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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

Discussion

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

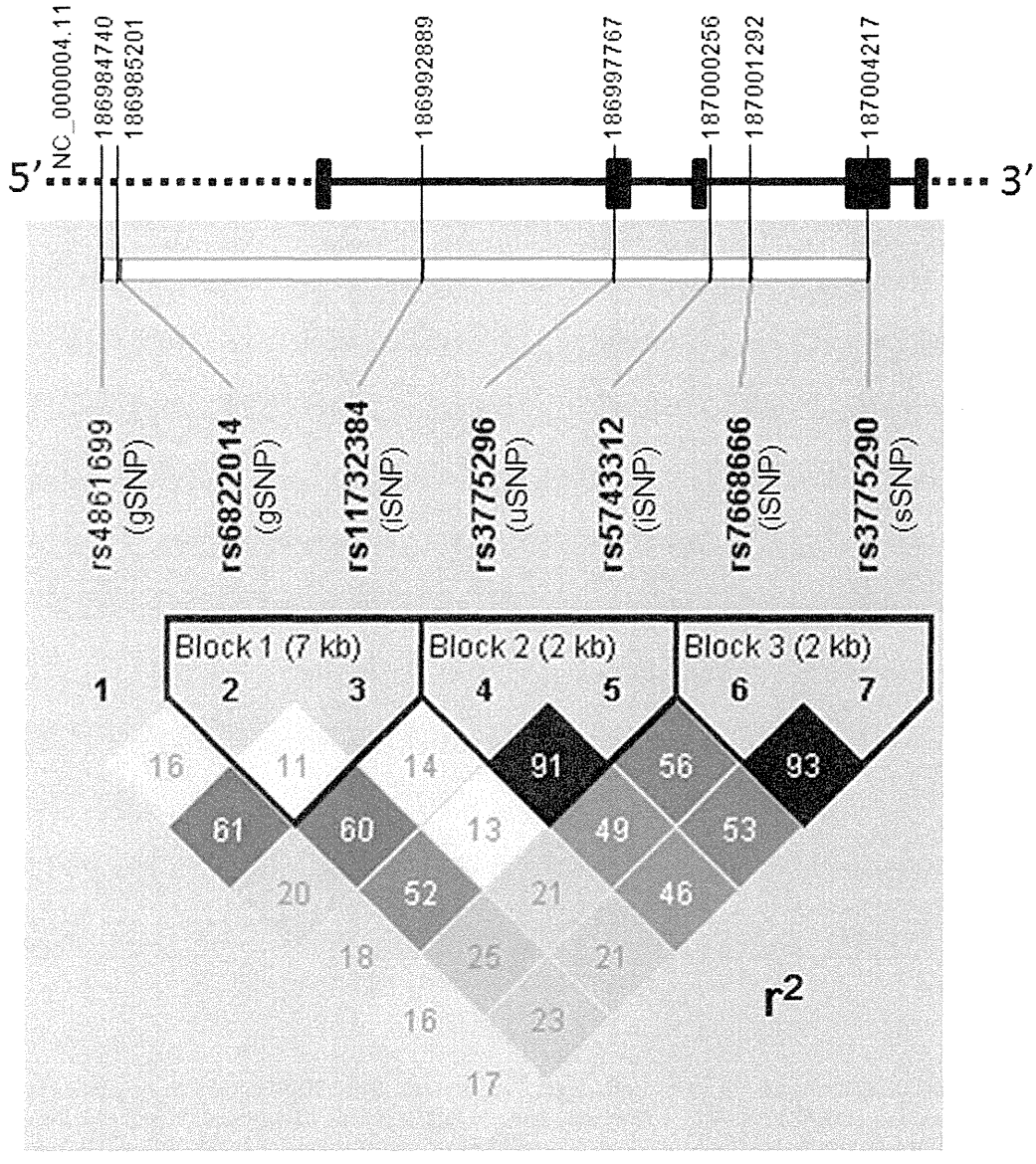

Figure 1. Linkage disequilibria among the 7 *TLR3* SNPs. Strong linkage disequilibrium was observed between rs.3775296 and rs.5743312, and between rs.7668666 and rs.3775290.
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with TLR3 rs.7668666, and TLR3 rs4861699G/G SNP exerted additive effects.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

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

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Patients

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

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

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

TLR3 SNPs Genotyping

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

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

HLA-A Genotyping

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

Statistical Analysis

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

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

Supporting Information

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

Author Contributions

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

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

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ABSTRACT

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

Design: Case-control study.

