial amounts during allergen exposure and disease development[18,19] and that COX enzymes are involved in the synthesis of prostaglandins.[20] COX-1 is an ubiquitous housekeeping enzyme and thought to produce a basal level of prostaglandins, whereas COX-2 is an inducible enzyme and thought to be involved in pathologic conditions. Consistently, we detected unchanged *COX-1* and markedly increased *COX-2* mRNA expression in conjunctiva after RW challenge. Furthermore, we also detected increased *PGES* mRNA values and PGE_2 contents in eyelids and mPGES-1 expression in conjunctival epithelium. Of 3 PGESs, cPGES and mPGES-2 are constitutive enzymes, whereas mPGES-1 is an induced enzyme and upregulated by proinflammatory stimuli.[21] Because the functional coupling of COX-2 to mPGES-1 for PGE_2 biosynthesis has been suggested,[22] the coordinate increased expression of COX-2 and mPGES-1 might contribute to an increase in PGE_2 production in the conjunctival epithelium during the elicitation phase of our EAC. Thus we propose the endogenous negative-feedback regulation of allergic inflammation; the PGE_2–EP3 pathway is activated on challenge and functions to suppress development of EAC. Notably, this endogenous feedback system can be augmented by stimulating EP3 with a selective agonist.

It is possible that the PGE_2–EP3 pathway might function to suppress the development of human allergic conjunctivitis because we have confirmed the expression of EP3 in human conjunctival epithelium at both the mRNA and protein levels (see Fig E4 in this article's Online Repository at www.jacionline.org).

In summary, we demonstrated that EP3 is expressed in the ocular surface and that the PGE_2–EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis. Although species differences between human subjects and mice should be considered cautiously and extrapolation from the mouse models to human pathologies must be performed with some reservation, stimulating the PGE_2–EP3 pathway with a selective agonist might be useful for treating allergic conjunctivitis in human subjects. Furthermore, we propose a new strategy for treating allergic conjunctivitis by modifying the epithelial cell functions in a similar way to the PGE_2–EP3 pathway.

We thank Dr T. Honda for discussion and Ms C. Mochida for technical assistance.

Clinical implications: EP3 in conjunctival epithelium can be a target for drug development to treat allergic conjunctivitis.

REFERENCES

1. Sears MR. Epidemiology of childhood asthma. Lancet 1997;350:1015-20.
2. Ono SJ, Abelson MB. Allergic conjunctivitis: update on pathophysiology and prospects for future treatment. J Allergy Clin Immunol 2005;115:118-22.
3. Matsuoka T, Hirata M, Tanaka H, Takahashi Y, Murata T, Kabashima K, et al. Prostaglandin D2 as a mediator of allergic asthma. Science 2000;287:2013-7.
4. Kunikata T, Yamane H, Segi E, Matsuoka T, Sugimoto Y, Tanaka S, et al. Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3. Nat Immunol 2005;6:524-31.
5. Chandler HL, Barden CA, Lu P, Kusewitt DF, Colitz CM. Prevention of posterior capsular opacification through cyclooxygenase-2 inhibition. Mol Vis 2007;13:677-91.
6. Hashimoto T, Igarashi A, Hoshina F, Yamada M, Toyoda Y, Notsu Y, et al. Effects of nonsteroidal anti-inflammatory drugs on experimental allergic conjunctivitis in Guinea pigs. J Ocul Pharmacol Ther 2003;19:569-77.
7. Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, et al. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. Nature 1998;395:281-4.
8. Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, et al. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. Endocrinology 2000;141:1554-9.
9. Ueta M, Nakamura T, Tanaka S, Kojima K, Kinoshita S. Development of eosinophilic conjunctival inflammation at late-phase reaction in mast cell-deficient mice. J Allergy Clin Immunol 2007;120:476-8.
10. Fukagawa K, Nakajima T, Tsubota K, Shimmura S, Saito H, Hirai K. Presence of eotaxin in tears of patients with atopic keratoconjunctivitis with severe corneal damage. J Allergy Clin Immunol 1999;103:1220-1.
11. Leonardi A, Jose PJ, Zhan H, Calder VL. Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis. Ophthalmology 2003;110:487-92.
12. Miyazaki D, Nakamura T, Komatsu N, Nawata N, Ikeda Y, Inoue Y, et al. Roles of chemokines in ocular allergy and possible therapeutic strategies. Cornea 2004;23(suppl):S48-54.
13. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97-120.
14. Broide DH. The pathophysiology of allergic rhinoconjunctivitis. Allergy Asthma Proc 2007;28:398-403.
15. Fukushima A. Roles of T-cells in the development of allergic conjunctival diseases. Cornea 2007;26(suppl):S36-40.
16. Fukuda K, Kumagai N, Fujitsu Y, Nishida T. Fibroblasts as local immune modulators in ocular allergic disease. Allergol Int 2006;55:121-9.
17. 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;84:1360-6.
18. Christodoulopoulos P, Cameron L, Durham S, Hamid Q. Molecular pathology of allergic disease. II: Upper airway disease. J Allergy Clin Immunol 2000;105:211-23.
19. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. J Allergy Clin Immunol 2000;105:193-204.
20. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999;79:1193-226.
21. Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin E synthase-1: a novel therapeutic target. Pharmacol Rev 2007;59:207-24.
22. Murakami M, Kudo I. Recent advances in molecular biology and physiology of the prostaglandin E2-biosynthetic pathway. Prog Lipid Res 2004;43:3-35.

