Table 1 Genotype frequencies of FasL SNPs among Japanese Stevens–Johnson syndrome/toxic epidermal necrolysis patients and healthy controls

	Control (%) (n = 160)	Stevens–Johnson syndrome/toxic epidermal necrolysis (%) (n = 76)	Allele 1 vs Allele 2		Genotype 11 vs 12+22		Genotype 11+12 vs 22	
			p Value (χ^2)	OR (95% CI)	p Value (χ^2)	OR (95% CI)	p Value (χ^2)	OR (95% CI)
rs.929087								
11 TT	49 (30.6)	18 (23.7)	0.667	–	0.269	–	0.645	–
12 TC	73 (45.6)	42 (55.3)						
22 CC	38 (23.8)	16 (21.1)						
rs.3830150								
AA	118 (73.8)	40 (52.6)	0.004	0.496 (0.3 to 0.8)	0.001	0.395 (0.2 to 0.7)	0.966	–
AG	40 (25.0)	35 (46.1)						
GG	2 (1.3)	1 (1.3)						
rs.2639614								
GG	131 (81.9)	52 (68.4)	0.025	0.526 (0.3 to 0.9)	0.021	0.480 (0.3 to 0.9)	0.589	–
GA	28 (17.5)	23 (30.3)						
AA	1 (0.6)	1 (1.3)						
rs.2859247								
GG	93 (58.1)	37 (48.7)	0.447	–	0.173	–	0.427	–
GA	54 (33.8)	35 (46.1)						
AA	13 (8.1)	4 (5.3)						

OR, odds ratio.

for rs. 929087, 5′-TGTATGCAGCGTTGTCGAA-3′ (sense) and 5′-TTTTGTTGAGGCTACACAGAGG-3′ (antisense) for rs. 3830150, 5′-ATCATTCAGGCCCACTCACC-3′ (sense) and 5′-TTGCTAGTCTCATCCCTTGCAC-3′ (antisense) for rs. 2639614 and rs. 2859247. All primers except rs. 3830150 were those recommended in the JSNP database. Genomic DNA was isolated from human peripheral blood at SRL (Tokyo). PCR amplification was with DNA polymerase (Takara, Shiga, Japan) for 35 cycles at 94°C for 1 min, annealing at 60°C for 1 min, and at 72°C for 1 min on a commercial PCR machine (GeneAmp; Perkin-Elmer Applied Biosystems, Waltham, MA). The PCR products were reacted with BigDye Terminator v3.1 (Perkin-Elmer Applied Biosystems), and sequence reactions were resolved on an ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

Statistical methods

Alleles were counted manually. Genotype patterns were also counted manually. The χ^2 test was used for Hardy–Weinberg equilibrium and statistical analysis to compare allelic and genotypic distributions. The odds ratio (OR) with 95% CI was calculated using Labo Server software (World Fusion, Tokyo). Each allele and genotype pattern was assessed as an independent variable, and separate p values were calculated for each polymorphism. A p value of <0.05 was regarded as significant. In addition, the p values were corrected for the number of alleles tested (Bonferroni method).

RESULTS

A summary of the case-control association study with the four genotyped SNPs is shown in table 1. All four SNPs examined were in Hardy–Weinberg equilibrium in our SJS/TEN patients and controls ($p>0.01$). SNP rs.3830150 A/G showed a significant strong inverse association with allele frequency (A versus G, raw and corrected p value 0.004 and 0.017, respectively; odds ratio (OR) 0.496) and with the dominant model (A/A versus A/G + G/G, raw and corrected p value 0.001 and 0.005, respectively; OR 0.395). SNP rs.2639614 G/A exhibited a significant inverse association with allele frequency (G versus A, raw p value 0.025; OR 0.526) and with the dominant model (G/G versus G/A + A/A, raw p value 0.021; OR 0.48) (table 1), although the results ceased to be significant when we corrected the p value for the number of alleles tested ($n = 4$).

We also analysed the genotype pattern of SNPs rs.3830150 and rs.2639614. Analysis of the genotype pattern of SNPs rs.3830150 and rs.2639614 (rs.3830150 A/A–rs.2639614 G/G) manifested a strong inverse association with SJS/TEN in Japanese patients (χ^2 test, $p = 0.001$, OR = 0.391, 95% CI 0.2 to 0.7) (table 2).

DISCUSSION

Our results suggest that polymorphisms in the FasL gene may be associated with SJS/TEN in the Japanese population. SNP rs.3830150 A/G showed a significant strong inverse association with allele frequency and with the dominant model. Analysis of

Table 2 Pattern structures and frequency of rs.3830150 and rs.2639614 SNPs of FasL Gene

Pattern type	rs.3830150	rs.2639614	Control (%) (n = 160)	Stevens–Johnson syndrome/ toxic epidermal necrolysis (%) (n = 76)	p Value (χ^2)	OR (95% CI)
1	A/A	G/G	115/160 (72.5)	38/76 (50.0)	0.001	0.39 (0.2 to 0.7)
2	A/G	A/G	25/160 (15.6)	21/76 (27.6)	0.03	2.1 (1.1 to 4.0)
3	A/G	G/G	14/160 (8.8)	14/76 (18.4)	0.03	2.4 (1.1 to 5.2)
4	A/A	A/G	3/160 (1.9)	2/76 (2.6)	NS	–
5	G/G	G/G	2/160 (1.3)	0/76 (0.0)	NS	–
6	A/G	A/A	1/160 (0.6)	0/76 (0.0)	NS	–
7	G/G	A/A	0/160 (0.0)	1/76 (1.3)	NS	–

the genotype pattern of SNPs rs.3830150 and rs.2639614 (rs.3830150 A/A–rs.2639614 G/G) also manifested a strong inverse association with SJS/TEN in Japanese patients. We have confirmed that the age–gender differences do not skew the data. All the controls and the patients are viable.

According to the International HapMap project, the rs.3830150 and rs.2639614 SNP, which showed a significant association with SJS/TEN, exists not only in Japanese-, but also in Han Chinese- and Caucasian populations, indicating that it is important to examine FasL SNPs in non-Japanese populations.

Previously, we have also documented the association with TLR3[1] and IL4R polymorphisms in Japanese SJS/TEN patients with ocular complications.[6] Because SJS/TEN is a rare condition, probably with a complex genetic-inheritance background, specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype.

It has been reported that the skin lesion of SJS/TEN in the acute stage is histologically characterised by marked keratinocyte apoptosis in the epidermis with dermo-epidermal separation, resulting in bullae.[8] Moreover, SJS/TEN patients in the acute stage manifested increased serum levels of FasL,[9 10] and the activation of Fas through FasL was reported to be an initial important step leading to the diffused apoptotic cell death of epidermal cells in SJS/TEN.[9 10]

Regarding eyes, it is reported that in the mouse eye, FasL is expressed by the corneal epithelium and endothelium,[11] and to maintain the cornea as a transparent barrier, the Fas-FasL pathway has a special significance in corneal immune privilege and in limiting inflammation.[12] However, no report has described the role of Fas/FasL-induced apoptosis in the pathogenesis of the ocular surface complications of SJS/TEN patients. The role of Fas/FasL signalling in chronic ocular surface inflammation remains elusive.

Drugs are probably the most widely accepted aetiological factor in SJS/TEN.[13] In addition, it is noteworthy that SJS/TEN patients often had the prodromata, including non-specific fever, coryza, and sore throat, that closely mimic upper-respiratory-tract infections commonly treated with antibiotics. These prodromata were evident from the clinical records of our SJS/TEN patients. Mycoplasma pneumoniae was responsible in five of 17 cases of childhood SJS,[14] and a viral aetiology involving herpes simplex-, Epstein–Barr-, cytomegalo-, and varicella zoster virus has been reported.[15 16]

On the other hand, apoptosis can reportedly be regulated by innate immunity.[17] Given the association between the onset of SJS/TEN and infections,[1 2] and the opportunistic infection of ocular surfaces by bacteria such as MRSA or MRSE,[18] we considered the possibility that there is an association between SJS/TEN and a disordered innate immune response. We postulated that drugs and/or viral infection may trigger a disorder in the host's innate immune response and that this event is followed by aggravated inflammation of the mucous membranes, ocular surface, and skin. The innate immune system may constitute a link between the environment and the adaptive immune system. We are continuing to examine the pathophysiology of SJS/TEN with ocular surface complications. A large international case-control study, called the Severe Cutaneous Adverse Reaction study, prospectively evaluated and supported the hypothesis that SJS and TEN are severity variants of a single entity.[19] The classification was based on the clinical appearance and pathology of skin lesions present in the acute stage. Although we were unable to accurately classify our patients into either SJS or TEN due to the fact that many of our patients were in the chronic stage, analysis of each condition independently may be worth pursuing in future studies.

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HLA class I and II gene polymorphisms in Stevens-Johnson syndrome with ocular complications in Japanese

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Purpose: Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are acute-onset mucocutaneous diseases induced by infectious agents and/or inciting drugs. Although the pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established, the extreme rarity of cutaneous and ocular surface reactions to drug therapies led us to suspect individual susceptibility. Our previous study of polymorphisms in the HLA-class I genes of 40 Japanese SJS/TEN patients with ocular surface complications showed that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN. In this study, we investigated the association between HLA class II antigens in addition to HLA class I antigens and SJS/TEN.

Methods: We studied the histocompatibility antigen genes, HLA-A, B, C, DRB1, and DQB1, of 71 Japanese SJS/TEN patients with ocular complications. We also genotyped 113 healthy volunteers for HLA-A, B, C, DRB1, and DQB1. We performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial bead-based typing kits.

Results: HLA-A*0206 was strongly associated with SJS/TEN. HLA-A*1101 was inversely associated. HLA-B*5901 exhibited a high odds ratio for SJS/TEN with ocular complications. However, when we corrected the p-value for the number of alleles detected (n=29), the results ceased to be significant. There was no association between HLA-C and SJS/TEN. There was also no significant association between HLA-DRB1 and SJS/TEN. HLA-DQB1*0502 was negatively and weakly associated with SJS/TEN although correction of the p-value for the number of alleles detected rendered the result not significant.

Conclusions: Because our findings are completely different from data reported on Caucasian patients, they suggest strong ethnic differences in the HLA-SJS associations.

Stevens-Johnson syndrome (SJS), an acute inflammatory vesiculobullous reaction of the skin and mucous membranes first described in 1922 [1], is commonly associated with infectious agents and/or inciting drugs [2,3]. In patients with extensive skin detachment and a poor prognosis, the condition is called toxic epidermal necrolysis (TEN) [4]. Although erythema multiforme (EM), SJS, and TEN were formerly accepted as part of a single "EM spectrum," a retrospective analysis of the type and distribution of skin lesions and the extent of epidermal detachment identified EM major and SJS/TEN as two separate clinical entities that differed with respect to histopathologic changes and etiology [5]. The annual incidence of SJS and TEN has been estimated as 0.4–1 and 1–6 cases per million persons, respectively [3,6]; the mortality rate is 3% and 27%, respectively [7]. Although rare, these reactions carry high morbidity and mortality rates and often result in severe and definitive sequelae such as vision loss. The pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established although the involvement of immune mechanisms [8,9], especially altered drug metabolism [10] and infections such as *Mycoplasma pneumoniae* [11], has been suggested. The extreme rarity of cutaneous and ocular surface reactions to drug therapies led us to suspect individual susceptibility.

