ベタメタゾン点眼で開始し、結膜切除術と角膜上皮形成術を施行。術後もベタメタゾン点眼を使用していたが、角膜菲薄化の進行が抑えられず、術後2週間目にシクロスポリン点眼を追加した。しかし術後 1 ヶ月目より、移植した lenticule が融解し、角膜輪部の菲薄化が認められたため、プレドニゾロン内服を追加した。しかし、その後も菲薄化と lenticule の融解が進行したため。術後2ヶ月目に自家作製タクロリムス眼軟膏（左眼1日3回）を追加した。その後、増悪傾向が抑えられ、ステロイド内服の漸減終了が可能となった。しかし、タクロリムス眼軟膏使用後に眼灼熱感と霧視の訴えがあり、また軟膏であるため使用感が悪く、次第にコンプライアンス不良となり、タクロリムス眼軟膏の継続が難しい状況となった。そのため加療を弱めると浸潤が悪化する状況が続き、潰瘍が4分の3周を越える状態となった。更に、左眼鼻下側の菲薄化が悪化し、侵入した結膜が角膜を蚕食する状態となったため、再度結膜切除術と角膜上皮形成術を施行した。術後は副腎皮質ステロイド薬の点眼・内服、シクロスポリン点眼を行い、強く消炎を図った。しかし、小浸潤の出没を認め、血管の勢いを抑制できないため、タクロリムス点眼（左眼1日2回）を追加した。タクロリムス点眼も点眼後の灼熱感の訴えがあったが、タクロリムス眼軟膏より使用感がよくなったことでコンプライアンスも改善した。これでようやく鎮静化し、プレドニゾロン内服・シクロスポリン点眼の漸減・終了が可能となった。

D. 考察

タクロリムスの作用機序は、T 細胞抗原受容体からの刺激伝達経路中のカルシニューリンの脱リン酸化を阻害することで、サイトカイン産生の抑制、細胞障害性 T 細胞の誘導の阻害、肥満細胞の脱顆粒の阻害などをもたらす。免疫抑制薬のシクロスポリンとタクロリムスを比較すると、2つの作用機序は同じで、免疫抑制効果は、タクロリムスがシクロスポリンの 10 から 100 倍あるとされている。タクロリムス眼軟膏や点眼は春季カタルでの有効性が報告されており、特にステロイドレスポンダーにおいては、ステロイド減量が可能であり、眼圧の下降が得られたと報告されている。タクロリムス局所投与は高用量の副腎皮質ステロイドによる加療あるいは外科的治療が考慮される症例における代替療法となり得ると考えられた。

タクロリムス局所投与の副作用として、投与開始直後の灼熱感があげられる。しかしこの症状は一時的であることが多く、連用し炎症が改善するにつれ、減少する傾向にある。春季カタルの治療にタクロリムスを眼軟膏として使用した例でも、1-2 週間以内にこの症状は消失したとの報告もある。本症例でもこの副作用の訴えはあり、加えてその使用感の悪さが影響してコンプライアンスが悪化した。タクロリムス点眼については春季カタルの治療薬として開発され、副作用に目の灼熱感や異物感が認められるが、多くはこれも一過性である。タクロリムスを眼軟膏として使用するよりも、使用方法が簡便になり、何よりも眼軟膏使用後の霧視が改善されることにより、本患者のコンプライアンスはある程度改善され、所見の改善が得られた。

今回の症例によって、コンプライアンスについては更なる改善が必要ながら、タクロリムス局所投与が特発性周辺部角膜潰瘍でも有効なことが実証された。今後は更に症例数を増やして検討したい。

E. 結論

特発性周辺部角膜潰瘍治療におけるタクロリムス局所投与の有効性が示唆された。眼軟膏よりもコンプライアンスのよい点眼の形がより適していると考えられた。

F. 健康危険情報

なし

G. 研究発表

論文発表

1. 唐下千寿、川口亜佐子、宮崎大、井上幸次、春木智子、佐々木慎一、縄田信彦：タクロリムスが奏効した難治性モーレン潰瘍の１例. 臨床眼科,67(7):in press,2013

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1. 唐下千寿、川口亜佐子、宮崎大、井上幸次、春木智子、佐々木慎一、縄田信彦：タクロリムスが奏効した難治性モーレン潰瘍の１例. 第 66 回日本臨床眼科学会, 京都, 2012.10.25-10/28

H. 知的所有権の取得状況

特許取得

なし

実用新案登録

なし

その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

特発性周辺部角膜潰瘍における角膜形状解析および視機能の評価

研究分担者　西田　幸二　　大阪大学眼科学　教授

研究要旨　特発性周辺部角膜潰瘍の診断基準（案）に基づいて診断した特発性周辺部角膜潰瘍症例について、前眼部光干渉断層計を用いて角膜形状解析を行い、その特徴と視機能との関連を評価した。C 字型が 9 眼（60%）、カニ爪型が 4 眼(26.7%)、分類不能型が 2 眼（13.2%）であり、C 字型と比較し、カニ爪型では病変の範囲が 180°を超えるものが有意に多かった(p=0.025, Chi-square test)。病変が角膜中心に近く、病変範囲が 180°以上と広いものが、カニ爪型の Axial power map を来す傾向にあり、病変距離と logMAR 視力に負の相関を認めた。

A.　研究目的

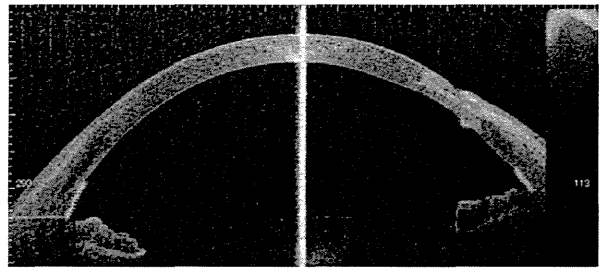
特発性周辺部角膜潰瘍（以下　Mooren 潰瘍）は、高度の充血、結膜浮腫に加えて、輪部に沿って炎症細胞浸潤を伴う円弧状の潰瘍を呈する難治な炎症性疾患である。昨年、我々は前眼部光干渉断層計（ASOCT）を用いて経過観察を行った、特発性周辺部角膜潰瘍の重症例 1 例について報告した。これまでに Mooren 潰瘍の角膜形状解析と視機能の評価を行った報告はなく、今回、Mooren 潰瘍患者を対象として ASOCT を用いて角膜形状解析を行い、その特徴と視機能との関連を評価したので報告する。

