the *HLA* types and 6p21 SNPs of the 141 Japanese SJS/TEN cases and an additional 65 Japanese individuals (non-SJS/TEN patients). The LD coefficient was calculated as previously described.[15,16]

Association analysis
Genome-wide SNPs data from allopurinol-related SJS/TEN cases and ethnically matched controls were used for association analysis using the Fisher's exact test based on the dominant genotype mode and minor allele frequencies of each SNP. Because there are no homozygotes of minor alleles of SNPs, which have significantly related to allopurinol-related SJS/TEN except rs3099844 and rs3131643 in 'Case group', other association analysis models such as trend test (Cochran–Armitage analysis) or recessive model analysis were not applied in this study. All association analyses were carried out with PLINK.[14] *P*-values were corrected for multiple testing according to the Bonferroni's correction. *P*-values $<5.62 \times 10^{-8}$ were regarded as statistically significant.

Results

Characteristics of study subjects
A total of 14 allopurinol-treated Japanese patients, who were diagnosed with definite SJS/TEN were recruited for the whole-genome association study (IDs 1–14 in Table 1). Patients, IDs 1, 2, 3, 9, 10, 13 and 14 were reported in our previous paper.[10] After the GWAS, an additional four allopurinol-treated Japanese SJS/TEN patients were recruited for *HLA* typing (IDs 15–18). Therefore, a total of 18 allopurinol-treated Japanese SJS/TEN patients participated in the study (Table 1). In all, 12 of 18 patients were male and 6 were female, and the average age was 72.3 ± 10.0 (mean ± s.d.) years. In all, 12 of 18 cases showed systemic complications of liver and/or renal dysfunction, and most patients had high fever. The average period of SJS/TEN onset after allopurinol treatment was 21.7 ± 11.9 days. Drug-induced lymphocyte stimulation tests were examined in 13 of 18 patients to determine the causative agent; however, in these tests, only two cases (IDs 1 and 5) were positive for allopurinol and only one (ID 16) was positive for oxipurinol, a metabolite of allopurinol. The patient (ID 1) who was positive for the drug-induced lymphocyte stimulation test for allopurinol was also positive for other co-administrated drugs (Table 1). On the other hand, patients who received a patch test showed positive reactions for allopurinol although only two patients were examined (ID 4, 10). The patient who was patch test positive for allopurinol (ID 4) was also patch test positive for other co-administrated drugs (Table 1). Four patients (ID 1, 2, 4 and 14) were co-administrated non-steroidal anti-inflammatory drugs, four (ID 7, 8, 11 and 15) were co-administrated angiotensin II receptor antagonists and three (ID 4, 7 and 17) were co-administrated statin anti-hyperlipemic agents.