In the acute stage, SJS/TEN patients manifest severe conjunctivitis and persistent corneal epithelial defects due to ocular surface inflammation with vesiculobullous skin lesions. In the chronic stage, ocular surface complications, such as conjunctival invasion into the cornea due to corneal epithelial stem cell deficiency, symblepharon, ankyloblepharon, and in some instances, keratinization of the ocular surface, persist despite the healing of the skin lesions [12]. Moreover, we observed that more than 95% of patients with SJS/TEN with ocular complications had lost their fingernails in the acute or sub-acute stage and that some continue to have transformed nails even after the healing of their skin lesions [2,13]. SJS/TEN is one of the most devastating ocular surface diseases, leading to corneal damage

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and loss of vision. The reported incidence of ocular complications in SJS/TEN is 50%–68% [3,7]. In this study, we focused on patients with SJS/TEN accompanied by ocular surface complications.

Our previous study of polymorphisms in the HLA-class I genes (HLA-A, B, C) of 40 Japanese SJS/TEN patients with ocular surface complications showed that in the Japanese, HLA-A*0206 was strongly associated with SJS/TEN with ocular surface complications [13]. We also documented that in Japanese SJS/TEN patients with ocular surface complications there was an association with TLR3 polymorphisms [2] and with IL4R polymorphisms [14]. Thus, genetic factors play an important role in an integrated etiology of SJS/TEN. However, HLA class II gene polymorphisms of Japanese SJS/TEN patients have not yet been reported.

Under the hypothesis of an immunologic reaction in susceptible individuals, we studied HLA class II (DRB1 and DQB1) gene polymorphisms in addition to HLA class I (HLA-A, B, C) in 71 Japanese SJS/TEN patients with ocular surface complications.

Table 1. HLA-A alleles and SJS/TEN with ocular complications.

HLA-A alleles	Carrier frequency				Allele frequency			
	SJS (n=71)	Normal (n=113)	p-value (X2)	Odds ratio	SJS (n=142)	Normal (n=226)	p-value (X2)	Odds ratio
*0101	0 (0/71)	0.035 (4/113)	0.16	-	0 (0/142)	0.018 (4/226)	0.3	-
*0201	0.268 (19/71)	0.221 (25/113)	0.47	-	0.155 (22/142)	0.111 (25/226)	0.22	-
*0206	0.423 (30/71)	0.15 (17/113)	0.00004 *	4.1	0.225 (32/142)	0.084 (19/226)	0.0001 *	3.2
*0207	0.07 (5/71)	0.08 (9/113)	1	-	0.035 (5/142)	0.04 (9/226)	1	-
*0301	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*1101	0.056 (4/71)	0.204 (23/113)	0.005	0.23	0.028 (4/142)	0.115 (26/226)	0.003 *	0.22
*2402	0.521 (37/71)	0.566 (64/113)	0.55	-	0.296 (42/142)	0.332 (75/226)	0.47	-
*2601	0.099 (7/71)	0.115 (13/113)	0.81	-	0.049 (7/142)	0.062 (14/226)	0.65	-
*2602	0.056 (4/71)	0.035 (4/113)	0.49	-	0.028 (4/142)	0.018 (4/226)	0.49	-
*2603	0.014 (1/71)	0.027 (3/113)	1	-	0.007 (1/142)	0.013 (3/226)	1	-
*3001	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*3101	0.099 (7/71)	0.186 (21/113)	0.14	-	0.049 (7/142)	0.093 (21/226)	0.16	-
*3303	0.225 (16/71)	0.212 (24/113)	0.84	-	0.113 (16/142)	0.111 (25/226)	0.95	-

HLA-A*0206 was strongly associated with SJS/TEN with ocular complications (carrier frequency: $p<0.00005$, corrected p value (Pc)<0.0005, OR=4.1; allele frequency: $p<0.0005$, $Pc<0.005$, OR=3.2). HLA-A*1101 was inversely associated (carrier frequency: $p<0.01$, $Pc=0.07$, OR=0.23; allele frequency: $p<0.005$, $Pc<0.05$, OR=0.22). SJS/TEN patients in this study consisted of 40 previously analyzed patients and 31 new patients. These results validate the strong association between HLA-A*0206 and SJS/TEN that we reported previously [13].

METHODS

Patients: This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants, and their written informed consent was obtained.

Table 2. HLA-B alleles and SJS/TEN with ocular complications.

HLA-B alleles	Carrier frequency				Allele frequency			
	SJS (n=71)	Normal (n=113)	p	Odds ratio	SJS (n=142)	Normal (n=226)	p	Odds ratio
*0702	0.113 (8/71)	0.106 (12/113)	1	-	0.056 (8/142)	0.053 (12/226)	1	-
*1301	0.07 (5/71)	0.027 (3/113)	0.26	-	0.035 (5/142)	0.013 (3/226)	0.27	-
*1302	0 (0/71)	0.009 (1/113)	1	-	0 (0/142)	0.004 (1/226)	1	-
*1501	0.042 (3/71)	0.115 (13/113)	0.11	-	0.028 (4/142)	0.058 (13/226)	0.21	-
*1511	0 (0/71)	0.009 (1/113)	1	-	0 (0/142)	0.004 (1/226)	1	-
*1518	0.014 (1/71)	0.035 (4/113)	0.65	-	0.007 (1/142)	0.018 (4/226)	0.65	-
*1527	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*2704	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*3501	0.211 (15/71)	0.15 (17/113)	0.29	-	0.106 (15/142)	0.084 (19/226)	0.49	-
*3701	0 (0/71)	0.027 (3/113)	0.29	-	0 (0/142)	0.013 (3/226)	0.29	-
*3802	0 (0/71)	0.009 (1/113)	1	-	0 (0/142)	0.004 (1/226)	1	-
*3901	0.085 (6/71)	0.071 (8/113)	0.78	-	0.042 (6/142)	0.035 (8/226)	0.78	-
*3902	0.014 (1/71)	0.018 (2/113)	1	-	0.007 (1/142)	0.009 (2/226)	1	-
*4001	0.169 (12/71)	0.124 (14/113)	0.39	-	0.092 (13/142)	0.062 (14/226)	0.29	-
*4002	0.127 (9/71)	0.133 (15/113)	1	-	0.07 (10/142)	0.066 (15/226)	0.88	-
*4003	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*4006	0.099 (7/71)	0.097 (11/113)	1	-	0.049 (7/142)	0.049 (11/226)	1	-
*4402	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*4403	0.225 (16/71)	0.204 (23/113)	0.72	-	0.12 (17/142)	0.106 (24/226)	0.69	-
*4601	0.085 (6/71)	0.115 (13/113)	0.62	-	0.042 (6/142)	0.062 (14/226)	0.49	-
*4801	0.028 (2/71)	0.088 (10/113)	0.13	-	0.014 (2/142)	0.044 (10/226)	0.14	-
*5101	0.197 (14/71)	0.124 (14/113)	0.18	-	0.113 (16/142)	0.066 (15/226)	0.12	-
*5102	0.028 (2/71)	0.009 (1/113)	0.56	-	0.014 (2/142)	0.004 (1/226)	0.56	-
*5201	0.127 (9/71)	0.212 (24/113)	0.17	-	0.07 (10/142)	0.115 (26/226)	0.16	-
*5401	0.042 (3/71)	0.133 (15/113)	0.07	-	0.021 (3/142)	0.066 (15/226)	0.08	-
*5502	0.028 (2/71)	0.044 (5/113)	0.71	-	0.014 (2/142)	0.022 (5/226)	0.71	-
*5601	0.028 (2/71)	0.027 (3/113)	1	-	0.014 (2/142)	0.013 (3/226)	1	-
*5901	0.113 (8/71)	0.018 (2/113)	0.01	7	0.056 (8/142)	0.009 (2/226)	0.02	6.7
*6701	0 (0/71)	0.035 (4/113)	0.16	-	0 (0/142)	0.018 (4/226)	0.3	-

HLA-B*5901 exhibited a high odds ratio for SJS/TEN with ocular complications (carrier frequency: $p<0.05$, $Pc=0.42$, OR=7.0; allele frequency: $p<0.05$, $Pc=0.46$, OR=6.7). However, when we corrected the p-value for the number of alleles detected (n=29), the results ceased to be significant.

For HLA genotyping, we enrolled 71 Japanese patients with SJS/TEN in the chronic or sub-acute phase; all presented with ocular surface complications. The diagnosis of SJS/TEN was based on a confirmed history of acute-onset high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least two mucosal sites including the ocular surface. The average patient age was 45.8 ± 17.4 years; the male:female ratio was 31:40.

Controls: The normal control group consisted of 113 healthy volunteer blood donors for HLA class I (A, B, C) and HLA class II (DRB1, DQB1) for genotyping. All volunteers were Japanese residing in Japan.

HLA genotyping: We studied the histocompatibility antigen genes HLA-A, B, C, DRB1, and DQB1 of 71 Japanese SJS/TEN patients with ocular complications. We also genotyped 113 healthy volunteers for HLA-A, B, C, DRB1, and DQB1. These alleles were detected by the polymerase chain reaction (PCR)-Luminex typing method using the WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan). First, the target DNA was amplified by polymerase chain reactions with biotinylated primers specifically designed for each HLA locus. Then, the PCR product was denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescent coded microsphere beads. At the same time, biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with the Luminex 100 system. Genotype determination and data analysis were performed automatically, using the WAKFlow typing software.

Statistical methods: For statistical analysis to compare carrier frequency and gene frequency, we used the χ^2-test for statistical analysis when the sample number was 10 and more than 10 and used the Fischer's exact test when the sample number was less than 10. The odds ratio (OR) with 95% confidence intervals (95% CI) was calculated using Labo Server software (World Fusion, Tokyo, Japan). Each allele was assessed as an independent variable and separate p values were calculated. A p value of <0.05 was regarded as significant. In addition, the p values were corrected for the number of alleles tested.

RESULTS

As shown in Table 1, HLA-A*0206 was strongly associated with SJS/TEN with ocular complications (carrier frequency: p<0.00005, corrected p value (Pc)<0.0005, OR=4.1; allele

Table 3. HLA-C alleles and SJS/TEN with ocular complications.

HLA-C alleles	Carrier frequency				Allele frequency			
	SJS (n=71)	Normal (n=113)	p	Odds ratio	SJS (n=142)	Normal (n=226)	p	Odds ratio
*0102	0.268 (19/71)	0.327 (37/113)	0.4	-	0.141 (20/142)	0.168 (38/226)	0.48	-
*0303	0.239 (17/71)	0.168 (19/113)	0.24	-	0.12 (17/142)	0.093 (21/226)	0.41	-
*0304	0.366 (26/71)	0.23 (26/113)	0.046	1.9	0.197 (28/142)	0.124 (28/226)	0.057	-
*0401	0.07 (5/71)	0.115 (13/113)	0.45	-	0.035 (5/142)	0.058 (13/226)	0.46	-
*0501	0.014 (1/71)	0 (0/113)	0.39	-	0.007 (1/142)	0 (0/226)	0.39	-
*0602	0 (0/71)	0.035 (4/113)	0.16	-	0 (0/142)	0.018 (4/226)	0.3	-
*0702	0.211 (15/71)	0.212 (24/113)	0.99	-	0.12 (17/142)	0.119 (27/226)	0.99	-
*0704	0.014 (1/71)	0.018 (2/113)	1	-	0.007 (1/142)	0.009 (2/226)	1	-
*0801	0.099 (7/71)	0.168 (19/113)	0.28	-	0.049 (7/142)	0.088 (20/226)	0.22	-
*0803	0.028 (2/71)	0.053 (6/113)	0.71	-	0.014 (2/142)	0.027 (6/226)	0.71	-
*1202	0.127 (9/71)	0.212 (24/113)	0.17	-	0.07 (10/142)	0.115 (26/226)	0.16	-
*1402	0.141 (10/71)	0.097 (11/113)	0.37	-	0.078 (11/142)	0.053 (12/226)	0.35	-
*1403	0.225 (16/71)	0.204 (23/113)	0.72	-	0.113 (16/142)	0.106 (24/226)	0.85	-
*1502	0.099 (7/71)	0.044 (5/113)	0.22	-	0.049 (7/142)	0.022 (5/226)	0.23	-

There was no association between HLA-C and SJS/TEN with ocular complications.