B.　研究方法

本研究は研究に関する倫理指針に従い、大学倫理審査委員会の承認を得て行った。

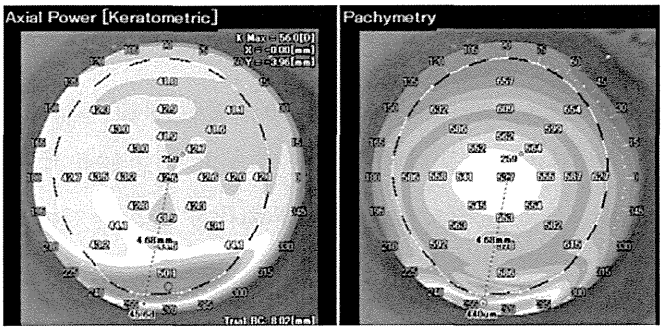
対象：2010 年 9 月～2012 年 11 月に大阪大学眼科を受診し、Mooren 潰瘍と診断された 10 例 15 眼（男性 5 例女性 5 例、平均年齢 62.3±3.9 歳）。高度の結膜侵入を来している症例および外科的治療が施された後の症例は除外した。

方法：前眼部光干渉断層計(CASIA,TOMEY 社)を用いて角膜断層像を最大 16mm 径で撮影した(16 スキャン)。



角膜形状解析：

得られたデータから Axial power map および Pachymetry map を作成し、角膜中心から病変最菲薄部までの距離を病変距離と定義し測定した。



病変範囲はスリット所見で判断した。

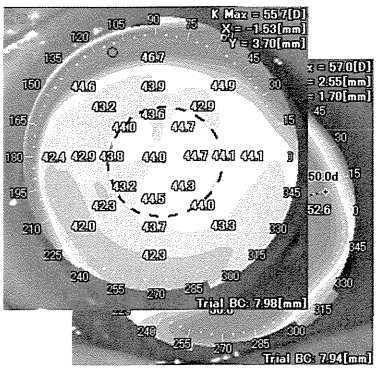
また、CASIA 搭載の解析ソフトを用いて角膜中心 3mm 領域での Fourier 解析を行った。

C. 研究結果

1 ：Axial power map pattern 分類

C 字型が 9 眼 (60%)、カニ爪型が 4 眼(26.7%)、分類不能型が 2 眼（13.2%）であった。C 字型とカニ爪型の代表症例を示す。 v

C 字型　　**カニ爪型**



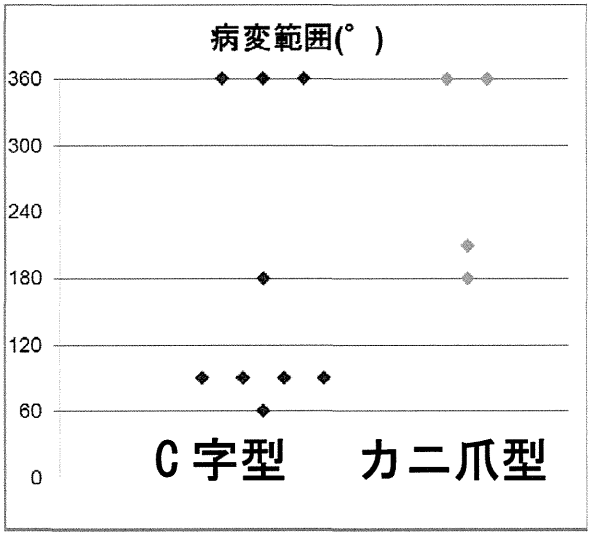
2－1 ：C 字型とカニ爪型の比較

	C字型	カニ爪型	p値
病変距離(mm)	4.747±0.117	3.648±0.358	0.007
最菲薄部角膜厚(μm)	454.2±23.63	338.3±53.23	0.076
病変範囲(°)	186.7±44.66	277.5±48.02	0.197
LogMAR (角膜以外の病変例除く)	0.007±0.064	0.496±0.451	0.115
球面成分(D)	43.40±0.484	46.50±1.856	0.143
非対称成分(D)	0.478±0.095	2.163±0.746	0.017
正乱視成分(D)	0.449±0.150	2.473±0.600	0.011
HOI (D)	0.232±0.032	0.545±0.147	0.030

・病変距離はカニ爪型が C 字型よりも有意に近位であった。

・角膜中心 3mm 領域における非対称成分、正乱視成分、HOI はカニ爪型が C 字型を有意に上回っていた。

2－2 ：C 字型とカニ爪型での病変範囲の比較

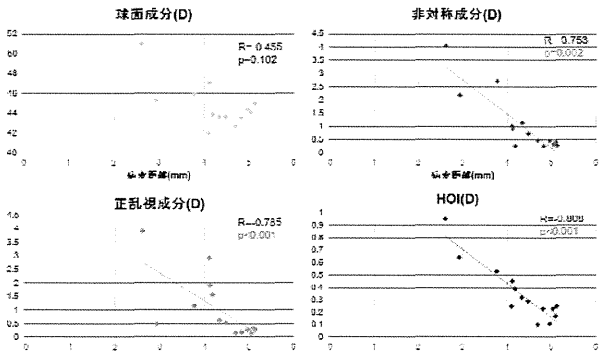


・C 字型と比較し、カニ爪型では病変の範囲が 180°を超えるものが有意に多かった(p=0.025, Chi-square test)。

・C 字型の病変範囲は 60°から 360°に至るまで

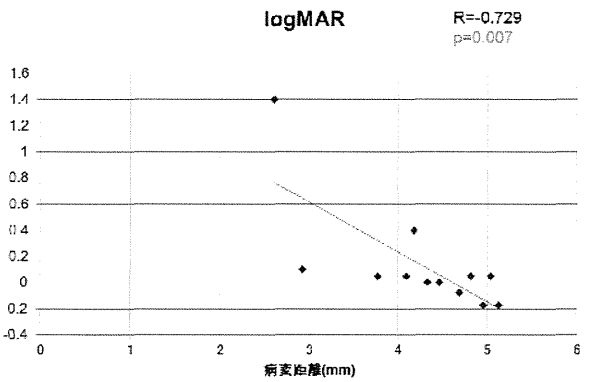
分散していた。

３－１：病変距離と屈折成分



•病変距離と角膜中心3mm領域の非対称成分、正乱視成分、HOIはいずれも有意な負の相関を示した。

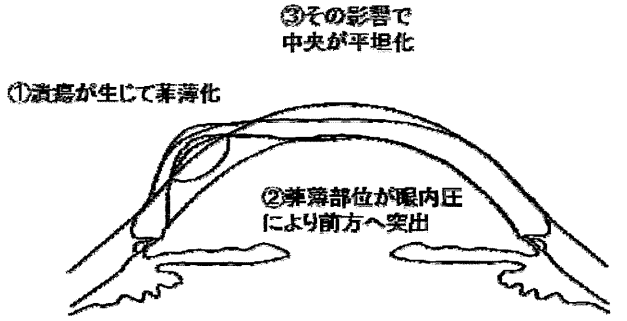
３－２：病変距離と logMAR(角膜以外の病変例除く)