Whole-genome association study of major determinants for allopurinol-related SJS/TEN
A total of 14 allopurinol-related SJS/TEN patients (IDs 1–14), who were diagnosed with definite SJS/TEN, and 991 ethnically matched controls, were genotyped with the use of the Illumina Human 1M-Duo BeadChip containing 11 63 218 SNPs. A series of quality-control steps resulted in the elimination of 272 897 polymorphisms. For each SNP, Fisher's exact tests were performed to compare the dominant genotype distributions and minor allelic frequencies in the allopurinol-related SJS/TEN patients (the case group) versus those in the ethnically matched healthy control group. The resulting *P*-values were adjusted with the Bonferroni's correction ($P<5.62 \times 10^{-8}$). The distribution of *P*-values from the Fisher's exact tests (dominant genotype mode) along each chromosome indicated that 21 SNPs were significantly associated with the cases, all of which were located on the chromosome 6: 6p21.3, 6p22.1 and 6p21.1 (Figures 1a and b). The quantile–quantile (Q–Q) plot for the distribution of *P*-values showed that observed *P*-values matched the expected *P*-values over the range of $0<-\log_{10}(p) < 4.0$ (Figure 2). A departure was observed at the extreme tail ($-\log_{10}(p)>4.0$) of the distribution of test statistics for the allopurinol-related Japanese SJS/TEN, suggesting that the identified associations are likely due to true variants rather than potential biases such as genotyping error. These SNPs, with their associated genes, are described in Table 2. As is observed in all SNPs in Table 2, minor allele frequencies in the controls were quite small, ranging around 0.5–0.6%. The genotypic distributions of the case and control groups are identical among groups with the same *P*-value, suggesting that these SNPs might be linked. These SNPs also have ORs that are much higher than the ORs of SNPs commonly observed in sporadic cancer and other complex diseases, suggesting they are of higher penetrance. For example, the most significant SNPs (rs2734583, rs3094011 and GA005234) had an OR of 66.8 (95% confidence interval, 19.8–225.0), and the twentieth most significant SNPs (rs9263827 and rs1634776) had an OR of 60.9 (95% confidence interval, 18.3–202.5). Most SNPs in Table 2 are associated with known or predicted genes; of these, 13 are in known genes. Three SNPs (rs17190526, rs9263726 and rs2233945) were found in *PSORS1C1* (psoriasis susceptibility 1 candidate 1), which is considered as one of the potential psoriasis genes.[17–19] The *CCHCR1* (coiled coil α helical rod protein 1), which is a regulator of keratinocyte proliferation or differentiation and is over-expressed in keratinocytes in psoriatic lesions,[20–23] contained four SNPs (rs9263745, rs130077, rs9263781 and rs9263785). *HCP5* (HLA complex P5), which is involved in hypersensitivity to abacavir,[24–26] had three SNPs (rs3094011, rs3099844 and rs31431643). *TCF19* (transcription factor 19), which is a potential trans-activating factor that might play an important role in the transcription of genes required for the later stages of cell cycle progression,[27] contained two SNPs (rs9263794 and rs10448701). Two SNPs (rs9263796 and rs9263800) were also found in *POU5F1* (POU class 5 homeobox; alternative names for Oct4). *BAT1* (HLA-B

Table 1 Summary of clinical characteristics of Japanese patients with allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis

Patient ID[a]	ADR type	Sex/age (years)	Highest BT (°C)	Total area of blistering skin (%)	Systemic complications	DLST to allopurinol (PT)	Period of onset (days) by allopurinol	Co-administered drugs: Drug name	Co-administered drugs: DLST result/period of onset
1	SJS	F/53	38.1	0.5	liver dysfunction	+	26	loxoprofen	+/9 days
					renal dysfunction			clarithromycin	+/26 days
2	TEN	M/58	37.1	15	neutropenia	–	ca 10 days	loxoprofen	–/1 day
					liver dysfunction			levofloxacin	–/1 day
3	SJS	M/77	unknown	unknown	none	not tested	16	none	
4	TEN	F/72	>37	20	none	–(PT+)	16	pitavastatin	–/16 days
								lansoprazole	–/179 days
								salicylamide, acetaminophen, caffeine, promethazine, methylenedisalicylate	–(PT+)/8 days
								serrapeptase	–/1 day
								loxoprofen	–/8 days
								acetaminophen	(PT+)/8 days
5	TEN	M/82	39	35	none	+	52	none	
6	SJS	M/67	1	1	liver dysfunction	not tested	14	none	
7	SJS	M/76	38.8	unknown	GI tract disturbance	not tested	<26 days	losartan	not tested/8 days
					liver dysfunction			furosemide	not tested/3 days
					renal dysfunction			carbon	not tested/7 days
								atorvastatin	not tested/8 days
8	SJS	M/83	>38	10	renal dysfunction	–	20	amlodipine	not tested/very long
								olmesartan medoxomil	not tested/very long
9	TEN	M/75	>38	20	neutropenia	–	6	none	
					liver dysfunction				
					renal dysfunction				
10	SJS	M/75	38.4	6	neutropenia	–(PT+)	14	none	
					liver dysfunction				
					renal dysfunction				
11	SJS	M/74	37.8	8	neutropenia	–	38	cefazolin	not tested/1 day
					liver dysfunction			Furosemide	not tested/53 day
					renal dysfunction			Sodium polystyrene sulfonate	not tested/51 day
								olmesartan medoxomil	not tested/59 day
12	SJS	M/67	38.9	2	liver dysfunction	not tested	17	none	
13	SJS	F/81	39.2	0.5	renal dysfunction	–	28	spironolactone	–/24 days
14	SJS	M/83	39	0	respiratory involvement	–	29	diclofenac	–/1 day
15	TEN	F/73	38	10	liver dysfunction	–	27	valsartan	–/18 days
					renal dysfunction			epoetin β	–/2 days
16	SJS	M/53	40	5	liver dysfunction	–(oxipurinol +)	19	none	
17	SJS	F/86	38	0	liver dysfunction	–	30	rosuvastatin	–/43 days
					renal dysfunction				
18	TEN	F/66	37.8	15	none	not tested	2	none	