METHODS

Measurement of immunoglobulins in serum

Twenty-four hours after RW challenge, blood was collected, and serum was prepared. Serum total IgE levels were assessed by means of ELISA with the OptEIA mouse IgE ELISA set (BD Biosciences PharMingen, San Diego, Calif), according to the manufacturer's recommendation. RW-specific immunoglobulin levels in the serum were assessed as previously reported (Kweon, 2000). Briefly, EIA plates (Costar, Corning, NY) were coated with RW extract (5 μg/mL) at 4°C overnight. After blocking with 1% BSA in PBS, serum samples were added and incubated for 4 hours at room temperature. The plates were washed with PBS plus 0.05% Tween 20 (Wako, Osaka, Japan) and incubated for 2 hours at room temperature with goat anti-mouse IgG1–horseradish peroxidase–conjugated antibody (Southern Biotechnology Associates, Inc, Birmingham, Ala) for RW-specific IgG1. In the case of IgE, biotin-conjugated rat anti-mouse IgE mAb (BD Bioscience PharMingen) was added to each well for 1 hour at room temperature, and then after washing, avidin–horseradish peroxidase conjugate (BD Bioscience PharMingen) was added to each well for 30 minutes at room temperature. After washing, the color reaction was developed with 3, 3′, 5, 5′-tetramethyl-benzidine (Moss, Inc, Pasadena, Calif). End point titers of RW-specific immunoglobulins were expressed as the reciprocal log2 of the last dilution that showed a level of OD units 2-fold higher than the background.

Human conjunctival 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 principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocol were explained to all patients, and their informed consent was obtained.

For RT-PCR, we obtained human conjunctival epithelial cells from healthy volunteers by means of impression cytology. For immunohistochemistry, serial sections of human conjunctivas were prepared from samples obtained at conjunctivochalasis surgery.

For the RT-PCR, the primers for human *EP3* and human *GADPH* were as follows: forward 5′- GCG CGC TGG TGC TGC GTC TGT ACA CTG CGG -3′ and reverse 5′- AGT GGC CGC TGC AGG GAG GTA GAG CTC CAG -3′ and forward 5′- CCA TCA CCA TCT TCC AGG AG-3′ and reverse 5′- CCT GCT TCA CCA CCT TCT TG-3′, respectively.