・角膜以外に病変を有さない 12 眼において、病変距離と logMAR 視力に負の相関を認めた。

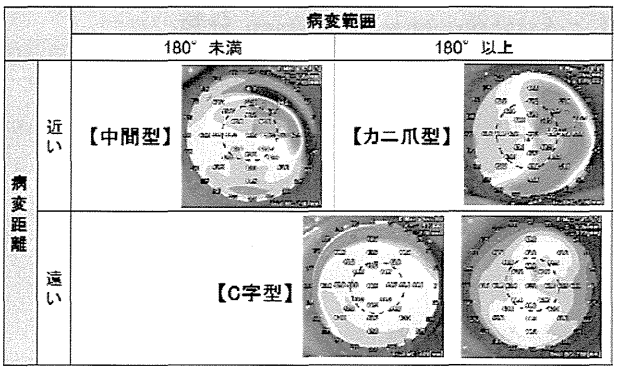
D. 考察

カニ爪型を来たす機序について以下に示す。



Mooren 潰瘍も Pellucid 角膜変性同様、菲薄部位が眼圧により前方へと突出して急峻化するが、その影響で中央部が平坦化することでカニ爪型を呈すると考えられた。今回の結果、病変が角膜中心に近く、病変範囲が 180°以上と広いものが、カニ爪型の Axial power map を来す傾向にあった。

Axial power map pattern についてまとめると以下のようになる。



最周辺部に存在すると測定範囲内の屈折力の影響が殆ど無くなり、乱視も少なく正常眼の pattern の map を示す。

ASOCT を用いて非侵襲的に Mooren 潰瘍の角膜・前眼部形状を評価することにより、視機能評価すること可能であった。今後、治療効果の判定を視機能もふまえて行うことが可能である。

E. 結論

Mooren 潰瘍は周辺部に病変を来たすことが多く、比較的視機能良好なものが多いが、病変が角膜中心に近い場合はカニ爪型の Axial power map を示すものがあり、乱視成分が増加し、視機能は低下する。

F．研究発表

論文発表　なし

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著書・総説　なし

G. 知的所有権の取得状況

特許取得

なし

実用新案登録

なし

その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

特発性周辺部角膜潰瘍の病態解析

研究分担者　上田　真由美　　同志社大学生命医科学部　准教授

研究要旨　特発性周辺部角膜潰瘍は、全身疾患を伴うことなく角膜周辺部に潰瘍を形成する難治性疾患である。その発症機序ならびに病態は明らかとなっていない。ステロイド、免疫抑制剤による保存療法がある程度有用であるが、これらの保存的治療が奏功しない重症例では、結膜切除術や角膜上皮形成術などの外科治療を必要とする。本研究では、手術時に切除される病変部結膜組織を解析し本症の病態解明を行った。まず、病変部結膜に浸潤する炎症細胞に着目し、免疫組織学的に解析し、病変部結膜組織に浸潤する免疫細胞はhelper T 細胞ならびにマクロファージが主体であることを明らかにした。さらに、近年、炎症に関与することが明らかとなってきた上皮細胞に着目し免疫組織学的に解析を行った。上皮細胞に発現し炎症を負に制御する因子プロスタグランジンE2 受容体サブタイプ EP3 が、瘢痕性眼表面炎症疾患では発現が著しく減弱しているのにもかかわらず、本疾患ではしっかり発現していることを明らかとした。さらに、プロスタグランジン E2 受容体サブタイプ EP4 においては、全症例ではないが、本疾患の一部の症例では発現が減少していることも明らかとなった。
これらの結果より、特発性周辺部角膜潰瘍の病態に免疫細胞を主体とする自己免疫性の反応が強く関与しているが可能性が示唆された。

A. 研究目的

特発性周辺部角膜潰瘍（Mooren 潰瘍）は、全身疾患を伴うことなく角膜周辺部に潰瘍を形成する難治性疾患である。角膜抗原性への自己免疫の関与(IOVS, 1995)、C型肝炎との関連（Eye, 2004)、寄生虫感染の関与（BJO, 1983）等を示唆する報告があるが、その発症機序ならびに病態は明らかでない。ステロイド、免疫抑制剤による保存療法がある程度有用であるが、保存的治療が奏功しない重症例では、結膜切除術などの外科治療が必要となる。本研究では、手術時に切除される病変部結膜組織を解析し本症の病態解明を行った。まず、病変部結膜に浸潤する炎症細胞に着目し解析を行い、次に、近年、炎症に関与することが明らかとなってきた上皮細胞に着目し解析を行ったので報告する。

B. 研究方法

対象は京都府立医科大学眼科で特発性周辺部角膜潰瘍の診断にて外科的治療を行った４例４眼で、手術時に採取された結膜組織を用いた。凍結包埋後薄切した結膜を用いて各種の免疫染色を行った。手術で採取された結膜弛緩症の結膜組織ならびにその他の眼表面疾患である

Stevens-Johnson 症候群や眼類天疱瘡、角膜化学外傷、翼状片の結膜組織と比較解析した。

① 病変部結膜に浸潤する炎症細胞の解析

抗 CD3、CD4、CD8、CD20cy、CD45RO、Mast cell tryptase、Neutrophil erastase、CD68 抗体を用いて免疫染色を行い、病変部結膜に浸潤する炎症細胞が、T 細胞、B 細胞、肥満細胞、好中球、マクロファージのいずれであるかを解析した。

② 病変部結膜組織におけるﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体ｻﾌﾞﾀｲﾌﾟ EP3 の発現の解析

我々は、眼類天疱瘡や Stevens-Johnson 症候群などの重症瘢痕性角結膜上皮症では、正常の結膜上皮では発現している EP3 が著しく減弱していることを報告している。本研究では、特発性周辺部角膜潰瘍の病変結膜組織における EP3 の発現を解析した。

③ ﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体ｻﾌﾞﾀｲﾌﾟ EP4 の病変部結膜組織における発現の解析

我々は、腸炎を抑制していると報告のあるﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体のもう一つのｻﾌﾞﾀｲﾌﾟ EP4 についても、特発性周辺部角膜潰瘍の病変結膜組織における発現を解析した。