Abbreviations: ADR, adverse drug reaction; BT, body temperature; DLST; drug-induced lymphocyte stimulation test; F, female; M, male; PT, patch test; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

[a]Patients ID 1–14 were applied for whole genome analysis. ID 1–18 were for the *HLA* typing and the analysis of linkage disequilibrium.

Patients IDs 1, 2, 3, 9, 10, 13, and 14 were reported in our previous paper.[10]

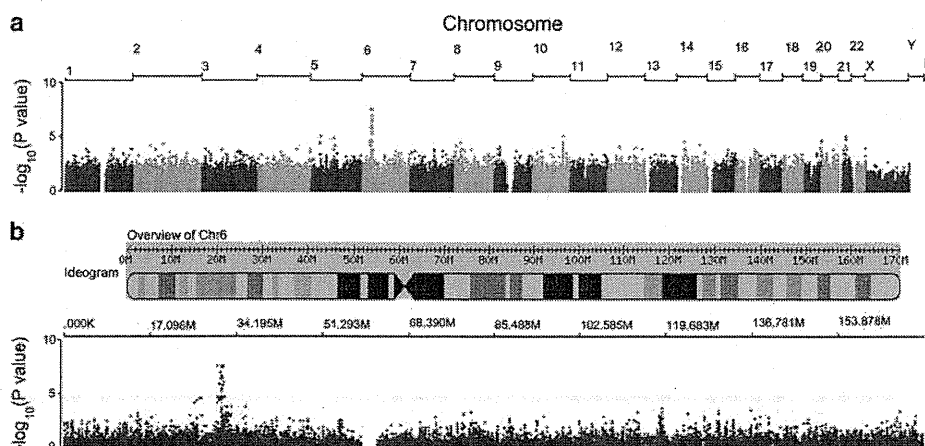

Figure 1 Genome-wide association study of allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis. Each dot represents a single nucleotide polymorphism (SNP). The x axis: the position of the SNP on chromosomes. The y axis: the $-\log_{10}$ of Fisher's exact test P-values (dominant genotype mode) of the SNP in the case–control association study. SNPs with P-values $<5.62\times10^{-8}$ are highlighted in red. (**a**) Whole genome. (**b**) Chromosome 6.

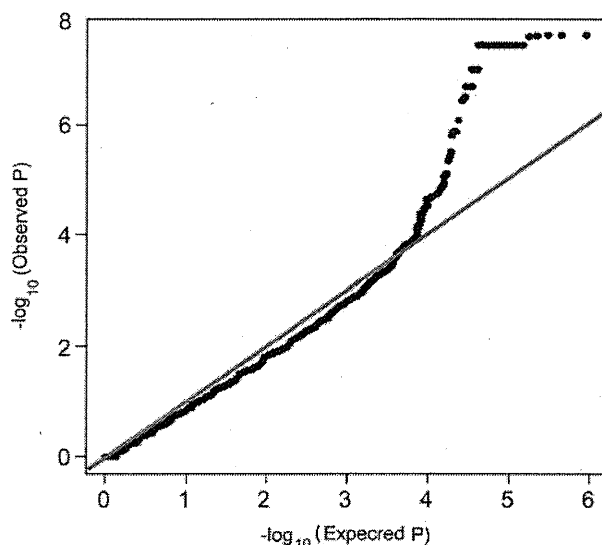

Figure 2 Quantile–quantile plot of Fisher's exact test statistics obtained from the genome-wide association study for allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis under dominant genotype mode. The solid red line represents the null model where observed Fisher's exact test values match the expected values. The dots represent observed versus the expected values from the case–control study.