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

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

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

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

ARTICLE SUMMARY

Article focus

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

Key messages

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

Strengths and limitations of this study

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Human conjunctival tissues

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

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

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

Quantitative RT-PCR

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

Immunohistochemistry

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

Data analysis

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

RESULTS

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

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

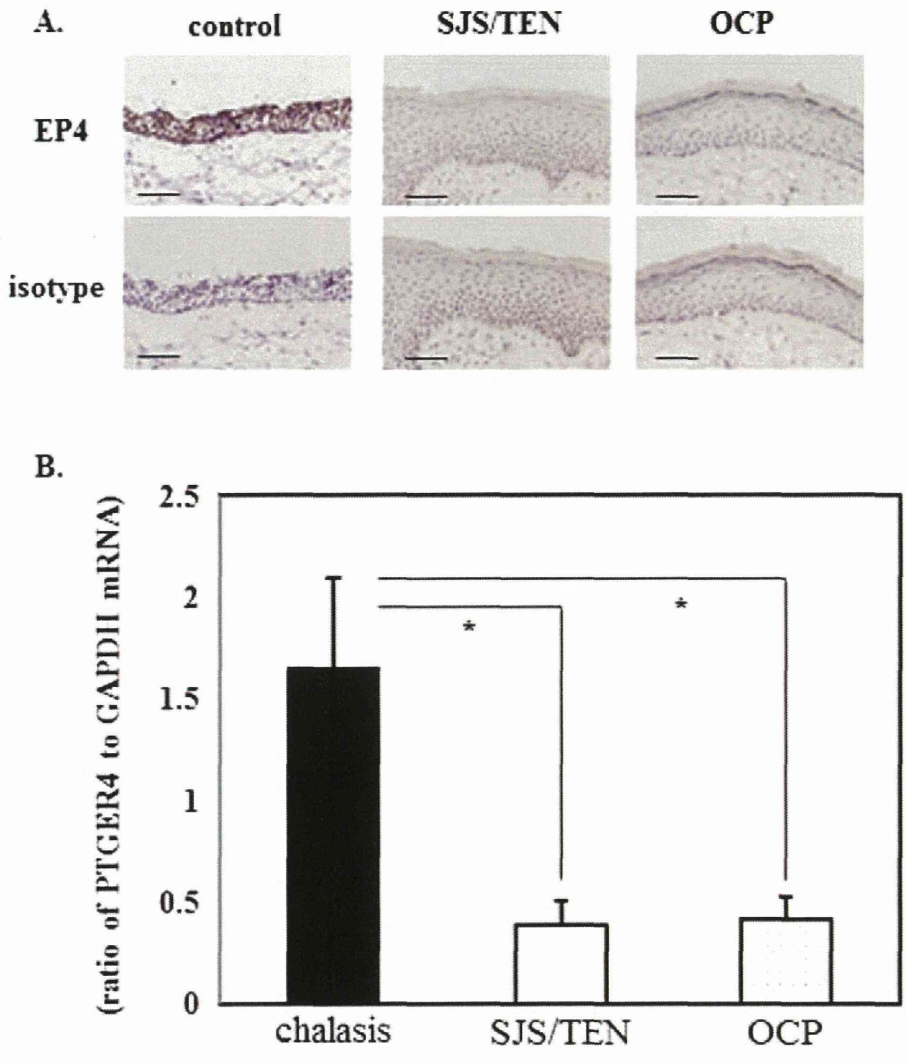

Figure 1 The expression of *PTGER4* mRNA in conjunctival tissues from patients with Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN), ocular cicatricial pemphigoid (OCP) and the controls. (A) Representative findings of EP4 immunoreactivity in each group (control, SJS/TEN, OCP). (B) Expression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