（倫理面への配慮）

本研究は厚生労働省による臨床研究に関する倫理指針および疫学研究に関する倫理指針に従い、大学倫理審査委員会の承認を得て行った。また患者由来の試料はすべて、インフォームドコンセントを得たうえで採取し、本研究に用いた。

C. 研究結果

① 病変部結膜に浸潤する炎症細胞の解析

結膜下には T リンパ球を主体とする細胞浸潤を認めた。一方、CD68 陽性のマクロファージも多く観察された。肥満細胞や好中球は少数観察されたのみであった。

② 病変部結膜組織におけるﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体ｻﾌﾞﾀｲﾌﾟ EP3 の発現の解析

重症瘢痕性角結膜上皮症では、結膜上皮細胞における EP3 の発現が著しく減弱しているのとは対照的に、モーレン潰瘍の病変部結膜組織では、正常結膜組織と同様に EP3 の発現が確認された。

③ ﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体ｻﾌﾞﾀｲﾌﾟ EP4 の病変部結膜組織における発現の解析

病変結膜組織を、抗 EP4 抗体で染色したところ、全症例ではないが一部の症例において発現の減弱が確認された。

D. 結論

眼類天疱瘡や Stevens-Johnson 症候群などの重症瘢痕性角結膜上皮症では、結膜上皮細胞におけるﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体ｻﾌﾞﾀｲﾌ EP3 ならびに EP4 の発現の減少がその病態大きく関与しているのとは対照的に、特発性周辺部角膜潰瘍の病態には、結膜上皮細胞、特にﾌﾟﾛｽﾀｸﾞﾗﾝｼﾞﾝ E 受容体は関与していないことが示唆された。また、結膜組織内の炎症細胞が T 細胞やマクロファージが主体であったことより、今まで報告されているように、免疫細胞を主体とする自己免疫性の反応がその病態に強く関与していると考えられた。

E. 研究発表

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   角膜カンファランス 2013（第 37 回日本角膜学会総会・第 29 回日本角膜移植学会）和歌山, 2013.2.15.

著書・総説

なし

F. 知的所有権の取得状況

特許取得

なし

実用新案登録

なし

その他

なし

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ueta,M, Sotozono C, Yokoi N, Kinoshita S	Downregulation of monocyte chemoattractant protein 1 expression by prostaglandin e2 in human ocular surface epithelium.	Arch Ophthalmol	130(2)	249-251	2012
Ueta,M, Matsuoka T, Sotozono C, Kinoshita S	Prostaglandin E2 Suppresses Poly I: C-Stimulated Cytokine Production Via EP2 and EP3 in Immortalized Human Corneal Epithelial Cells.	Cornea	31(11)	1294-1298	2012
Hata M, Nakamura T, Sotozono C, Kumagai K, Kinoshita S, Kurimoto Y	Atypical Continuous Keratitis in a Case of Rheumatoid Arthritis Accompanying Severe Scleritis.	Cornea	31(12)	1493-1496	2012
Ueta M, Sotozono C, Yamada K, Yokoi N, Inatomi T, Kinoshita S	Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders: case-control study.	BMJ Open	2(5)	e001330	2012
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唐下千寿、川口亜佐子、宮崎大、井上幸次、春木智子、佐々木慎一、縄田信彦	タクロリムスが奏効した難治性モーレン潰瘍の１例	臨床眼科	67(7)	In press	2013

IV. 研究成果の刊行物・別刷

RESEARCH LETTERS

Downregulation of Monocyte Chemoattractant Protein 1 Expression by Prostaglandin E_2 in Human Ocular Surface Epithelium

Elsewhere, we reported that in the tears and serum of patients with acute-stage Stevens-Johnson syndrome or toxic epidermal necrolysis, the levels of interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) were dramatically increased.[1] We also reported that Stevens-Johnson syndrome or toxic epidermal necrolysis with severe ocular complications was associated with polymorphism of the prostaglandin E receptor 3 (EP_3) gene (*PTGER3*).[2]

Prostanoids are a group of lipid mediators that form in response to various stimuli. They include prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2. There are 4 subtypes of the PGE receptor: EP_1, EP_2, EP_3, and EP_4. We previously reported that PGE_2 suppresses polyinosine–polycytidylic acid (polyI:C)–stimulated cytokine production via EP_2 and/or EP_3 in human ocular surface epithelial cells.[3,4] PolyI:C is a ligand of Toll-like receptor 3, which is strongly expressed in ocular surface epithelium.[5] We found that PGE_2 suppresses the production of IL-6, chemokine (C-X-C motif) ligand 10, chemokine (C-X-C motif) ligand 11, and chemokine (C-C motif) ligand 5 but not IL-8 by epithelial cells on the human ocular surface[3]; it remains to be determined whether it also suppresses MCP-1 production. Monocyte chemoattractant protein 1 plays a significant role in the recruitment of monocytes and lymphocytes to the site of cellular immune reactions. In this study, we investigated whether PGE_2 downregulates polyI:C-induced MCP-1 production.

All experiments were conducted in accordance with the principles set forth in the Declaration of Helsinki. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction were performed with primary human conjunctival epithelial cells and immortalized human corneal-limbal epithelial cells using previously described methods (eAppendix, http://www.archophthalmol.com).[3]

First, we examined whether PGE_2 downregulated the production and messenger RNA (mRNA) expression of MCP-1 induced by polyI:C stimulation in human conjunctival and corneal epithelial cells. We found that it significantly attenuated the production of MCP-1 (**Figure**, A). Quantitative real-time polymerase chain reaction confirmed that the mRNA expression of MCP-1 was significantly downregulated by PGE_2 (Figure, A).

Next, we examined which PGE_2 receptor(s) contributed to the downregulation of polyI:C-induced MCP-1. We used the EP_2 agonist ONO-AE-259, the EP_3 agonist ONO-AE-248, and the EP_4 agonist ONO-AE-329. Enzyme-linked immunosorbent assay showed that the EP_2 and EP_3 agonists significantly suppressed the polyI:C-induced production of MCP-1, while the EP_4 agonist did not exert suppression (Figure, B). Quantitative real-time polymerase chain reaction confirmed that the EP_2 and EP_3 agonists significantly downregulated the mRNA expression of MCP-1 (Figure, C). Thus, our results document that PGE_2 attenuated the mRNA expression and production of MCP-1 via both EP_2 and EP_3.