Table 4. HLA-DRB1 alleles and SJS/TEN with ocular complications.

HLA-DRB1 alleles	Carrier frequency				Allele frequency			
	SJS (n=71)	Normal (n=113)	p	Odds ratio	SJS (n=142)	Normal (n=226)	p	Odds ratio
*0101	0.113 (8/71)	0.097 (11/113)	0.81	-	0.056 (8/142)	0.049 (11/226)	0.81	-
*0401	0.028 (2/71)	0.018 (2/113)	0.64	-	0.014 (2/142)	0.009 (2/226)	0.64	-
*0403	0.028 (2/71)	0.053 (6/113)	0.71	-	0.014 (2/142)	0.031 (7/226)	0.49	-
*0404	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*0405	0.197 (14/71)	0.248 (28/113)	0.43	-	0.106 (15/142)	0.131 (30/226)	0.44	-
*0406	0.028 (2/71)	0.062 (7/113)	0.49	-	0.014 (2/142)	0.031 (7/226)	0.49	-
*0407	0 (0/71)	0.035 (4/113)	0.16	-	0 (0/142)	0.018 (4/226)	0.3	-
*0410	0.028 (2/71)	0.018 (2/113)	0.64	-	0.014 (2/142)	0.009 (2/226)	0.64	-
*0701	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*0802	0.113 (8/71)	0.062 (7/113)	0.27	-	0.056 (8/142)	0.031 (7/226)	0.28	-
*0803	0.239 (17/71)	0.133 (15/113)	0.06	-	0.12 (17/142)	0.071 (16/226)	0.11	-
*0901	0.301 (22/71)	0.31 (35/113)	1	-	0.176 (25/142)	0.168 (38/226)	0.84	-
*1001	0 (0/71)	0.009 (1/113)	1	-	0 (0/142)	0.004 (1/226)	1	-
*1101	0.07 (5/71)	0.027 (3/113)	0.26	-	0.035 (5/142)	0.013 (3/226)	0.27	-
*1201	0.028 (2/71)	0.062 (7/113)	0.49	-	0.014 (2/142)	0.031 (7/226)	0.49	-
*1202	0.085 (6/71)	0.035 (4/113)	0.19	-	0.042 (6/142)	0.018 (4/226)	0.19	-
*1301	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*1302	0.169 (12/71)	0.195 (22/113)	0.66	-	0.085 (12/142)	0.097 (22/226)	0.68	-
*1401	0.028 (2/71)	0.053 (6/113)	0.71	-	0.014 (2/142)	0.027 (6/226)	0.72	-
*1403	0.042 (3/71)	0.062 (7/113)	0.74	-	0.021 (3/142)	0.031 (7/226)	0.75	-
*1405	0.056 (4/71)	0.062 (7/113)	1	-	0.028 (4/142)	0.031 (7/226)	1	-
*1406	0.014 (1/71)	0.018 (2/113)	1	-	0.007 (1/142)	0.009 (2/226)	1	-
*1501	0.183 (13/71)	0.106 (12/113)	0.14	-	0.092 (13/142)	0.058 (13/226)	0.21	-
*1502	0.127 (9/71)	0.186 (21/113)	0.31	-	0.07 (10/142)	0.102 (23/226)	0.31	-
*1602	0 (0/71)	0.035 (4/113)	0.16	-	0 (0/142)	0.018 (4/226)	0.3	-

There was no significant association between HLA-DRB1 and SJS/TEN with ocular complications.

Table 5. HLA-DQB1 alleles and SJS/TEN with ocular complications.

HLA-DQB1 alleles	Carrier frequency				Allele frequency			
	SJS (n=71)	Normal (n=113)	p	Odds ratio	SJS (n=142)	Normal (n=226)	p	Odds ratio
*0201	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*0301	0.254 (18/71)	0.212 (24/113)	0.52	-	0.141 (20/142)	0.106 (24/226)	0.32	-
*0302	0.169 (12/71)	0.177 (20/113)	0.89	-	0.085 (12/142)	0.093 (21/226)	0.78	-
*0303	0.296 (21/71)	0.354 (40/113)	0.41	-	0.169 (24/142)	0.195 (44/226)	0.54	-
*0401	0.197 (14/71)	0.239 (27/113)	0.51	-	0.106 (15/142)	0.128 (29/226)	0.51	-
*0402	0.056 (4/71)	0.053 (6/113)	1	-	0.028 (4/142)	0.027 (6/226)	1	-
*0501	0.113 (8/71)	0.097 (11/113)	0.81	-	0.056 (8/142)	0.053 (12/226)	1	-
*0502	0 (0/71)	0.08 (9/113)	0.01	0	0 (0/142)	0.04 (9/226)	0.01	0
*0503	0.085 (6/71)	0.062 (7/113)	0.57	-	0.042 (6/142)	0.031 (7/226)	0.57	-
*0601	0.352 (25/71)	0.274 (31/113)	0.26	-	0.19 (26/142)	0.164 (37/226)	0.63	-
*0602	0.155 (11/71)	0.106 (12/113)	0.33	-	0.078 (11/142)	0.058 (13/226)	0.45	-
*0603	0.014 (1/71)	0.009 (1/113)	1	-	0.007 (1/142)	0.004 (1/226)	1	-
*0604	0.169 (12/71)	0.195 (22/113)	0.66	-	0.085 (12/142)	0.097 (22/226)	0.68	-

HLA-DQB1*0502 showed a tendency of negative association with SJS/TEN with ocular complications (carrier frequency: $p<0.05$, $Pc=0.17$, OR=0; allele frequency: $p<0.05$, $Pc=0.19$, OR=0). Although none of the 71 SJS/TEN patients and 10 of the 117 healthy volunteers (8.5%) had the HLA-DQB1*0502 allele, the correction of the p-value for the number of alleles detected (n=14) rendered the result not significant.

frequency: $p<0.0005$, $Pc<0.005$, OR=3.2). HLA-A*1101 was inversely associated (carrier frequency: $p<0.01$, $Pc=0.07$, OR=0.23; allele frequency: $p<0.005$, $Pc<0.05$, OR=0.22). SJS/TEN patients in this study consisted of 40 previously analyzed patients and 31 new patients. These results validate the strong association between HLA-A*0206 and SJS/TEN that we reported previously [13].

Table 2 shows the results on HLA-B alleles. HLA-B*5901 exhibited a high odds ratio for SJS/TEN with ocular complications (carrier frequency: $p<0.05$, $Pc=0.42$, OR=7.0; allele frequency: $p<0.05$, $Pc=0.46$, OR=6.7). However, when we corrected the p-value for the number of alleles detected (n=29), the results ceased to be significant.

There was no association between HLA-C and SJS/TEN with ocular complications (Table 3). There was also no significant association between HLA-DRB1 and SJS/TEN with ocular complications (Table 4).

HLA-DQB1*0502 showed a tendency of negative association with SJS/TEN with ocular complications (carrier frequency: $p<0.05$, $Pc=0.17$, OR=0; allele frequency: $p<0.05$, $Pc=0.19$, OR=0); Table 5). Although none of the 71 SJS/TEN patients and 10 of the 117 healthy volunteers (8.5%) had the HLA-DQB1*0502 allele, the correction of the p-value for the number of alleles detected (n=14) rendered the result not significant.

We used the χ^2-test for statistical analysis when the sample number was 10 and more than 10 and used the Fischer's exact test when the sample number was less than 10. Carrier frequency is "frequency of the person with the allele

TABLE 6. ETHNIC DIFFERENCES IN THE ASSOCIATION OF SJS/TEN WITH HLA.

Allele	Japanese		Caucasian [19]		Taiwanese [18]	
	SJS	Control	SJS	Control	Carbamazepine induced SJS	Control
A*0206	0.423	0.15	-	(0 ~1.4%)	-	(3.1 ~24%)
B*1502	0	0		(0 ~0.2%)	1	0.086
B*4402	0.014	0	-	(6.7 ~26.5%)	-	0
B*4403	0.225	0.204	-	(6.6 ~20.0%)	-	(0 ~2%)
DQB1*0601	0.352	0.274	0.17	0.03	-	-

Carrier frequency of SJS-associated alleles in Japanese and Caucasian. Data in parentheses are from "Allele Frequency in Worldwide Populations."

at the population level," and allele frequency is "frequency of alleles at the population level" [15].

DISCUSSION

Analysis of our 71 Japanese patients showed that HLA-A*0206 was strongly associated with SJS/TEN with ocular complications. On the other hand, HLA-A*1101 was negatively associated. We postulate that the decreased B*5401 frequency in the patients is attributable to its linkage disequilibrium with A*1101. We also found that HLA-B*5901 was weakly associated with SJS/TEN with ocular complications although when we corrected the p-value for the number of alleles detected, the result ceased to be significant. We postulate that B*5901 could be a risk factor independent of A*0206 because only one patient had both alleles. Interestingly, none of the 71 SJS/TEN patients but 10 of the 117 volunteers (8.5%) had the HLA-DQB1*0502 allele. HLA-DQB1*0502 was also weakly associated with SJS/TEN with ocular complications although correction of the p-value for the number of alleles detected rendered the result not significant.

Regarding HLA-class I, previous reports from the United States [16] and France [17] showed that the HLA-B12 (HLA-Bw44) antigen was significantly increased in Caucasian SJS patients. In our study population, we did not find an association with HLA-B12 probably because in Caucasians, the HLA-B12 antigen is primarily coded by HLA-B*4402 whereas in the Japanese, it is almost exclusively coded by HLA-B*4403 [15]. HLA-A*0206, strongly associated with SJS/TEN with ocular complications in the Japanese, is absent in Caucasians. While we were unable to identify the causative drug(s) unequivocally, we suspect that antibiotics, cold remedies, or non-steroid anti-inflammatory drugs were involved in some of our patients. Limiting carbamazepine-induced SJS, the HLA-B*1502 allele was documented to show a very strong association [18].

With respect to HLA-class II, Power et al. [19] reported that HLA-DQB1*0601 was associated with Caucasian patients with ocular complications of SJS. In French SJS/TEN patients, HLA-DR antigens (DR) was not associated at all [16]. Different from the findings of others, we found that in our Japanese patients, there is no significant association between SJS/TEN and HLA-DQB1*0601.