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

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

DISCUSSION

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

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

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

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

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

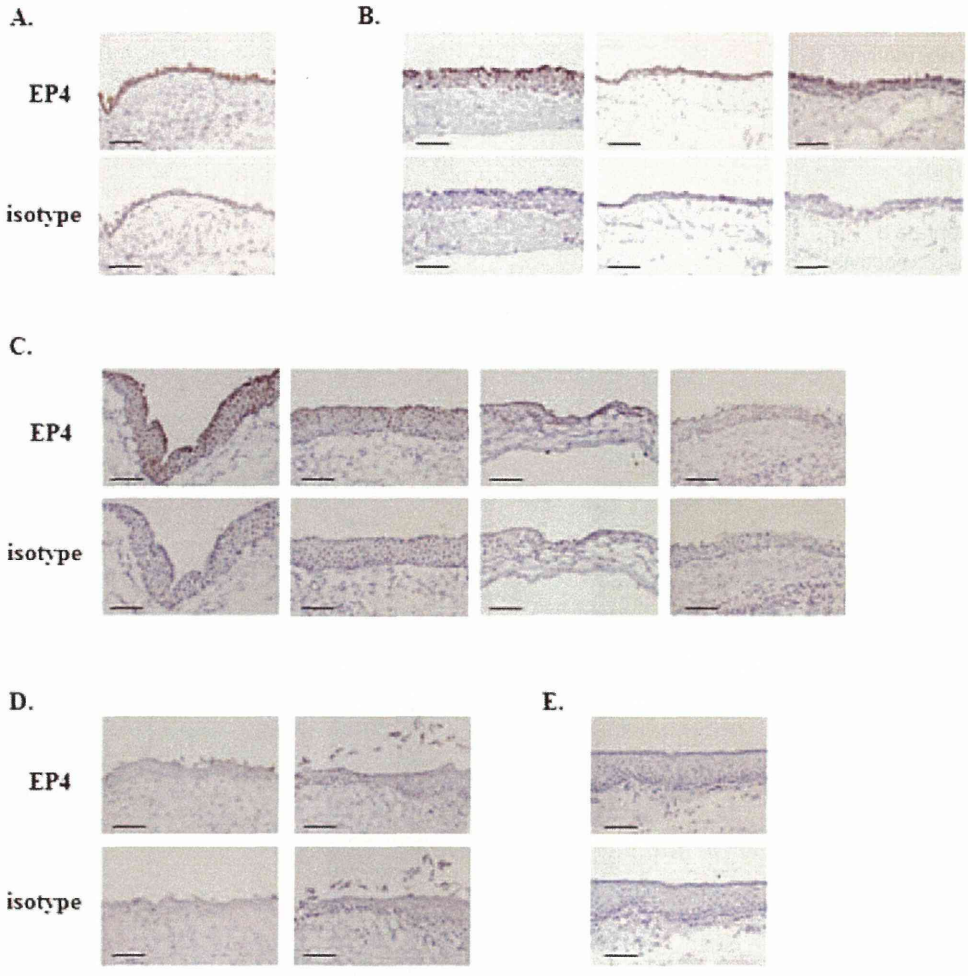

Figure 2 Immunohistological analysis of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface diseases. (A) Nearly normal conjunctival tissues from patients with conjunctivochalasis. (B) Conjunctival tissues from patients with chemical eye burn requiring ocular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

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

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

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

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

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Contributors All the authors substantially contributed to the conception and design, acquisition of data, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be published.

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

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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;0:1–5)