In human macrophages, PGE_2 attenuated the lipopolysaccharide-induced mRNA and protein expression of chemokines including MCP-1 through EP_4.[6] On the other hand, we demonstrated that in human ocular surface epithelial cells, PGE_2 attenuated the polyI:C-induced mRNA and protein expression of MCP-1 through EP_2 and EP_3 but not EP_4. Our findings suggest that EP_2 and EP_3 play important roles in the regulation of inflammation in epithelial cells, while EP_2 and EP_4 have important roles in immune cells such as macrophages.

In the tears and serum of patients with acute-stage Stevens-Johnson syndrome or toxic epidermal necrolysis, the levels of IL-6, IL-8, and MCP-1 were dramatically increased.[1] Although IL-8 was not regulated by PGE_2, IL-6 was regulated by PGE_2 via EP_3 in human ocular surface epithelial cells.[3] Herein, we demonstrated that MCP-1 could be regulated by PGE_2 via EP_2 and EP_3. The regulation of cytokine production by PGE_2 may be associated with the pathogenesis of Stevens-Johnson syndrome or toxic epidermal necrolysis with severe ocular complications because it was associated with polymorphism of the EP_3 gene (*PTGER3*), one of the PGE receptors (EP_1, EP_2, EP_3, EP_4).[2]

In summary, our results show that MCP-1 produced by human ocular surface epithelial cells could be downregulated by PGE_2 via EP_2 and EP_3.

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Correspondence: Dr Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Ka-

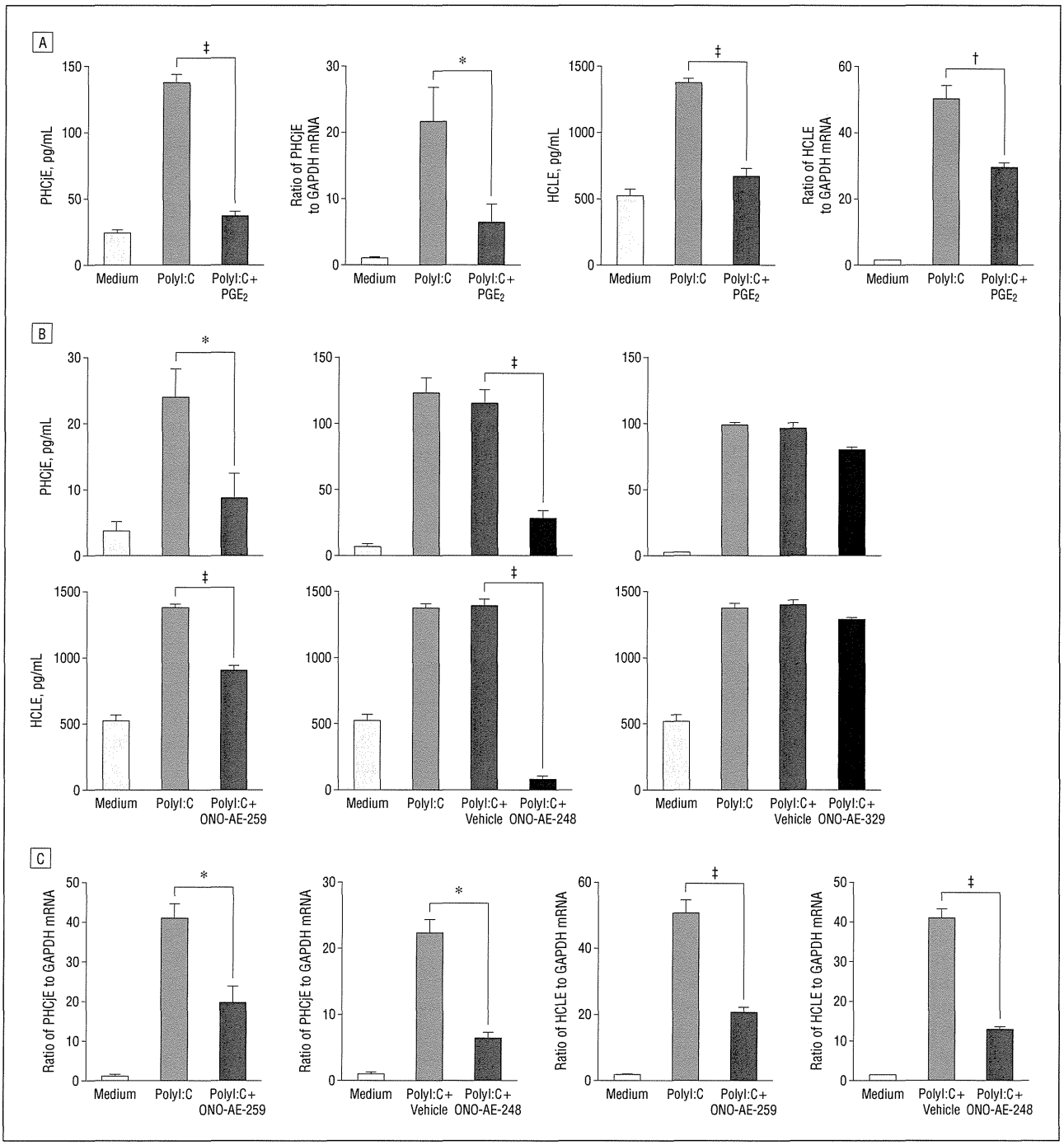

Figure. Prostaglandin E_2 (PGE_2) attenuated the messenger RNA (mRNA) expression and production of monocyte chemoattractant protein 1 via both prostaglandin E receptor 2 (EP_2) and EP_3. A, Primary human conjunctival epithelial cells (PHCjE) and human corneal-limbal epithelial cells (HCLE) were exposed to 10 µg/mL of polyinosine–polycytidylic acid (poly I:C) and 100 µg/mL of PGE_2 for 24 hours (enzyme-linked immunosorbent assay) or 6 hours (quantitative real-time polymerase chain reaction). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase. B and C, The PHCjE and HCLE were exposed to 10 µg/mL of poly I:C and 10 µg/mL of the EP_2, EP_3, or EP_4 agonist for 24 hours (enzyme-linked immunosorbent assay) (B) or 6 hours (quantitative real-time polymerase chain reaction) (C). Data are representative of 3 separate experiments and are given as the mean (SEM) from 1 experiment carried out in 6 to 8 wells (enzyme-linked immunosorbent assay) (B) or 4 to 6 wells (quantitative real-time polymerase chain reaction) (C) per group. *$P<.05$; †$P<.005$; ‡$P<.001$.

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Author Contributions: Dr Ueta had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: The work described in this article was carried out in collaboration with Ono Pharmaceutical Co Ltd, who supplied ONO-AE-248 used in this study.

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Shimizu Foundation for Immunological Research Grant.
Online-Only Material: The eAppendix is available at http://www.archophthalmol.com.
Additional Contributions: Chikako Endo provided technical assistance.