Thus, our findings suggest strong ethnic differences in the association of SJS/TEN with HLA (Table 6). Because SJS/TEN is a rare condition probably with a complex genetic inheritance background, specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype. Since the strong association of specific HLA antigens with SJS with ocular complications may be a clue to understanding its basic pathobiology, we are attempting to develop a reliable test for identifying individuals susceptible to SJS with ocular complications.

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Human conjunctival epithelial cells express functional Toll-like receptor 5

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ABSTRACT

Purpose: The expression and function of Toll-like receptor 5 (TLR5) was analysed in human conjunctival epithelial cells (HCjEC).

Methods: The expression of TLR5 in HCjEC was studied by reverse transcriptase (RT) PCR and flow cytometry. The amount of interleukin (IL) 6 and IL-8 proteins was determined by ELISA. Messenger RNA expression elicited by stimulation with flagellins derived from *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella typhimurium*, and *Bacillus subtilis* was assayed by quantitative RT–PCR. The localisation of TLR5 protein in human conjunctival epithelium was detected immunohistochemically.

Results: HCjEC expressed TLR5-specific mRNA and TLR5 protein. In HCjEC stimulated with flagellins derived from *P aeruginosa* and *S marcescens*, IL-6 and IL-8 production was increased and IL-6 and IL-8 mRNA was upregulated. Flagellins from *S typhimurium* and *B subtilis* did not induce the upregulation of these genes and proteins. TLR5 protein was detected on the basolateral but not the apical side of human conjunctival epithelium.

Conclusions: Human conjunctival epithelium harbours functional TLR5. Considering the spatially selective basolateral localisation of TLR5 protein, it was postulated that flagellins from ocular pathogenic bacteria induce inflammatory responses when disruption of the epithelial barrier permits their transmigration to the basolateral side but not under healthy physiological conditions on the ocular surface.

The ocular surface consists of mucosal epithelium composed of corneal and conjunctival epithelial cells. As are other mucosal epithelia, the ocular mucosal epithelium is continuously challenged by pathogenic and non-pathogenic microbes. The mucosal epithelium is thought to represent a first line of defence against diverse microbes, not only by providing a physical barrier, but also by producing pro-inflammatory cytokines and antimicrobial peptides.[1] Ocular surface epithelium such as corneal and conjunctival epithelium produce antimicrobial peptides similar to other mucosal epithelium.[2 3]

Toll-like receptors (TLR), pattern recognition receptors that sense conserved pathogen-associated molecular patterns, are the key receptors for the recognition of microbes.[4 5] TLR were initially shown to be expressed and functional in immunocompetent cells such as macrophages and dendritic cells (DC); subsequently, their expression in a variety of cells including intestinal and airway epithelial cells was documented.[6 7] Signalling through TLR induces the activation of transcription factors for antimicrobial genes and cytokines.[6] TLR5 recognises bacterial flagellin,[6] a component protein of bacterial flagella. Flagella are present in both Gram-positive and Gram-negative bacteria; they are essential for bacterial motility, invasion, and chemotaxis. *Pseudomonas aeruginosa* and *Serratia marcescens* are the bacteria associated with ocular surface with flagella.[10 11]

We previously reported that human corneal epithelial cells (HCEC) express TLR2 and TLR4 intracellularly but not on their cell surface, and that HCEC do not secrete interleukin (IL) 6 or IL-8 in response to peptidoglycan and lipopolysaccharide stimulation. This defective response may contribute to the immunosilent environment in the epithelium for commensal bacteria inhabiting the ocular surface.[12] We documented that TLR3 on poly I : C-treated HCEC responded by producing pro-inflammatory cytokines and interferon β.[13] Others[14–16] reported that functional TLR in HCEC play a pivotal role in innate immunity to pathogen-associated molecular patterns. Elucidation of the unique innate immune responses of HCEC that are distinct from those of immune-competent cells will lead to a better understanding of the host–commensal symbiosis on the ocular surface.

P aeruginosa flagellin elicits the inflammatory responses of HCEC in a TLR5-dependent manner.[17] We reported that HCEC express TLR5 and secrete pro-inflammatory cytokines in response to flagellin derived from ocular bacteria that are pathogenic, but not to non-pathogenic ocular bacteria.[18] We found that flagellin derived from *S. typhimurium*, a bacterium pathogenic in the intestine but not on the ocular surface, evoked a response by human intestinal epithelial cells but not HCEC.[18]

Whereas the critical homeostatic role of TLR in corneal epithelium has been discussed extensively in the literature, there are few reports on the role of TLR in conjunctival epithelium. Cook *et al*[19] and Bonini *et al*[20] documented the expression of TLR2, TLR4, and TLR9 in human conjunctival epithelial cells (HCjEC). The expression and function of TLR5 in conjunctival epithelium has not, however, been clarified yet.

In this study we confirmed the expression and function of TLR5 in HCjEC. We document that flagellins derived from *P aeruginosa* and *S marcescens* can trigger the innate immune response in HCjEC expressing TLR5, and TLR5 was selectively expressed on the basolateral but not the apical side of conjunctival epithelium.

MATERIALS AND METHODS

Human conjunctival epithelial cells

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, in Kyoto, Japan. All experimental

procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocol were explained to all patients and their informed consent was obtained.

For reverse transcriptase (RT) PCR, we obtained HCjEC from healthy volunteers by brush cytology. A tiny brush (Cytobrush S; Medscand AM, Malmo, Sweden) was used to scrape epithelial cells from the bulbar conjunctiva. For ELISA, real-time quantitative PCR, and flow cytometric analysis, we harvested primary HCjEC from conjunctival tissue obtained at conjunctivochalasis surgery.[21] The cells were cultured using a modification of previously described methods.[22] Briefly, conjunctival tissues were washed and immersed for 1 h at 37°C in 1.2 U ml^{-1} purified dispase (Roche Diagnostic Ltd, Basel, Switzerland). Epithelial cells were detached, collected, and cultured in low-calcium k-SFM medium supplemented with 0.2 ng ml^{-1} human recombinant epidermal growth factor (Invitrogen, Carlsbad, California, USA), 25 mg ml^{-1} bovine pituitary extract (Invitrogen), and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within three or four days. After reaching 80% confluence in seven to 10 days, the cells were seeded and after their subconfluence, they were used in subsequent procedures.

Bacterial flagellins used in this study

Flagellins derived from *P aeruginosa* and *S marcescens* were obtained from Inotek Pharmaceuticals (Beverly, Massachussetts, USA); flagellins from *Salmonella typhimurium* and *Bacillus subtilis* were from InvivoGen (San Diego, California, USA). *S typhimurium* flagellin is a strong pathogen that gains entrance into intestinal mucosa by penetrating enterocytes;[23] it does not penetrate to the ocular surface mucosa. The pathogen *S marcescens* is implicated in approximately 5–10% of Gram-negative corneal ulcers related to contact lens wear.[10 11] The pathogenic potential of *B subtilis* is reportedly weak or absent.[24] *P aeruginosa* is an opportunistic ocular pathogen that can initiate a highly destructive corneal infection in humans[10 11] and is involved in up to 70% of contact lens-related bacterial keratitis cases.[25]

Purification of mononuclear cells from peripheral blood

The procedures were described elsewhere.[13] Briefly, we obtained peripheral venous blood samples from volunteers who had given their previous informed consent. Mononuclear cells isolated in lymphoprep tubes (Daiichi Pure Chemicals, Tokyo, Japan) were stimulated for 24 h with the different flagellins.

Reverse transcriptase PCR

We analysed HCjEC for TLR1–10 RNA expression as described in our previous study.[13] Briefly, total RNA was isolated from HCjEC and human peripheral mononuclear cells using Trizol Reagent (Life Technologies, New York, New York, USA) according to the manufacturer's instructions. For the RT reaction we used the SuperScript Preamplification Kit (Invitrogen). PCR amplification was with DNA polymerase (cTaq; Toyobo, Japan); the conditions were 38 cycles at 94°C for one minute, annealing for one minute, and 72°C for one minute on a commercial PCR machine (GeneAmp; Perkin-Elmer Applied Biosystems, Foster City, California, USA). The primers were as described in our previous study (table 1).[13] RNA integrity was assessed electrophoretically in ethidium bromide-stained 1.5% agarose gels.

Enzyme-linked immunosorbent assay

Primary HCjEC were examined to quantify cytokine secretion as described in our previous report.[13] Briefly, primary HCjEC were plated in 24-well plates and after reaching subconfluence were either left untreated or incubated for 24 h with each flagellin at a final concentration of 100 ng/ml or with 10 ng/ml human IL-1α (R&D Systems, Inc, Minneapolis, Minnesota, USA). The amount of IL-6 and IL-8 secreted in response to flagellin exposure increased with the flagellin concentration; we used 100 ng/ml. As early as 24 h, we found high levels of IL-6 and IL-8 in supernatants from flagellin-exposed cultures of primary HCjEC. IL-6 and IL-8 release into culture supernatants was quantitated using the Opteia IL-6 and IL-8 set (BD Pharmingen, San Diego, California, USA) according to the manufacturer's instructions.

Table 1 The primer list of Toll-like receptor (TLR)

Gene (accession no)	Primer sense antisense	Product size	Annealing
TLR1 (NM003263)	5'–TGCCCTGCCTATATGCAA–3' 5'–GAACACATCGCTGACAACT–3'	555 bp	54
TLR2 (XM003304)	5'–GCCAAAGTCTTGATTGATTGG–3' 5'–TTGAAGTTCTCCAGCTCCTG–3'	346 bp	52
TLR3 (NM003265)	5'–CGCCAACTTCACAAGGTA–3' 5'–GGAAGCCAAGCAAAGGAA–3'	689 bp	54
TLR4 (XM005336)	5'–TGGATACGTTTCCTTATAAG–3' 5'–GAAATGGAGGCACCCCTTC–3'	506 bp	52
TLR5 (NM003268)	5'–ATCTGACTGCATTAAGGGGAC–3' 5'–TTGAGCAAAGCATTCTGCAC–3'	567 bp	52
TLR6 (NM006068)	5'–CCTCAACCACATAGAAACGAC–3' 5'–CACCACTATACTCTCAACCCAA–3'	531 bp	50
TLR7 (NM016562)	5'–AGTGTCTAAAGAACCTGG–3' 5'–CCTGGCCTTACAGAAATG–3'	544 bp	50
TLR8 (NM016610)	5'–CAGAATAGCAGGCGTAACACATCA–3' 5'–AATGTCACAGGTGCATTCAAAGGG–3'	639 bp	56
TLR9 (NM017442)	5'–GTGCCCCACTTCTCCATG–3' 5'–GGCACAGTCATGATGTTGTTG–3'	259 bp	50
TLR10 (NM030956)	5'–CTTTGATCTGCCCTGGTATCTC–3' 5'–AGCCCACATTTACGCCTATCCT–3'	497 bp	52
GAPDH (XM033263)	5'–CCATCACCATCTTCCAGGAG–3' 5'–CCTGCTTCACCACCTTCTTG–3'	575 bp	60

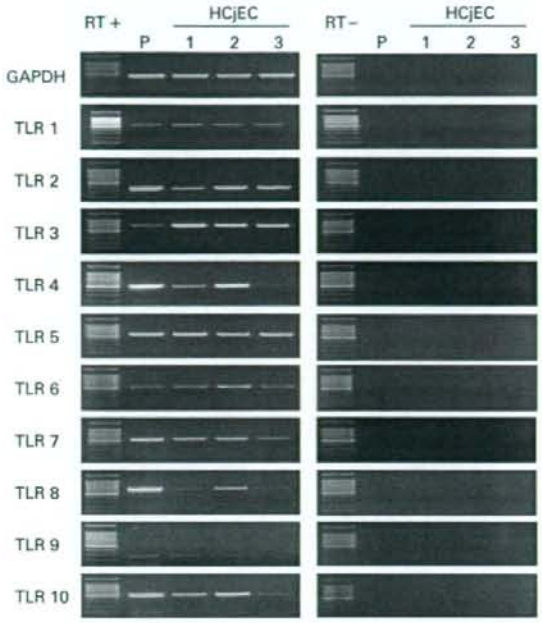

Figure 1 Human conjunctival epithelial cells (HCjEC) express Toll-like receptor (TLR) 1–10-specific mRNA. Total RNA was isolated from human conjunctival epithelial cells. For the reverse transcriptase (RT) reaction, we used the SuperScript preamplification system. PCR amplification was with DNA polymerase; primers are listed in table 1. Human mononuclear cells were the positive control (P).