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

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

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

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

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

MATERIALS AND METHODS

Human Corneal Epithelial Cells

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

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

Reverse Transcription–Polymerase Chain Reaction

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

Enzyme-Linked Immunosorbent Assay

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

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

Quantitative RT-PCR

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

Data Analysis

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

RESULTS

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

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

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

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

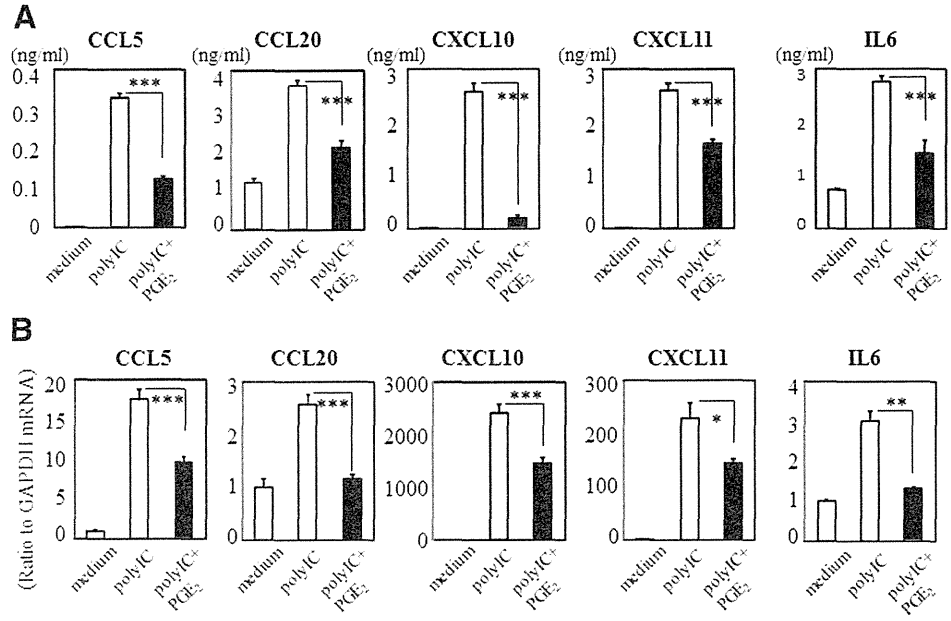

FIGURE 1. A, Suppression of the production of CCL5, CCl20, CXCL10, CXCL11, and IL-6 by PGE_2. HCLE were exposed to 10 μg/mL poly I:C and 100 μg/mL PGE_2 for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out in 6 wells per group. 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).

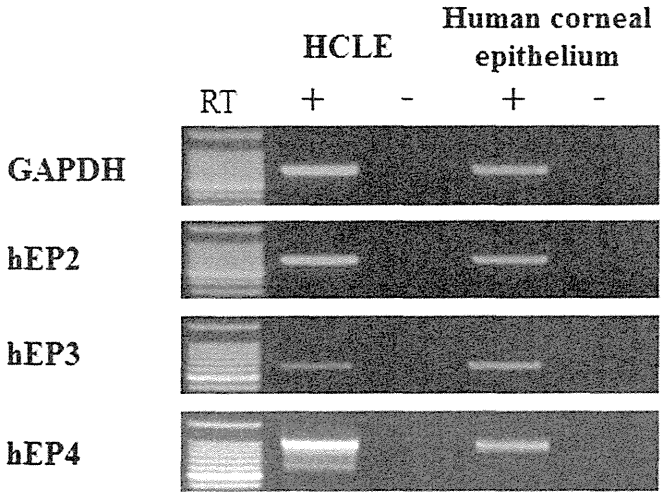

FIGURE 2. mRNA expression of the PGE_2 receptors EP2, EP3, and EP4. RT-PCR assay of the expression of PGE_2 receptor EP2, EP3, and EP4-specific mRNA in HLCE and human corneal epithelium. RT identifies data that were obtained without reverse transcription (controls).