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Depth Profile Study of Abnormal Collagen Orientation in Keratoconus Corneas

In a previous study,[1] we used femtosecond laser technology to cut ex vivo human corneas into anterior, mid, and posterior sections, after which x-ray scatter patterns were obtained at fine intervals over each specimen. Data analysis revealed the predominant orientation of collagen at each sampling site, which was assembled to show the variation in collagen orientation between central and peripheral regions of the cornea and as a function of tissue depth. We hypothesized that the predominantly orthogonal arrangement of collagen (directed toward opposing sets of rectus muscles) in the mid and posterior stroma may help to distribute strain in the cornea by allowing it to withstand the pull of the extraocular muscles. It was also suggested that the more isotropic arrangement in the anterior stroma may play a role in tissue biomechanics by resisting intraocular pressure while at the same time maintaining corneal curvature. This article, in conjunction with our findings of abnormal collagen orientation in full-thickness keratoconus corneas,[2,3] received a great deal of interest from the scientific community and prompted the following question: how does collagen orientation change as a function of tissue depth when the anterior curvature of the cornea is abnormal, as in keratoconus? Herein, we report findings from our investigation aimed at answering this question.

Methods. The Baron chamber used in our previous study[1] was adapted to enable corneal buttons to be clamped in place and inflated (by pumping physiological saline into the posterior compartment) to restore their natural curvature. A button diameter of 8 mm or larger was deemed necessary to ensure tissue stability during this process.

The next step, obtaining fresh, full-thickness, keratoconus buttons of sufficient diameter, proved to be problematic owing to the increasing popularity of deep anterior lamellar keratoplasty. Recently, however, the opportunity arose to examine an 8-mm full-thickness (300-340 μm minus epithelium) keratoconus corneal button with some central scarring and a mean power greater than 51.8 diopters (**Figure 1**). The tissue was obtained in accordance with the tenets of the Declaration of Helsinki and with full informed consent from a 31-year-old patient at the time of penetrating keratoplasty. Using techniques detailed previously,[1] the corneal button was clamped in the chamber and inflated. The central 6.3-mm region of the button was then flattened by the applanation cone and a single cut was made at a depth of 150 μm from the surface using an IntraLase 60-kHz femtosecond laser (Abbott Medical Optics Inc),[1] thus splitting the cornea into anterior and posterior sections of roughly equal thickness. Wide-angle x-ray scattering patterns were collected at 0.25-mm intervals over each cor-

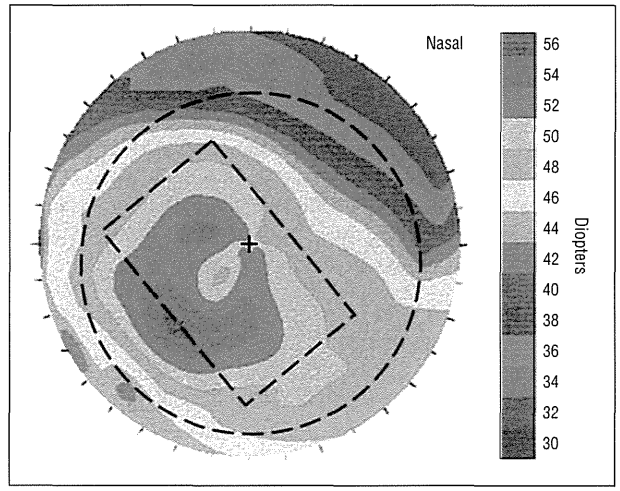

Figure 1. Corneal topography of the keratoconus cornea (recorded 12 years previously).[3] The broken lines show the 6.3-mm region of the cornea cut with the femtosecond laser (circle) and the region of greatest corneal steepening depicted in Figure 2 (rectangle).

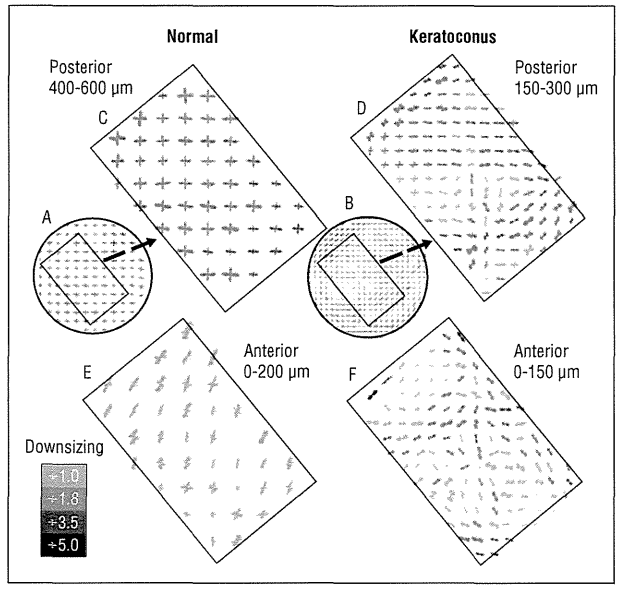

Figure 2. Collagen orientation in the normal (A) and keratoconus (B) posterior stroma (central 6.3 mm). The highlighted regions of the posterior (C and D) and anterior (E and F) stroma are expanded. Large vector plots showing high collagen alignment are downsized (key).

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

(*Cornea* 2012;31:1294–1298)

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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The work described in the present article was carried out in collaboration with Ono Pharmaceutical Co, Ltd, who supplied ONO-AE-259, ONO-AE-248, and ONO-AE-329 used in this study. The authors have no other competing financial interests.

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.corneajrnl.com).