Flow cytometric analysis

HCjEC were analysed for TLR5 expression by flow cytometry as previously described.[11 18] We purchased mouse anti-human TLR5 monoclonal antibody from Abcam (Cambridge, UK); it recognises an intracellular epitope in the cytoplasmic domain of TLR5.

Immunohistochemical study of TLR5 in human conjunctival sections

Serial sections of human conjunctiva were prepared from samples obtained at conjunctivochalasis surgery. These were fixed for 30 minures with methanol, incubated overnight in a moist chamber at 4°C with mouse anti-human TLR5 monoclonal antibody (Abcam) or isotype control mouse IgG2a (DakoCytomation, Kyoto, Japan), and washed in phosphate buffered saline. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, Oregon, USA) was applied for 1 h at room temperature, the slides were washed and then antifade mounting medium with propidium iodide was applied (Vectashield; Vector Laboratories, Burlingame, California, USA).

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems) according to previously described procedures.[13 18] The initial amount of RNA used for reverse transcribing to complimentary DNA was approximately 1 μg and the cDNA was used at the original concentration for quantitative PCR. The stimulation time used in this study (1 h) was optimal for the maximum induction of IL-6 and IL-8 mRNA expressions (see supplemental fig 2 published online only).

The primers and probes for human IL-6 (Hs00174131), IL-8 (Hs00174103) and human GAPDH (Hs 4326317E) were from Perkin-Elmer Applied Biosystems. Quantitative PCR was used to measure the expression of IL-6 and IL-8 mRNA in HCjEC treated for 1 h with flagellin derived from four different microbial bacteria (100 ng/ml) or human IL-α. The quantification data were normalised to the expression of the housekeeping gene GAPDH.

Data analysis

Data were expressed as the mean ± SE and evaluated by Student's t-test using the Excel program.

RESULTS

TLR-specific mRNA and TLR5 protein expression in HCjEC

TLR1–10-specific mRNA expression was present in HCjEC harvested by impression cytology from healthy volunteers; TLR5-specific mRNA was expressed at levels comparable to mononuclear cells (fig 1). This finding demonstrates that the TLR5 gene is constitutively expressed in HCjEC. The TLR5 protein was also expressed by primary HCjEC harvested from conjunctival tissue at levels comparable to lymphocytes (fig 2).

Primary HCjEC respond only to flagellin derived from ocular pathogenic bacteria

To assess whether the flagellins used were able to induce an inflammatory response in immunocompetent cells, we stimulated fractionated human peripheral mononuclear cells for 24 h

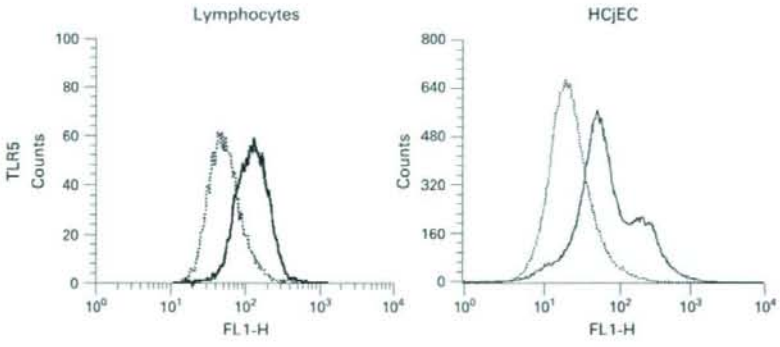

Figure 2 Human conjunctival epithelial cells (HCjEC) express Toll-like receptor (TLR) 5 protein. Intracellular fluorescence-activated cell sorter analysis showed that TLR5 is expressed in HCjEC. As the TLR5 antibody recognises the intracellular domain of the TLR5 complex, we performed intracellular staining. Lymphocytes were the positive control. Histogram data are representative of three separate experiments (solid line, TLR5 antibody; dotted line, isotype control).

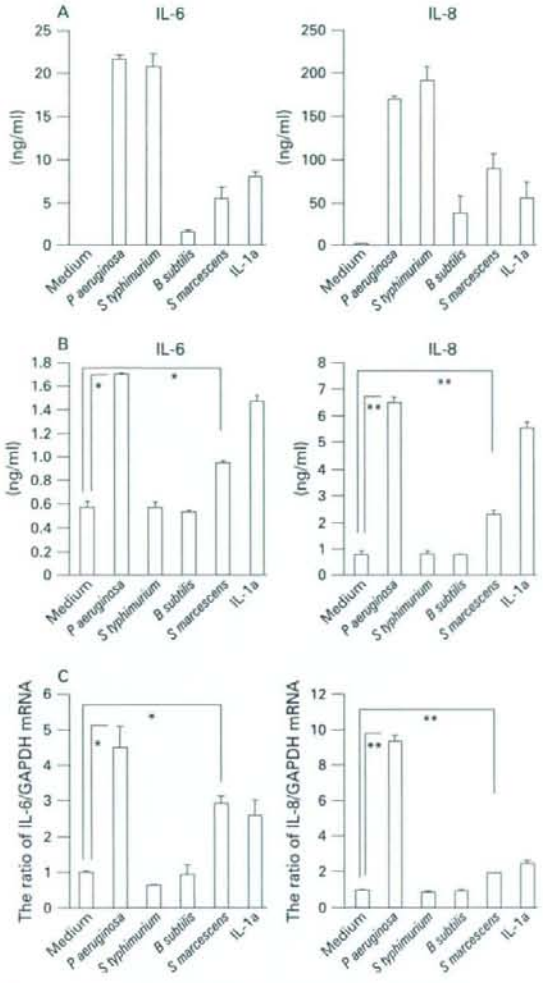

Figure 3 Production and mRNA expression of IL-6 and IL-8 in human conjunctival epithelial cells exposed to various flagellins. (A) To determine whether the flagellins induce an inflammatory response, fractionated human periphera mononuclear cells were stimulated for 24 h with *P aeruginosa, S typhimurium, B subtilis*, or *S marcescens*-derived flagellin at a concentration of 100 ng/ml. The supernatants were harvested and the concentration of IL-6 and IL-8 was measured. (B) To quantify inflammatory cytokine secretion, human conjunctival epithelial cells (HCjEC) were plated in 24-well plates. Upon reaching subconfluence they were left untreated or exposed for 24 h to flagellins derived from the four different microbial bacteria (100 ng/ml) or 10 ng/ml human IL-1a. The supernatants were harvested to measure IL-6 and IL-8 concentrations. (C) Quantitative reverse transcriptase PCR was used to measure the expression of IL-6 and IL-8 mRNA in HCjEC treated for 1 h with flagellin derived from four different microbial bacteria (100 ng/ml) or 10 ng/ml human IL-α. The quantification data were normalised to the expression of the housekeeping gene GAPDH. The Y axis shows the increase of specific mRNA over unstimulated samples. Data show the representative results from two independent experiments (A) or three independent experiments (B, C). Data represent mean $\pm$ SEM from the representative experiments with triplicated dishes (*$<$0.05, **$<$0.005).

with *P aeruginosa, S typhimurium, B subtilis* or *S marcescens*-derived flagellin. All flagellins induced the production of significant levels of the inflammatory cytokines IL-6, and IL-8 (fig 3A).

Our results indicated that the flagellins derived from the four distinct microbes were able to induce pro-inflammatory cytokine production by human mononuclear cells. As we previously documented that HCEC produced IL-6 and IL-8 in response to flagellins derived from ocular pathogenic, but not non-pathogenic, bacteria,[18] we investigated whether HCjEC exhibited a similar response. We found that in primary HCjEC, flagellins derived from *P aeruginosa* and *S marcescens*, but not those derived from *S typhimurium* and *B subtilis* elicited a significant increase in the secretion of IL-6 and IL-8 protein (fig 3B). The mRNA expression levels specific for IL-6 and IL-8 were considerably elevated in primary HCjEC stimulated with *P aeruginosa* and *S marcescens*-derived flagellin. In contrast, neither IL-6 nor IL-8-specific mRNA levels were significantly elevated in primary HCjEC stimulated with *S typhimurium* and *B subtilis*-derived flagellin (fig 3C). These results suggest that, like HCEC, primary HCjEC respond to ocular pathogenic but not to ocular non-pathogenic flagellins.

We decided on the dose for ELISA and real-time quantitative PCR according to our dose analysis and the recommended concentration of flagellin written in the manufacturer's instructions. As per the manufacturer's instructions, the recommended concentration to achieve TLR5 stimulation is 0.1–100 ng/ml, and the strongest response was achieved with 100 ng/ml *P aeruginosa* and *S marcescens*-derived flagellin (see supplemental fig 1 published online only).

TLR5 is expressed basolaterally in conjunctival epithelium

We subjected conjunctival tissues obtained at conjunctivochalasis surgery to immunohistochemical study to determine the presence and localisation of TLR5 expression in stratified conjunctival epithelium. TLR5 protein was consistently and abundantly expressed in human conjunctival epithelium. It was detected only at basal and wing sites, indicating its spatially selective presence on the basolateral but not the apical side (fig 4).

DISCUSSION

This is the report of the expression and function of TLR5 on HCjEC. HCjEC stimulated with flagellins derived from *P aeruginosa* and *S marcescens* but not from *S typhimurium* and *B subtilis* induced the elevated production and mRNA upregulation of IL-6 and IL-8. TLR5 was selectively expressed on the basolateral but not the apical side.