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,

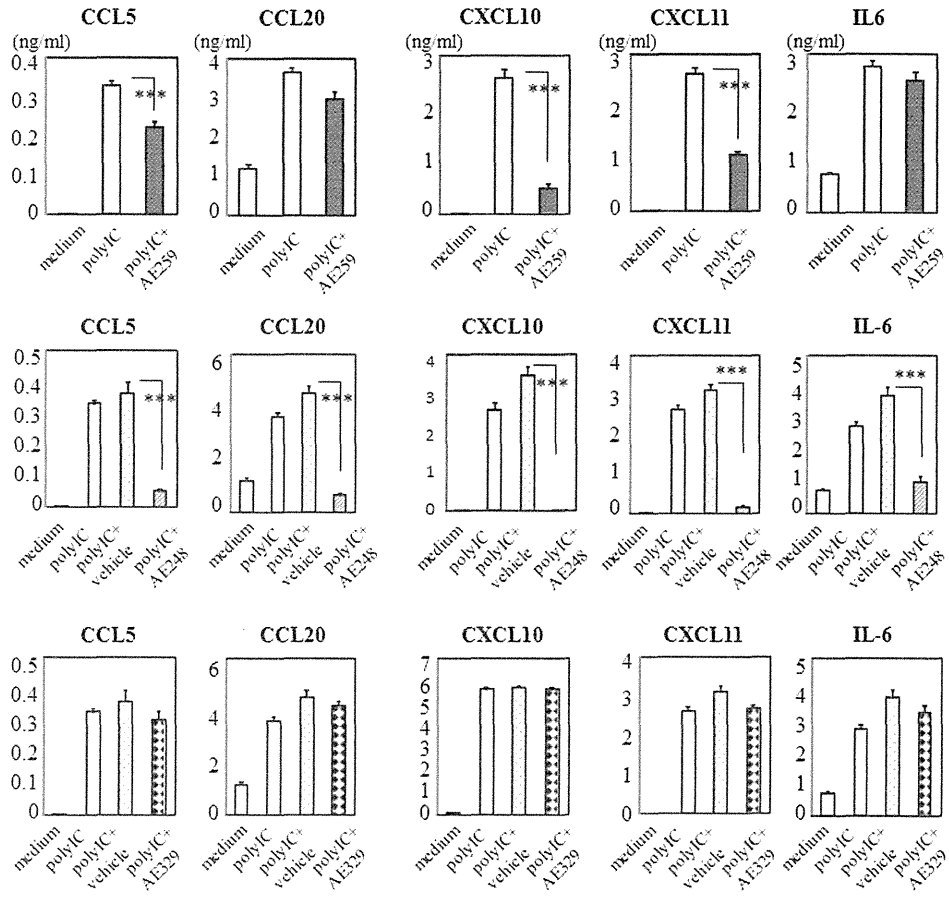

FIGURE 3. Effect of the PGE_2 receptors EP2, EP3, and EP4 on poly I:C-induced cytokine production. HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2, EP3, or EP4 agonist for 24 hours. Data are representative of 3 separate experiments and are given as the mean ± SEM from one experiment carried out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

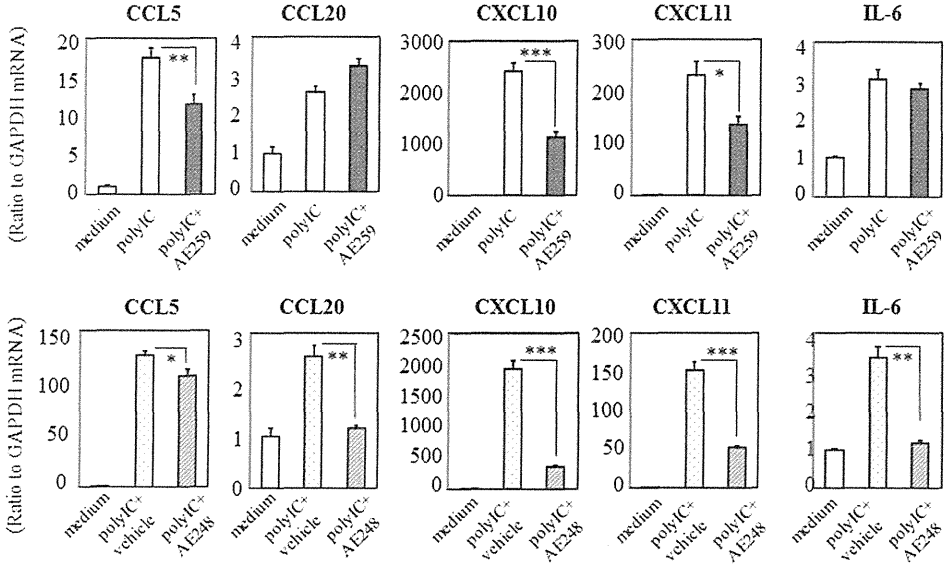

FIGURE 4. Effect of the PGE_2 receptors EP2 and EP3 on the poly I:C-induced mRNA expression of cytokines: HCLE were exposed to 10 μg/mL poly I:C and 10 μg/mL EP2 or EP3 agonist for 6 hours. The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. The *y* axis shows 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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Letter to the Editor

Epistatic interaction between Toll-like receptor 3 *(TLR3)* and prostaglandin E receptor 3 *(PTGER3)* genes

To the Editor:

We previously reported that conjunctival eosinophilic infiltration in murine experimental allergic conjunctivitis (EAC) was significantly less marked in Toll-like receptor 3 gene *(TLR3)* knockout (KO) mice[1] and significantly more marked in prostaglandin E receptor 3 (EP3) gene *(PTGER3)* KO mice than in wild-type mice.[2] Considering the opposite roles of TLR3 and EP3 in allergic conjunctivitis, we speculate the possibility of unknown functional interaction between TLR3 and EP3.