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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

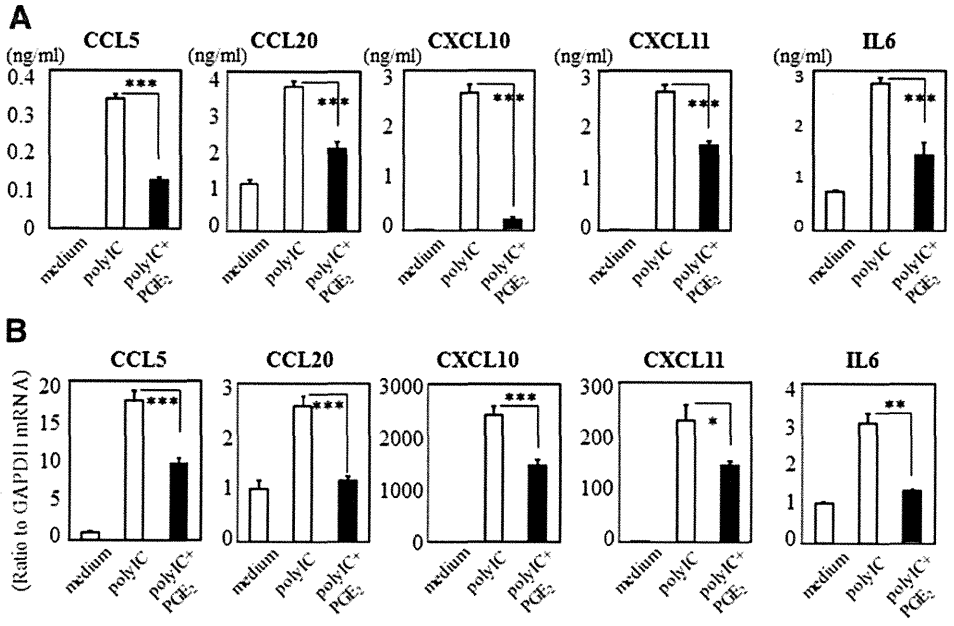

FIGURE 1. A, Suppression of the production of CCL5, CCl20, CXCL10, CXCL11, and IL-6 by PGE_2. HCLE were exposed to 10 μg/mL poly I:C and 100 μg/mL PGE_2 for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group. B, Suppression of mRNA expression of CCL5, CCL20, CXCL10, CXCL11, and IL-6 by PGE_2. HCLE were exposed to 10 μg/mL poly I:C and 100 μg/mL PGE_2 for 6 hours. The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. The *y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

lengths were obtained for EP2 (683 bp), EP3 (622 bp), and EP4 (956 bp) (Fig. 2), but not for EP1 (723 bp) (data not shown), from HCLE and *in vivo* human corneal epithelial cells, suggesting that the human corneal epithelium expresses EP2, EP3, and EP4 mRNAs. To confirm the specificity for the detection of EP2-, EP3-, and EP4 mRNA, we isolated and sequenced the PCR products. The obtained sequences were identical to the human EP2-, EP3-, and EP4 cDNA sequences. Moreover, we could detect EP2, EP3 and EP4 proteins using immunoblotting (see **Figure, Supplemental Digital Content 1**, http://links.lww.com/ICO/A42).

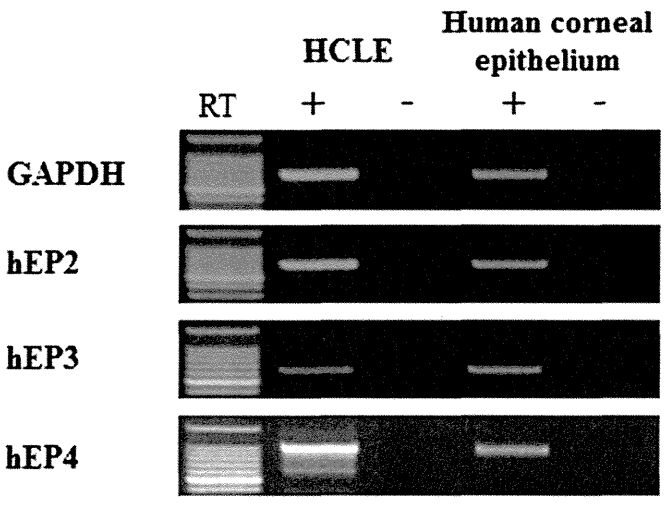

FIGURE 2. mRNA expression of the PGE_2 receptors EP2, EP3, and EP4. RT-PCR assay of the expression of PGE_2 receptor EP2, EP3, and EP4-specific mRNA in HLCE and human corneal epithelium. RT identifies data that were obtained without reverse transcription (controls).

EP2 and EP3, but not EP4 Agonists Downregulated the Production of Cytokines Induced by Poly I:C Stimulation

Using the EP2, EP3, and EP4 agonists, ONO-AE-259, ONO-AE-248, and ONO-AE-329, respectively, we also examined which PGE_2 receptor(s) contributed to their polyI:C-induced downregulation. HCLE were exposed to 10 μg/mL polyI:C and 10 μg/mL of the EP2, EP3, or EP4 agonist for 24 hours (ELISA) or 6 hours (quantitative RT-PCR). ELISA showed that the EP2 agonist significantly suppressed the polyI:C-induced production of CCL5, CXCL10, and CXCL11 (all, $P < 0.0005$) but not of CCL20 and IL-6, and that the EP3 agonist significantly suppressed the production of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (all, $P < 0.0005$). However, the EP4 agonist failed to suppress the cytokine production induced by polyI:C stimulation (Fig. 3). Quantitative RT-PCR confirmed that the EP2 agonist significantly downregulated the mRNA expression of CCL5, CXCL10, and CXCL11 (respectively, $P < 0.005$, $P < 0.0005$ and $P < 0.05$), but not of CCL20 and IL-6, and that the EP3 agonist significantly downregulated the mRNA expression of all examined cytokines (CCL5, $P < 0.05$; CCL20, $P < 0.005$; CXCL10, $P < 0.0005$; CXCL11, $P < 0.0005$; and IL-6, $P < 0{,}005$) (Fig. 4). Thus, our results show that PGE_2 attenuated the mRNA expression and production of CCL5, CXCL10, and CXCL11 via both EP2 and EP3, and that the CCL20 and IL-6 mRNA expression and production were attenuated only by EP3 in human corneal epithelial cells.

DISCUSSION

Lipid mediators like PGE_2 regulate immune and inflammatory responses by modulating the production of cytokines and chemokines.[11] In macrophages, PGE_2 suppressed the proinflammatory gene expression induced by LPS,