The ability to detect microbes is an important task of the immune system. Exaggerated host defence reactions of the epithelium to endogenous bacterial flora may start and perpetuate inflammatory mucosal responses.[26] Among ocular surface-related bacteria, only a few common causative ocular pathogens, ie *P aeruginosa* and *S marscesens*, have flagella; *Staphylococcus epidermidis* and *Pseudomonas acnes*, commensal ocular bacteria,[27 28] do not. Most, if not all, of the responses to bacterial flagellin are thought to be mediated by TLR5.[29] *S typhimurium* flagellin, strongly pathogenic and pro-inflammatory to intestinal epithelial cells,[28] had little effect on HCjEC. Although *B subtilis* is one of the related species of *Bacillus cereus*, which is a major cause of severe keratitis, endophthalmitis, and

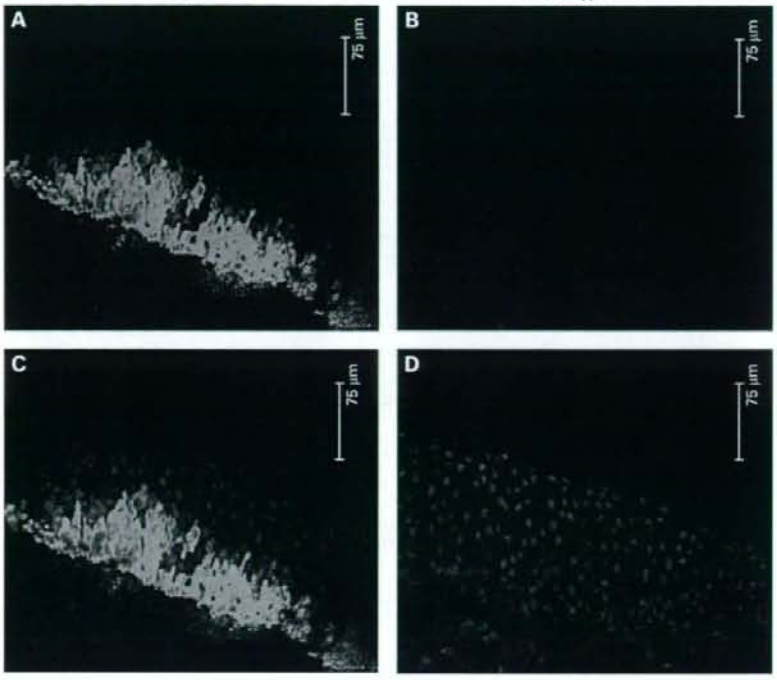

Figure 4 Localisation of Toll-like receptor (TLR) 5 in a human conjuncitval epithelium. TLR5 was detected by immunofluorescence staining. Frozen cryostat sections of a human conjunctiva were incubated with anti-TLR5 antibody (A, C). Isotype control incubation was the negative control (B, D). Bound antibodies were visualised by Alexa Fluor 488 goat anti-mouse IgG, nuclei by propidium iodide (PI) staining. Merged staining of TLR5 and PI shows no TLR5 staining associated with the apical layer of the epithelium.

panophthalmitis,[30] *B subtilis* flagellin elicited no immune response in HCjEC. This suggests that TLR5 in HCjEC may discriminate specifically between bacteria that are ocular pathogenic and non-pathogenic.

Whereas *P aeruginosa* and *S marcescens* are common pathogens in keratitis,[10 11] they do not usually cause conjunctivitis. Conjunctivitis attributable to these bacteria tends to be seen primarily in neonates and immunocompromised hosts.[31 32] The lamina propria, abundant in immunocompetent cells and lymphatic organs, is located beneath the conjunctival epithelium.[33] On the other hand, the avascular stroma under the corneal epithelium harbours few immunocompetent cells. The host defence in the conjunctival and corneal component may be different and this may account for the difference in the pathogens that result in keratitis and conjunctivitis.

Immunohistochemical data presented here and elsewhere[18] indicate that TLR5 is basolaterally expressed in both conjunctival and corneal epithelium. Similarly, TLR5 is expressed on the basolateral surface of intestinal epithelium and detects invasive flagellated bacteria.[34] TLR5 in ocular surface epithelium may be crucial for the sensing of invasive flagellated bacteria. It is possible that the flagellins of *P aeruginosa* and *S marcescens* induce an inflammatory reaction only if the integrity of the epithelial barrier is breached, thereby allowing their transepithelial transport to the basolateral site.

Interestingly, the ocular surface epithelium did not respond to flagellin derived from the enteropathogenic, ocular non-pathogenic bacterium *S typhimurium*. Murine intestinal lamina propria cells can differentiate between pathogenic and non-pathogenic bacteria.[34] It remains unknown, however, how TLR can distinguish between pathogenic and non-pathogenic microbes. A collaboration between co-receptors and TLR may be a possible explanation; a receptor called fimbriae cooperates with TLR4 to distinguish between pathogenic and non-pathogenic bacteria in the urinary tract.[35] Alternatively, there may be some minor molecular differences at the site of recognition in TLR5 expressed on different cell types. Mouse and human TLR5 discriminates between different flagellins, and the recognition site on TLR5 has been mapped to the diversified extracellular domain.[36] Studies are underway in our laboratory to identify the molecular mechanisms that allow HCjEC to discriminate between flagellins derived from ocular pathogenic and non-pathogenic bacteria.

Another group recently reported that primary HCjEC express TLR5 and respond to *S typhimurium*-derived flagellin.[37] Their result differs from the result obtained in our study. The cause of the difference is not evident but it might be due to the dose of flagellin used. That group used an extremely large amount of flagellin in their experiment. Our study confirmed that a much smaller amount (100 ng/ml) of *S typhimurium*-derived flagellin could upregulate IL-8 production in human colonic carcinoma cell line HT29[18] but not HCjEC, and that the same amount of *P aeruginosa*-derived flagellin could also significantly upregulate IL-6 and IL-8 production in HCjEC.

We document that HCjEC possesses functional TLR5, and that conjunctival epithelial cells respond to exposure to flagellins derived from ocular pathogenic but not non-pathogenic bacteria by secreting pro-inflammatory cytokines. Considering the spatially selective localisation of TLR5 protein on the basolateral side of human conjunctival epithelium, an inflammatory reaction may be induced only when flagellin from ocular pathogenic bacteria cross the conjunctival epithelium to the basolateral side.

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Competing interests: None.

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The use of trehalose-treated freeze-dried amniotic membrane for ocular surface reconstruction

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ABSTRACT

The aim of this study was to evaluate the efficacy and safety of trehalose-treated freeze-dried amniotic membrane (TT-FDAM) for ocular surface reconstruction. Human AM deprived of amniotic epithelial cells was first incubated with 10% trehalose solution, and then freeze-dried, vacuum-packed, and sterilized with gamma-irradiation. The resultant newly developed TT-FDAM was characterized for its physical, biological, and morphological properties by comprehensive physical assays, immunohistochemistry, electron microscopy, cell adhesion assay, 3D cell culture, and an *in vivo* biocompatibility test. The adaptability of TT-FDAM was markedly improved as compared to FDAM. Immunohistochemistry for several extracellular matrix molecules revealed that the process of freeze-drying and irradiation apparently did not affect its biological properties, however, electron microscopy revealed that the detailed morphological appearance of TT-FDAM is more similar to that of native AM than to FDAM. Intracorneal and scleral-surface transplantation of TT-FDAM showed excellent biocompatibility with ocular surface tissues. Thus, TT-FDAM retained most of the physical, biological, and morphological characteristics of native AM, consequently it is a useful biomaterial for ocular surface reconstruction.

1. Introduction

The amniotic membrane (AM), whose function is to protect the embryo and to surround and contain the amniotic fluid, has been used as surgical biomaterial in a variety of clinical applications [1,2]. It has been shown to possess various kinds of biological effects such as an anti-inflammatory effect [3,4], anti-fibroblastic activity [5], anti-microbial [6] and anti-angiogeneic [7] properties, very limited immunogenicity [8], and a proper substrate for cells. Recently, particular attention has been focused on the AM in the ophthalmologic field, since its variety of characteristics make it ideally suited for use in ocular surface reconstruction. However, some serious biological and logistical problems still remained, such as the deficiency of appropriate sterilization and difficulties in transport and storage.

Recently, we have resolved such serious problems by developing a sterilized, freeze-dried amniotic membrane (FDAM) using a special vacuum-packaging process and gamma-irradiation, and have successfully developed and adapted it for clinical application [9,10]. Even though FDAM retained most of the biological characteristics of native AM, the manufacturing process (especially the freeze-drying process) ultimately affects some of the characteristics of the AM; therefore it is not completely identical to native AM both physically and biologically [9–11]. Therefore, further improvement of the physical, biological, and morphological characteristics of FDAM is needed in order to adapt it to a wider variety of clinical applications.

Trehalose is a nonreducing disaccharide ($C_{12}H_{22}O_{11}$) found in high concentrations in a wide variety of organisms that are capable of surviving almost complete dehydration. It is thought to be a storage sugar in the cells, and evidence is accumulating that trehalose production may be a universal stress response. Its presence confers desiccation resistance to bacterial and human cells [12]. Its remarkable effectiveness is clearly due to its ability to replace some of the water in the cell, thereby stabilizing and protecting the cellular membrane and proteins during the freezing process [13]. These findings led us to the interesting hypothesis that trehalose might protect AM from damage during the freeze-drying process and consequently improve the biological quality of FDAM.

In this report, we present the efficacy and safety of trehalose-treated freeze-dried amniotic membrane (TT-FDAM) for ocular surface reconstruction.

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2. Materials and methods

2.1. Preparation of TT-FDAM

Human AM was prepared following our previously reported standard method [14]. Briefly, after obtaining proper informed consent in accordance with the tenets of the Declaration of Helsinki and with approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AMs were obtained at the time of elective cesarean section from volunteers who were seronegative donors. Under sterile conditions, the AM was washed with sterile phosphate buffered saline (PBS) containing antibiotics and antimycotics and cut into pieces measuring approximately 5 × 5 cm. The AM was then deprived of amniotic epithelial cells by incubation with 0.02% ethylene diamine tetraacetic acid (EDTA; Nacalai Tesqu Co., Kyoto, Japan) at 37 °C for 2 h. To protect the structure of denuded AM from damage during the freeze-drying process, denuded AM was further incubated with 10% trehalose solution (TREHA, Hayashibara, Okayama, Japan) at 37 °C for 2 h. This trehalose solution was attentively pre-treated with an ultrasonic machine to remove the micro-bubbles. After that, TT-FDAM was fixed and stretched in the plastic holder to prevent it from crinkling. It was then freeze-dried under vacuum conditions using vacuum freeze-drying apparatuses (TAITEC, Saitama, Japan) for 1 h and then vacuum-packed at room temperature (RT) as soon as possible. Finally, gamma-irradiation (20 kGy) was used to sterilize the resultant TT-FDAM. The manufacturing process of FDAM [9] is almost the same as that of TT-FDAM except for the pretreatment of the trehalose solution.

2.2. Physical examination of TT-FDAM

To investigate the physical characteristics of TT-FDAM, we examined its thickness, haze, tensile strength, and adaptability. Samples examined in this study were AM without epithelium, FDAM, and TT-FDAM ($N = 5$).

The thickness of each sample (measuring 25 × 25 mm each) was measured by a surface-scanning laser confocal displacement meter (LT-9010M; Keyence, Osaka, Japan). Haze was measured by a turbidimeter (NDH2000, Nippon Denshoku, Tokyo, Japan). Briefly, each 25 × 25 mm sample under the wet condition was placed and fixed on the measuring holder without folds or bubbles. Use of the turbidimeter allowed for the calculation of total light transmittance, diffused transmittance, and parallel transmittance. Haze was calculated as follows: haze = diffused transmittance/total light transmittance. Tensile strength was examined by stretch–stress (SS) tests performed on 10 × 30 mm samples using our previous protocol [9]. Briefly, both ends of each 10 × 30 mm sample under the wet condition were held with a clip. Each sample was then pulled vertically with a uniaxial stretching device. Cross-head speed was set at 10 mm/min. Adaptability was examined by putting the samples on the rabbit corneal surface under the wet condition and calculating the number of AM folds on the cornea under a microscope by three different individuals.