Intriguingly, we have also reported that Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) accompanied by severe ocular surface complications was associated with *TLR3* gene polymorphisms[3] and *PTGER3* polymorphisms.[4] SJS is an acute inflammatory vesiculobullous reaction of the skin and mucosa, often including the ocular surface,[5] and TEN occurs with its progression. SJS/TEN with ocular surface complications often results in severe and definitive sequelae, such as vision loss (see Fig E1 in this article's Online Repository at www.jacionline.org).[6]

For the past decade, single nucleotide polymorphisms (SNPs) have been widely used as genetic markers for identifying human disease-susceptibility genes. However, it has become apparent that gene-gene interactions should be considered in addition to major single-locus effects.[7] In particular, nonadditive (epistatic) models for some complex diseases fit to actual observations, suggesting interactions involving multiple loci.

In this study we examined whether there are functional interaction between TLR3 and EP3. Moreover, we also examined whether there is an epistatic interaction between *TLR3* and *PTGER3* polymorphisms in patients with SJS/TEN with ocular surface complications.

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine and the University of Tokyo, Graduate School of Medicine. All experimental procedures were conducted in accordance with the principles of the Helsinki Declaration. Details of the patients and methods are described in the Methods section in this article's Online Repository at www.jacionline.org. The primers and probes used in this study are shown in Table E1 in this article's Online Repository at www.jacionline.org.

First, we examined the functional interaction between TLR3 and EP3 by using *TLR3* KO, *PTGER3* KO, and *TLR3/PTGER3* double-knockout (DKO) mice in addition to our EAC model. We compared conjunctival eosinophil infiltration in wild-type, *TLR3* KO, *PTGER3* KO, and *TLR3/PTGER3* DKO mice. Although sensitization (intracutaneous and intraperitoneal injection of short ragweed pollen [RW; Polysciences, Inc, Warrington, Pa] adsorbed on aluminum hydroxide [200 μg of RW and 2.6 mg of alum]) without challenge (RW eye drop) did not affect the number of eosinophils after sensitization and challenge, the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in all of them compared with those in PBS-challenged control animals, and the number after sensitization and challenge in *PTGER3* KO mice was significantly larger and significantly lower in *TLR3* KO than in wild-type mice, as

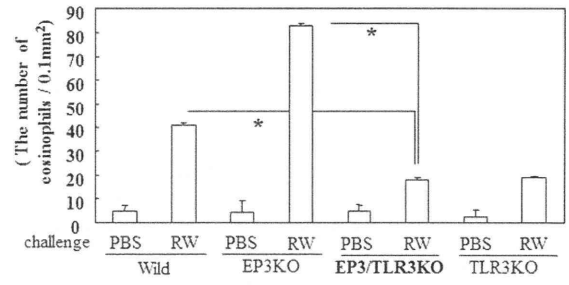

FIG 1. Functional interaction between EP3 and TLR3. In *TLR3/PTGER3* DKO mice the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to that seen in *TLR3* KO mice and was significantly lower than that seen in either *PTGER3* KO or wild-type mice. Data are shown as means ± SEMs of samples from all the mice examined (wild-type: phosphate-buffered saline, n = 24; RW, n = 28; *PTGER3* KO mice: phosphate-buffered saline, n = 23; RW, n = 25; EP3/TLR3 DKO mice: phosphate-buffered saline, n = 4; RW, n = 11; *TLR3* KO mice: phosphate-buffered saline, n = 12; RW, n = 12). *$P < .0005$.

we have reported previously.[1,2] Because TLR3 could regulate allergic inflammation in the absence of exogenous viral infection or the TLR3 ligand, it is possible that in our allergic conjunctivitis model endogenous RNA from tissues or cells stimulates TLR3.[1] With respect to EP3, one of the prostaglandin E receptors (EP1-EP4), our earlier observations suggested that during the elicitation phase of our EAC model, prostaglandin E_2 is synthesized in the conjunctival epithelium through microsomal prostaglandin E synthase 1.[2]