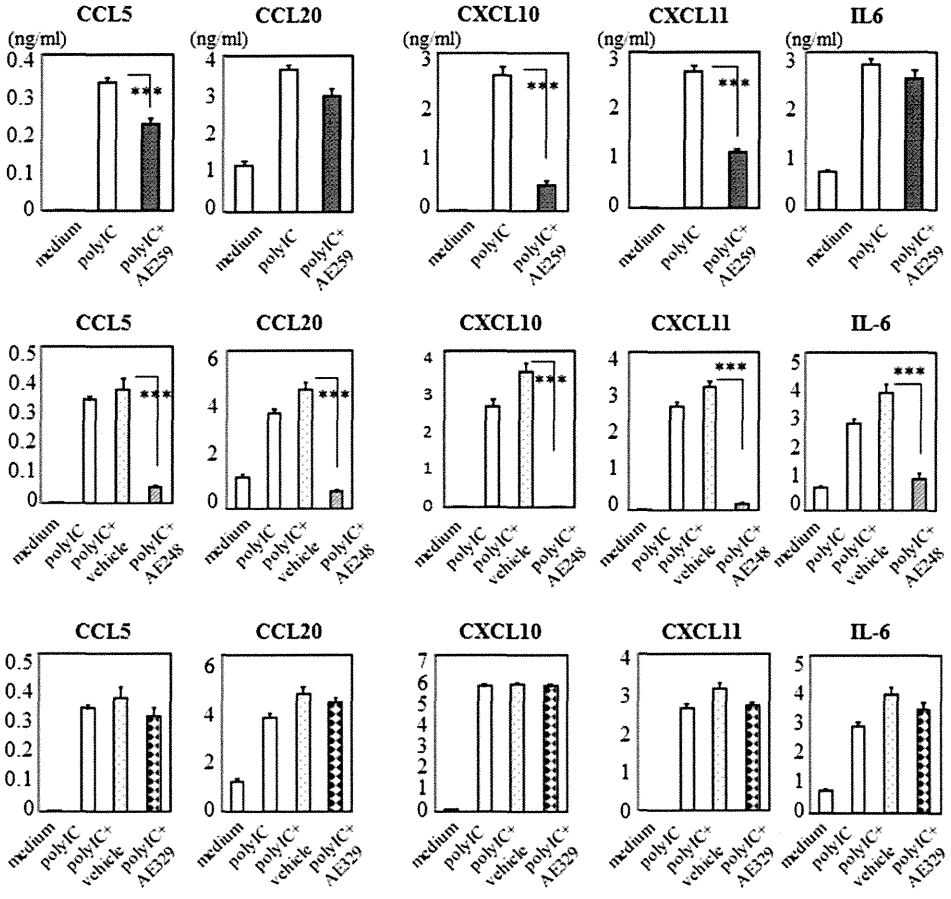

FIGURE 3. Effect of the PGE_2 receptors EP2, EP3, and EP4 on poly I:C-induced cytokine production. HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2, EP3, or EP4 agonist for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group (***$P < 0.0005$).

including macrophage inflammatory protein (MIP)-1α, MIP-1β, CCL5, CXCL10, and IL-8.[9] Here we document that PGE_2 modulates the expression and production of polyI:C-induced proinflammatory genes in not only human conjunctival epithelial cells but also corneal epithelial cells. It exerted an inhibitory effect on polyI:C-induced CCL5, CCL20, CXCL10, CXCL11, and IL-6 mRNAs (respectively, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$, $P < 0.05$ and $P < 0.005$) and on protein production in HCLE (all, $P < 0.0005$). PGE_2 exerts its biological actions by binding to EP located primarily on the plasma membrane. We confirmed the presence of the PGE_2 receptor subtypes, EP2,

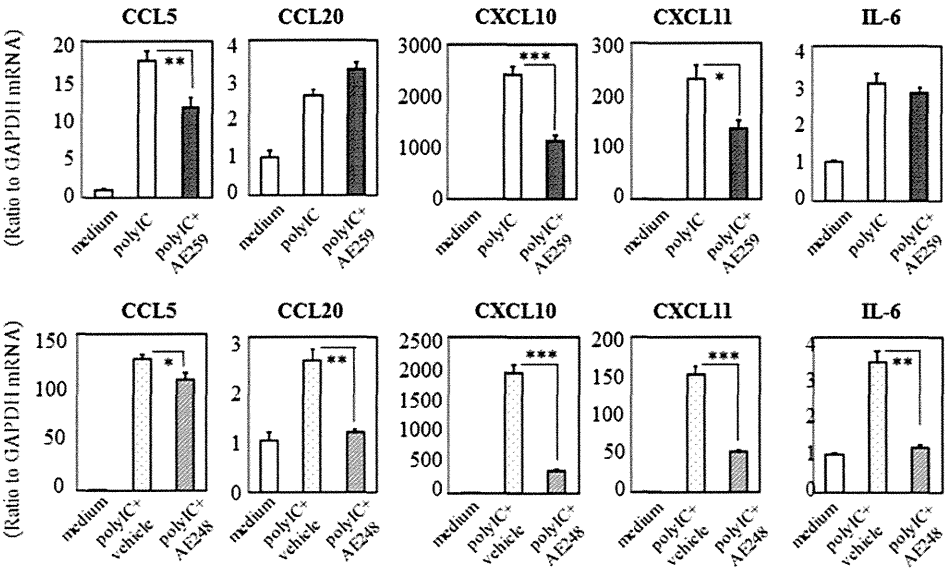

FIGURE 4. Effect of the PGE_2 receptors EP2 and EP3 on the poly I:C-induced mRNA expression of cytokines: HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2 or EP3 agonist for 6 hours. The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. The *y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

EP3, and EP4, in human corneal epithelial cells. Stimulation with either EP2- or EP3-specific agonists had a suppressive effect on polyI:C-induced CCL5, CXCL10, and CXCL11 production (both EP2- and EP3-specific agonists: all, $P < 0.0005$) , but only the EP3-specific agonist had a suppressive effect on the production of CCL20 and IL-6 (both, $P < 0.0005$).

Stimulation with PGE_2 exhibits immunosuppressive effects in various cell types including macrophages and dendritic cells via EP2 and/or EP4.[9–11] This phenomenon is explicable by the elevation of intracellular cyclic adenosine monophosphate (cAMP) via the activation of adenylcyclase.[9,10] Although PGE_2 acts on EP2 and EP4 and activates adenylcyclase, resulting in the elevation of intracellular cAMP, its action on EP3 suppresses adenylcyclase, resulting in a decrease in intracellular cAMP. In human conjunctival and corneal epithelial cells, both EP2 and EP3 contribute to the immunosuppressive effect against polyI:C stimulation; therefore, the suppressive effect cannot be explained by the elevation of intracellular cAMP. The precise molecular mechanisms underlying the immunosuppressive effects of PGE_2 in epithelial cells remain to be elucidated.

Release of PGE_2 is associated with ocular inflammation, but the exact role in inflammation has not been identified, rather PGE_2 might have been considered as inflammation-related molecules in the cornea. In this study, it is evident that PGE_2 could contribute to suppressing the production of various cytokines and chemokines in the ocular surface. Elsewhere we reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells and that it downregulates the progression of murine experimental allergic conjunctivitis,[7] suggesting the possibility of the PGE_2 and EP3 selective agonists as antiinflammatory drugs.

In summary, our results suggest that PGE_2 and its receptors in ocular surface (conjunctival and corneal) epithelium contribute to the regulation of ocular surface inflammation.

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