2.3. Immunohistochemistry

Immunohistochemical examinations in this study were carried out using our previously described method [15,16]. Briefly, semi-thin (7 μm) cryostat sections were obtained from unfixed tissue embedded in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN, USA). After fixation with cold acetone for 10 min, the sections were incubated with 1% bovine serum albumin for 30 min. Subsequently, the sections were incubated at RT for 1 h with the primary antibody (Table 1), then washed three times in PBS containing 0.15% TritonX-100 (PBST) for 10 min. The controls consisted of replacing the primary antibody with the appropriate non-specific normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration. As an additional control, the primary antibody was omitted. After incubation with the primary antibody, the sections were then incubated at RT for 1 h with appropriate secondary antibodies, Alexa Fluor 488 conjugated anti-mouse and rabbit IgG antibody (Molecular Probes Inc., Eugene, OR, USA). After several washings with PBS, the sections were coverslipped using anti-fading mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA, USA) and examined by confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Table 1
Primary antibodies and source

Antibodies	Category	Dilution	Source
Collagen 1	Rabbit polyclonal	X300	LSL, Japan
Collagen 3	Rabbit polyclonal	X300	LSL, Japan
Collagen 4	Rabbit polyclonal	X300	LSL, Japan
Collagen 5	Rabbit polyclonal	X300	LSL, Japan
Collagen 7	Mouse monoclonal	X100	Chemicon, USA
Fibronectin	Mouse monoclonal	X100	Novo Castra, UK
Laminin 5	Mouse monoclonal	X100	Chemicon
Keratin 1	Mouse monoclonal	X20	Novo Castra
Keratin 3	Mouse monoclonal	X100	Progen, Germany
Keratin 4	Mouse monoclonal	X200	Novo Castra
Keratin 10	Mouse monoclonal	X50	Novo Castra
Keratin 13	Mouse monoclonal	X200	Novo Castra
Muc5ac	Mouse monoclonal	X200	Zymed, USA
CD4	Mouse monoclonal	X50	Dako, Japan
CD8	Mouse monoclonal	X50	Dako, Japan
CD68	Mouse monoclonal	X50	Dako, Japan
Neutrophil elastase	Mouse monoclonal	X50	Dako, Japan

2.4. Electron microscopy

TT-FDAM was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). AM without epithelium and FDAM were also examined for comparison. Specimens were fixed in 2.5% glutaraldehyde in 0.1 M PBS, washed three times for 15 min in PBS, and post-fixed for 2 h in 2% aqueous osmium tetroxide. They were washed three more times in PBS before being passed through a graded ethanol series. For SEM preparation, specimens were transferred to hexamethyldisilazane (TAAB Laboratories Equipment Ltd., UK) for 10 min and allowed to air-dry. When dry, specimens were mounted on aluminum stubs and sputter-coated with gold before examination in a digital scanning electron microscope (JEOL JSM 5600; JEOL Ltd., Tokyo, Japan). For TEM preparation, the specimens were embedded in Agar 100-epoxy resin (Agar Scientific, UK). Ultrathin (70 nm) sections were collected on copper grids and stained for 1 h each with uranyl acetate and 1% phosphotungstic acid, then for 20 min with Reynold's lead citrate prior to examination on a transmission electron microscope (JEOL JEM 1010; JEOL Ltd.).

2.5. Adhesion of rabbit corneal epithelial cells to the AM

To evaluate the cell adhesion properties of each different AM matrix, a single cell suspension of rabbit corneal epithelial cells was seeded onto the AM without epithelium, FDAM, and TT-FDAM in an incubator at 37 °C for two different lengths of time: 60 min and overnight. Briefly, rabbit corneal epithelial cells including the limbal region were incubated at 37 °C for 1 h with 1.2 IU dispase and then trypsinized at 37 °C for 30 min. The resultant single cell suspension of corneal epithelial cells was then seeded onto each AM matrix spread on the bottom of culture inserts (5×10^4 cells/six well culture insert, $N = 5$). After incubation, non-adherent cells were washed with the culture medium (defined keratinocyte growth medium (ArBlast Co., Ltd., Kobe, Japan) supplemented with 5% FBS) and then adherent cells were trypsinized and the cell number was then counted. In our series, we confirmed macroscopically and histologically that no cells remained attached to the three different types of AM.

2.6. 3D culture of rabbit corneal epithelial cells on TT-FDAM

We cultured rabbit corneal epithelial cells using a previously reported system with some modifications [9]. Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/ml of mitomycin C (MMC) for 2 h at 37 °C under 5% CO_2 to inactivate proliferation. They were then rinsed with PBS, trypsinized, and plated onto plastic dishes at a density of 2×10^4 cells/cm^2. The TT-FDAM was spread, epithelial-basement-membrane side up, on the bottom of culture inserts (Corning Inc., NY, USA); these inserts were then placed in plastic dishes containing treated 3T3 fibroblasts without direct cell–cell contact. Rabbit corneal epithelial cells including the limbal region were incubated at 37 °C for 1 h with 1.2 IU dispase and then trypsinized at 37 °C for 30 min. The resultant cell suspension of corneal epithelial cells was then seeded onto TT-FDAM spread on the bottom of culture inserts and co-cultured with MMC-inactivated 3T3 fibroblasts without direct cell–cell contact. The culture was submerged in medium (defined keratinocyte growth medium (ArBlast Co., Ltd., Kobe, Japan) supplemented with 5% FBS) for 2 weeks and then exposed to air by lowering the medium level (airlifting) for the final day; the medium was changed daily.

2.7. Biocompatibility of TT-FDAM in vivo

Animals examined in this study were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine.

To investigate the compatibility of TT-FDAM with corneal tissue, we transplanted it into the intracorneal stroma ($N = 3$). This was carried out by marking the rabbit cornea 3.0 mm inside the limbus, after which a semilayer incision of the corneal stroma was performed using a micro-knife and a spatula. The TT-FDAM (4 × 4 mm) was then inserted into the intrastromal layer (Supplementary data, Fig. 1). One suture of 10–0 nylon was placed around the corneal wound. Corneal transparency and neovascularization were assessed by slit-lamp microscopy. The transplanted cornea (1 month after transplantation) was stained with hematoxylin and eosin (HE).

To further investigate the biocompatibility of TT-FDAM with the ocular surface, we transplanted it onto the bare sclera surface of a rabbit ($N = 3$). FDAM was also examined for comparison. The transplantation of both TT-FDAM and FDAM was carried out by the following method: first, we removed the rabbit conjunctiva (15 × 10 mm) with surgical scissors. The remaining severed edge of the conjunctiva was secured onto the sclera with 10–0 nylon, and after wiping the scleral surface with a micro-sponge the TT-FDAM and FDAM were then simply put on the sclera

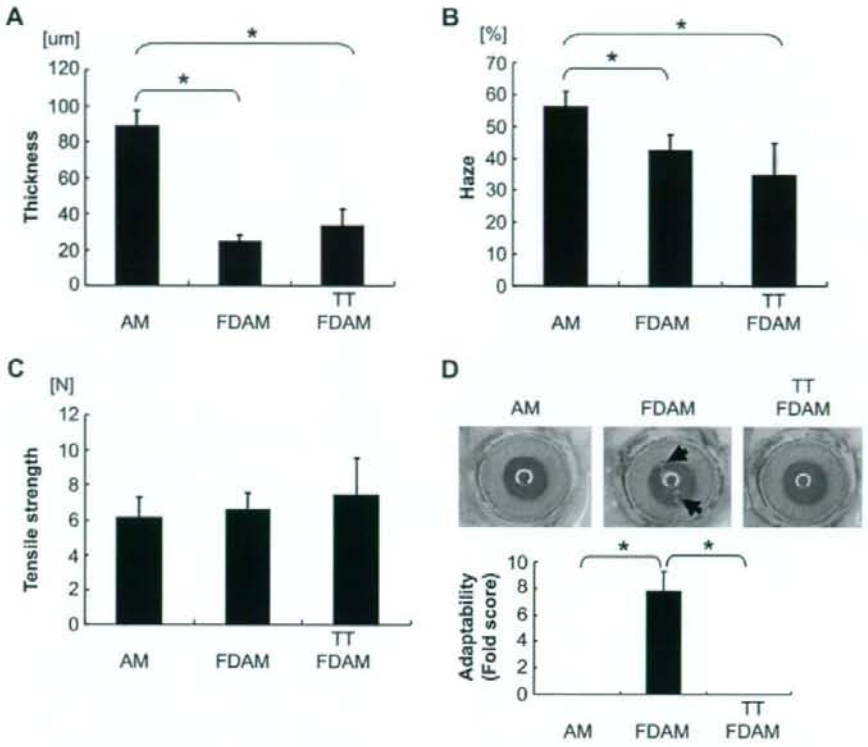

Fig. 1. The graphs show the results of thickness (A), haze (B), tensile strength (C), and adaptability (D) of AM, FDAM, and TT-FDAM, respectively. The thickness of FDAM and TT-FDAM was significantly reduced as compared to native AM (A: t-test, $^*p < 0.01$). The haze of FDAM and TT-FDAM tended to improve as compared to AM (B: t-test, $^*p < 0.01$). There were no statistically significant differences in physical tensile strength between AM, FDAM, and TT-FDAM (C: t-test, $p > 0.05$). TT-FDAM was smoother and more adaptable than FDAM (D: Mann–Whitney U-test, $^*p < 0.01$). TT-FDAM was almost identical to native AM.

surface with the epithelial-basement-membrane side facing up. At 4 weeks after transplantation, we examined the epithelialization and hyperemia of the surgical area by slit-lamp microscopy ($N = 3$).

2.8. Statistical analysis

For statistical assessment of the physical characteristics of AM, five different samples were analyzed by Student t-test or Mann–Whitney U-test. For statistical assessment of adherent cell ratios, five different samples were also analyzed by Student t-test.

3. Results

3.1. Appearance and morphological features of TT-FDAM

Visually, FDAM and TT-FDAM were so similar to each other in the dry condition that it was macroscopically difficult to distinguish any difference between the two. The FDAM and TT-FDAM were both wafer-like in appearance, very light and thin, and easy to handle and suture without tearing. Moreover, they both became smooth and flexible upon hydration, however, the TT-FDAM was smoother and more flexible than FDAM in the wet condition (Supplementary data, Movie 1). The results of the bacteriology tests of FDAM and TT-FDAM were all negative in our series.

3.2. Physical characteristics of TT-FDAM

The surface-scanning laser confocal displacement meter showed that the thickness of the AM, FDAM, and TT-FDAM was 88.48 ± 8.81, 24.24 ± 3.89, and 33.09 ± 9.32 μm (standard deviations (SD)), respectively, indicating that the freeze-drying process markedly reduced the thickness of the native AM (Fig. 1A, t-test, $p < 0.01$). The turbidimeter showed that the haze of the AM, FDAM, and TT-FDAM was 56.18 ± 5.00, 42.36 ± 5.22, and 34.75 ± 10.24 (SD), respectively, suggesting that the freeze-drying process decreased the haze of the AM (Fig. 1B, t-test, $p < 0.01$). The SS tests were performed on 10×30 mm samples to determine the maximum tear resistance of all membranes. Under wet conditions, AM, FDAM, and TT-FDAM showed a tearing strength of 6.12 ± 1.17, 6.59 ± 0.97, and 7.39 ± 2.18 gf (SD), respectively (Fig. 1C). There were no statistically significant differences in the physical tensile strength among AM, FDAM, and TT-FDAM (Fig. 1C, t-test, $p > 0.05$). Adaptability assay indicated that there was no AM fold when both AM and TT-FDAM were placed on the cornea. In contrast, there were some AM folds (7.8 ± 1.48, SD) when FDAM was placed on the cornea (Fig. 1D, Mann–Whitney U-test, $p < 0.01$). These results clearly indicated that TT-FDAM has better adaptability than FDAM and is almost identical to native AM.