Furthermore, in *TLR3/PTGER3* DKO mice the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to the number of eosinophils in the lamina propria mucosae of the conjunctiva in *TLR3* KO mice and was significantly lower than the number of eosinophils in the lamina propria mucosae of the conjunctiva in not only *PTGER3* KO mice but also in wild-type mice (Fig 1). In addition, we previously reported that in human conjunctival epithelial cells the EP3 agonist suppressed the production of cytokines, such as thymic stromal lymphopoietin[8] and RANTES,[9] induced by polyinosinic:polycytidylic acid, a TLR3 ligand. Thymic stromal lymphopoietin and RANTES play important roles in the recruitment of eosinophils. These results suggest that EP3 negatively regulates the eosinophilic infiltration of EAC induced by TLR3, which causes reduced eosinophilic conjunctival inflammation in *TLR3/PTGER3* DKO mice, despite the pronounced eosinophilic conjunctival inflammation seen in *PTGER3* KO mice.

We have reported that the frequency of carriers of the HLA-A*0206 allele is significantly higher among Japanese patients with severe ocular surface complications.[10] We have also performed SNP association analysis of candidate genes and documented the associated polymorphisms of several immune-related genes, including *TLR3*,[3] IL-4 receptor *(IL4R)*,[11] *IL13*, and Fas ligand *(FasL)* in Japanese patients with SJS/TEN. Furthermore, we have performed a genome-wide association study of the patients with SJS/TEN and found associations between 6 SNPs in the *PTGER3* gene and the Japanese patients with SJS/TEN.[4]

We carried out a statistical search for interactions between all possible pairs of loci by applying high-dimensional variable selection methods, such as Sure Independence Screening (SIS)