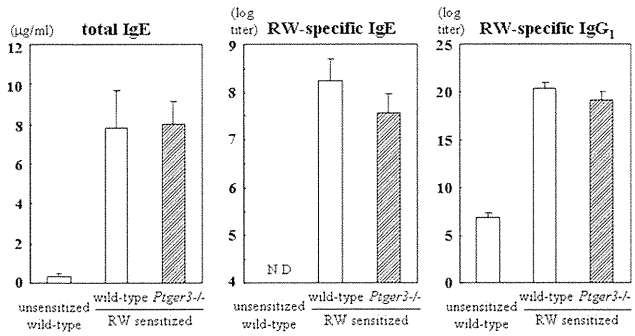

FIG E1. Antibody responses to RW sensitization and challenge. The serum concentration of total IgE *(left)* and the log titer of RW-specific IgE *(middle)* and RW-specific IgG1 *(right)* assessed by means of ELISA are shown. Sera were prepared from sensitized wild-type and *Ptger3*$^{-/-}$ mice at 24 hours after RW challenge. Sera from unsensitized wild-type mice were used as controls. Data are shown as the means ± SEMs of 8 samples.

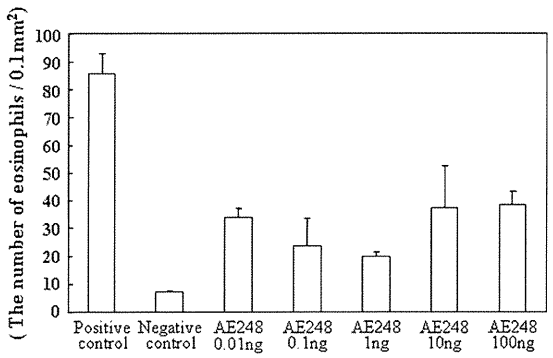

FIG E2. The optimal dose of ONO-AE-248. ONO-AE-248 was administered at various doses (0.01 ng, 0.1, 1, 10, 100 ng) in 5 μL of PBS per eye to wild-type mice at 1, 3, and 7 hours after RW challenge. The quantified eosinophil numbers in conjunctivas of wild-type mice were shown. The data from the mice without both RW challenge and topical treatment and the mice without topical treatment were used as negative and positive controls, respectively. Data are shown as the means ± SEMs of samples from all mice examined. Positive control (RW sensitization with challenge), n = 19; negative control (RW sensitization without challenge), n = 19. AE248: 0.01 ng, n = 18; 0.1 ng, n = 6; 1 ng, n = 27; 10 ng, n = 6; 100 ng, n = 17.

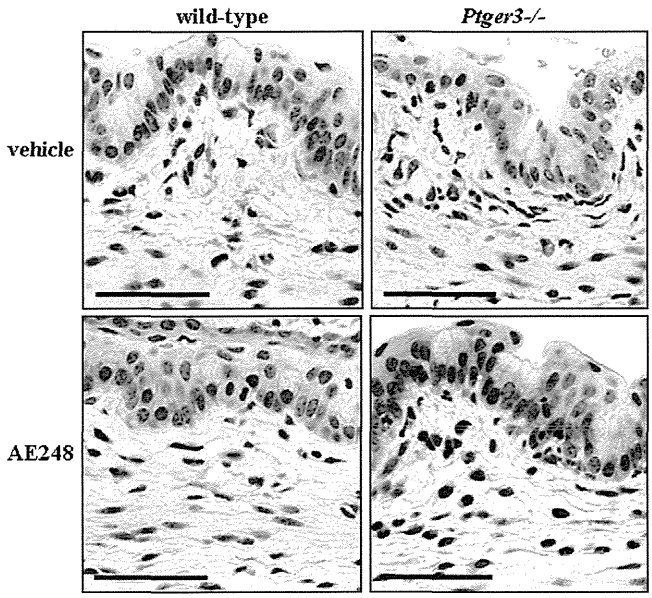

FIG E3. Infiltration of eosinophils into the conjunctiva treated with an EP3-selective agonist. Infiltrations of eosinophils were detected by using the Luna method, which stained eosinophil granules with a distinctive red. *Scale bars* = 50 μm.