3.3. Immunofluorescence of extracellular matrix molecules

Three individual AM, FDAM, and TT-FDAM samples were examined. The patterns of extracellular matrix molecule expression in the samples were investigated with immunohistochemistry. Negative control sections, incubated with normal mouse and rabbit IgG and without primary antibody, exhibited no discernible specific

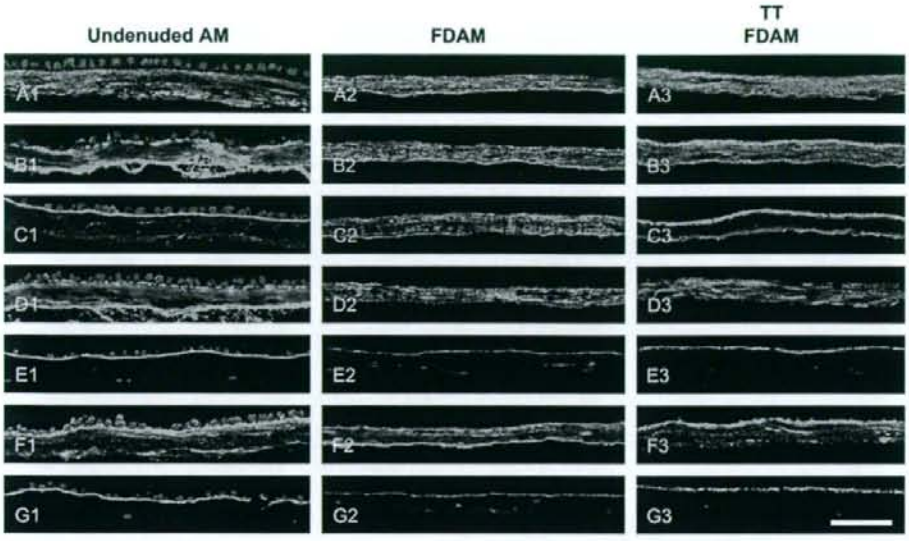

Fig. 2. Representative immunohistochemical staining of collagen 1 (A1–A3), collagen 3 (B1–B3), collagen 4 (C1–C3), collagen 5 (D1–D3), collagen 7 (E1–E3), fibronectin (F1–F3), and laminin 5 (G1–G3) in undenuded AM (A1–G1), FDAM (A2–G2), and TT-FDAM (A3–G3). Collagens 1, 3–5, and fibronectin were expressed throughout the entire AM, FDAM, and TT-FDAM samples. In contrast, collagen 7 and laminin 5 were expressed in the basement membrane side of AM. Nuclei were stained with propidium iodide (red). Scale bar: 100 μm.

immunoreactivity throughout the entire regions. The immunoreactivity observed in each of the three individual samples was compared with these controls. Immunohistochemistry showed the presence of collagens 1, 3–5, and fibronectin throughout the entire AM, FDAM, and TT-FDAM samples (Fig. 2A–D,F). In contrast, collagen 7 and laminin 5 were expressed on the basement membrane side of AM, FDAM, and TT-FDAM (Fig. 2E,G).

3.4. Electron microscopic examination of TT-FDAM

TEM showed the denuded AM surface to have the basal lamina remaining (Fig. 3A). The AM stroma contained widely spaced collagen fibrils and a few scattered cells. SEM showed the AM to be smooth, but under high magnification minute traces of the denuded cells were visible, giving the surface a slightly textured appearance (Fig. 3D). Next, TEM showed the denuded surface of FDAM to have the basal lamina remaining, but this appeared thinner than in the native AM (Fig. 3B). The FDAM stroma appeared very compressed with the collagen fibrils packed very tightly together. SEM showed the FDAM to be flat and featureless (Fig. 3E). All remaining traces of denuded cells on the FDAM were shrunk down, thus producing a flat featureless surface. Finally, TEM showed the denuded surface of TT-FDAM to have the basal lamina remaining (Fig. 3C). The appearance of the basal lamina was very similar to that of native AM. The stroma contained widely spaced collagen fibrils and a few scattered cells. SEM showed the TT-FDAM

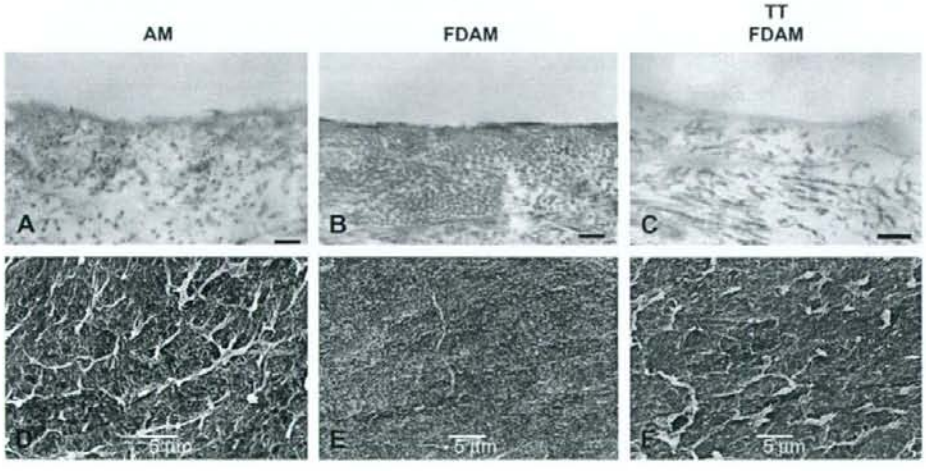

Fig. 3. Representative scanning and transmission electron micrographs of AM, FDAM, and TT-FDAM. The appearance of TT-FDAM (C, F) was more similar to that of AM (A, D) than to FDAM (B, E). In particular, the stroma of AM and TT-FDAM is less compacted than that of FDAM (A–C). Scale bar = 200 nm (A–C) and 5 μm (D–F).

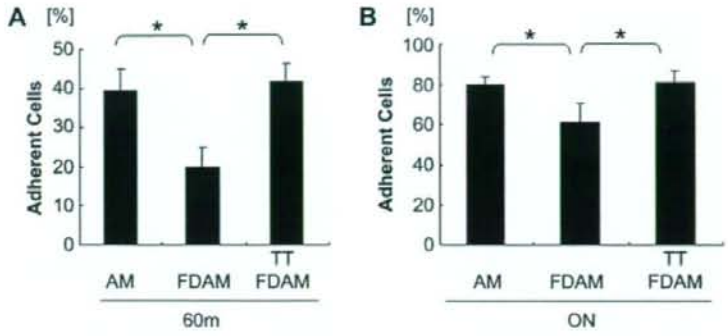

Fig. 4. The adhesion property of AM, FDAM, and TT-FDAM was evaluated by incubating rabbit corneal limbal epithelial cells for different lengths of time including 60 min (A: *t*-test, $^{*}p < 0.01$) and overnight (B: *t*-test, $^{*}p < 0.01$). The adhesion property of TT-FDAM was almost identical to that of native AM.

to be generally smooth, but at high magnification slight traces of the denuded cells remained, giving a textured appearance similar to that observed in native AM (Fig. 3F).

3.5. Adhesion of corneal epithelial cells to AM matrix

The adhesion property of AM, FDAM, and TT-FDAM was evaluated by incubating rabbit corneal epithelial cells for different lengths of time (60 min and overnight). For the 60-min incubation, the percentage of adherent cells on AM, FDAM, and TT-FDAM was 39.3 ± 5.88, 19.9 ± 5.1, and 41.7 ± 4.63 (SD), respectively (Fig. 4A). For the overnight incubation, the percentage of adherent cells on AM, FDAM, and TT-FDAM was 79.8 ± 4.11, 61.4 ± 9.92, and 81.1 ± 5.93 (SD), respectively (Fig. 4B). These findings indicated that the adhesion property of TT-FDAM was better than that of FDAM, and was almost identical to native AM (Fig. 4, *t*-test, $p < 0.01$).

3.6. Fabrication of cultivated corneal epithelial sheet on TT-FDAM

Corneal epithelial cells began to form colonies on the TT-FDAM within 3 days. After 7 days in culture, a confluent primary culture of corneal epithelial cells had been established that covered the entire TT-FDAM. At 2 weeks, the cultivated corneal epithelial cells showed 4–5 layers of stratification, were well differentiated, and appeared very similar to normal corneal epithelium (Fig. 5A). These sheets showed immunoreactivity for cornea-specific keratin 3, but not epidermal-specific keratin 10 or mucosal-specific keratin 13 (Fig. 5B–D).

3.7. Biocompatibility of TT-FDAM

One month after intracorneal TT-FDAM transplantation, we observed the transplanted rabbit corneal surface by slit-lamp microscopy. The TT-FDAM clarity was markedly improved and there was no neovascularization on the corneal surface (Fig. 6A,B). All of

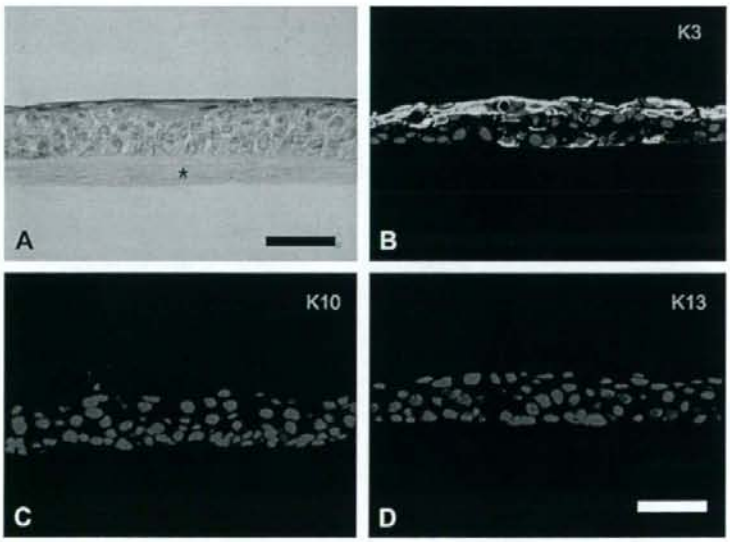

Fig. 5. Light micrograph showing a representative cross-section of the cultivated corneal epithelial cells on TT-FDAM stained with hematoxylin and eosin (A). The cultivated corneal epithelial sheet had 4–5 layers of stratified, well-differentiated cells and appeared very similar to *in vivo* normal corneal epithelium (A). Asterisks indicated TT-FDAM (A). Light micrographs showing immunohistochemical staining for keratin 3 (B), keratin 10 (C), and keratin 13 (D). These sheets showed immunoreactivity for cornea-specific keratin 3, but not epidermal-specific keratin 10 or mucosal-specific keratin 13. Scale bar: 50 μm (A–D).