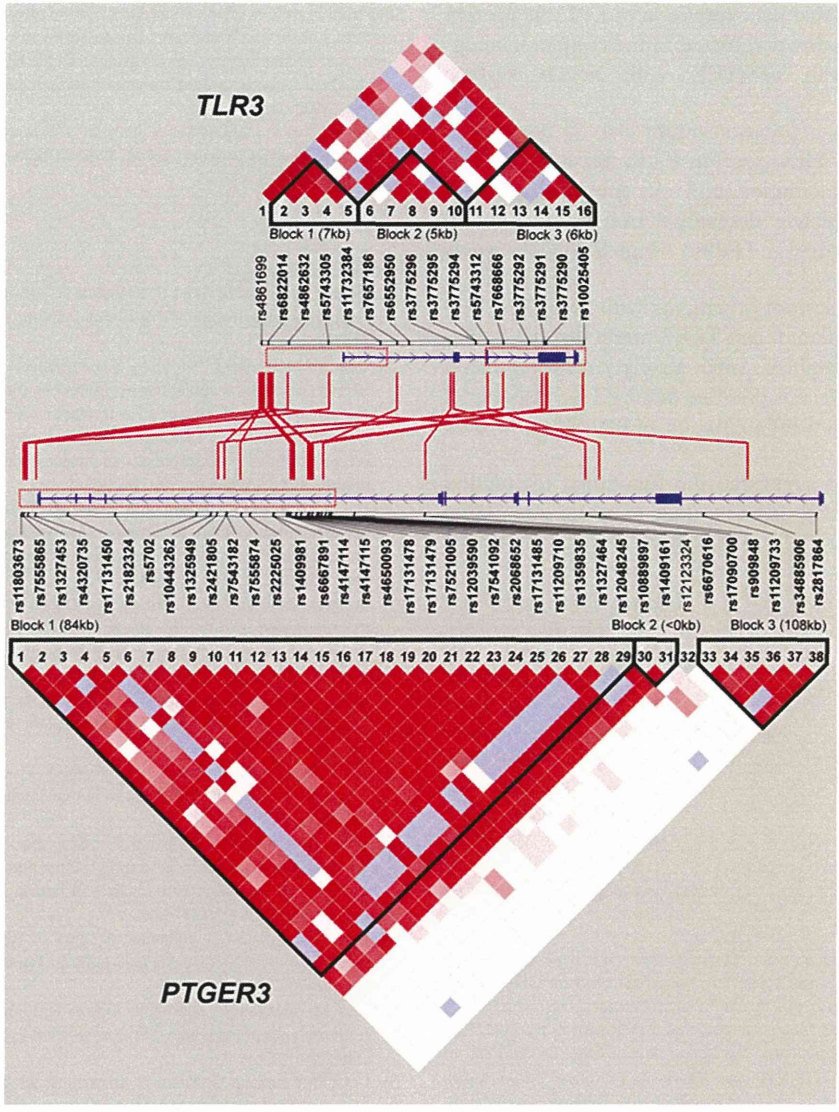

FIG 2. LD in EP3 and TLR3 regions. LD in the *TLR3* and *PTGER3* regions show 3 solid-spine LD blocks in each region. Iterative SIS reported 14 variables with nonzero regression coefficients, as if connecting the 5′ region of *TLR3* and the 3′ region of *PTGER3*.

and LASSO, to the comprehensive dataset obtained from our previous studies for a total of 14 immune-related genes (see Table E2 in this article's Online Repository at www.jacionline.org), including *PTGER3* and *TLR3*. After filtering with the standard SNP quality control filter, 36 SNPs were used for SIS to scan a total of 5778 ($3 \times 36 + 9 \times 36 \times [36\text{-}1]/2$) dummy variables. As a result, iterative SIS reported 2 variables with susceptible effects on SJS, which were involved in locus pairs of *PTGER3-TLR3* and *HLA-A-IL1A*, respectively (see Table E3 in this article's Online Repository at www.jacionline.org). The result showed that the *PTGER3* rs.4147114G/C SNP with the *TLR3* rs.3775296T/T SNP exhibited a higher odds ratio (OR, 25.3; $P = .0000527$) than only the *PTGER3* rs.4147114G/C SNP (OR, 2.66; $P = .0023$) or only the *TLR3* rs.3775296T/T SNP (OR, 5.35; $P = .00025$). These 2 susceptible interactions were also confirmed by using LASSO.

Next, we focused on the epistatic interaction between *TLR3* and *PTGER3* and analyzed the additional 10 SNPs of *TLR3* and 32 SNPs of *PTGER3*, resulting in a total of 17 SNPs of *TLR3* and 38 SNPs of *PTGER3*. All genotyping results agreed with Hardy-Weinberg equilibrium ($P > .01$) in both the case and control samples. These results showed that 5 additional SNPs of *TLR3* and 14 SNPs of *PTGER3*, a total of 7 SNPs of *TLR3* and 20 SNPs of *PTGER3*, were associated with SJS/TEN with ocular complications in addition to the previously reported 2 SNPs of *TLR3* and 6 SNPs of *PTGER3* (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Moreover, we investigated linkage disequilibrium (LD) in *TLR3* and *PTGER3* regions by using the squared correlation coefficient (1 SNP of *TLR3* [rs3775293] for which the minor allele frequency in both cases and control subjects was less than 5% was excluded) and identified 3 solid-spine LD blocks in each region. Iterative SIS reported 14 variables with nonzero regression coefficients as if connecting the 5′ region of *TLR3* (block 1) and the 3′ region of *PTGER3* (block 1, Fig 2).

We previously reported that the expression of EP3, the protein of the *PTGER3* gene, was downregulated in the conjunctival epithelium of patients with SJS/TEN with ocular surface complications.[4,12]

Although *TLR3* mRNA expression might also be downregulated in patients with SJS/TEN (see Fig E2 in this article's Online Repository at www.jacionline.org), our immunohistologic analysis did not clearly detect downregulation of the protein (see Fig E3 in this article's Online Repository at www.jacionline.org).

In the conjunctival epithelium of patients with SJS/TEN, EP3 was remarkably downregulated, and TLR3 might also be downregulated. Because EP3 might be more strongly downregulated than TLR3 in these patients, it is possible that EP3 is incapable of preventing TLR3-associated inflammation in patients with SJS/TEN.

In conclusion, we have suggested the functional interaction between TLR3 and EP3 supported by their epistatic interaction that confers an increased risk for SJS with severe ocular surface complications.

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