associated transcript 1) and *PSORS1C3* each carried one SNP (rs2734583 and rs9263827). The SNPs, rs1634776 and rs4084090, were located in more than 10 kb away from the *HLA-B* and *HLA-C* genes, respectively. Two pseudo genes, *MICC* (major histocompatibility complex class I polypeptide-related sequence) and *PPIAP9* (peptidylprolyl isomerase A (cyclophilin A) pseudogene 9), had one SNP each (GA005234 and rs9267445). Previous report using Han-Chinese patients with allopurinol-induced SCAR indicated rs3117583 of *BAT3*, rs1150793 of *MSH5* and rs2855804 of *MICB*, which are located in *HLA* region, showed significant P-values ($P<1\times10^{-7}$).[7] In this study using Japanese patients, both rs3117583 and rs1150793 showed $P=6.34\times10^{-3}$ (allele frequency mode) and $P=6.14\times10^{-3}$ (dominant genotype mode). There was no data of rs2855804 in the Illumina Human 1M-Duo BeadChip.

HLA types of allopurinol-related SJS/TEN patients

Classical class I *HLA* types (*A*, *B* and *Cw*) of allopurinol-related SJS/TEN patients were determined because the *HLA-B*5801* type is associated with allopurinol-related SCARs in Han Chinese,[7] Caucasians[9] and Japanese[10] (Table 3). In this analysis, four patients with allopurinol-related SJS/TEN (IDs 15–18), who were recruited after BeadChip analysis, joined the case group (total of 18 allopurinol-related SJS/TEN patients). Eight cases of *HLA-A*3303* (allele frequency = 22.2%), 10 cases of *HLA-B*5801* (allele frequency = 27.8%) and 10 cases of *HLA-Cw*0302* (allele frequency = 27.8%) were found in 18 allopurinol-related SJS/TEN patients (Table 3). By comparison, the allelic frequencies of *HLA-A*3303*, *HLA-B*5801* and *HLA-Cw*0302* were 7.9%, 0.6% and 0%, respectively in Japanese general population (Tables 4a–c). The OR of *HLA-A*3303* was calculated as 3.32 (Table 4a). The OR of *HLA-B*5801* was calculated as 62.8 (Table 4b), which was a little larger than the previously reported OR in Japanese patients.[10] *HLA-Cw*0302* also showed significant association with allopurinol-related SJS/TEN (Table 4c). *HLA-A*3303* and *HLA-Cw*0302* are in LD with *HLA-B*5801* in the Japanese although the general frequency of *HLA-A*3303* is higher than other two types. Other *HLA-A*, *B* and *Cw* types, which were not listed in Tables 4a–c, showed very low frequencies in the general Japanese population, or were not found in 18 allopurinol-related SJS/TEN patients.

Table 2 The association of single nucleotide polymorphism with allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

Order	SNP	Chromosome	Closest gene	Distance to gene (bp)	Case[a]	Control[a]	Dominant genotype mode		Allelic frequency mode	MAF (%)
							P	Odds ratio (95% CI)	P	
1	rs2734583	6p21.3	*BAT1*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	rs3094011	6p21.3	*HCP5*	6553	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	GA005234	6p22.1	*MICC*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
4	rs3099844	6p21.3	*HCP5*	3693	1/5/8	0/11/978	2.47×10^{-8}	66.7 (19.8–224.5)	1.33×10^{-9}	0.56
5	rs9267445	6p21.1	*PPIAP9*	3776	0/6/8	0/11/971	2.58×10^{-8}	66.2 (19.7–222.9)	4.87×10^{-8}	0.56
6	rs17190526	6p21.3	*PSORS1C1*	−446	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263726	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs2233945	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263733	6p21.3	*POLR2LP*	139	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263745	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs130077	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263781	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263785	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263794	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.47×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs1044870	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.58×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263796	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263800	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs4084090	6p21.3	*HLA-C*	17691	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
19	rs3131643	6p21.3	*HCP5*	0	1/5/8	0/12/977	3.68×10^{-8}	61.1 (18.4–203.1)	2.08×10^{-9}	0.61
20	rs9263827	6p21.3	*PSORS1C3*	−3369	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61
20	rs1634776	6p21.3	*HLA-B*	12661	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61

Abbreviations: CI, confidence interval; MAF; minor allelic frequency; SNP, ingle nucleotide polymorphism.
[a]Number of subjects in minor homo/hetero/major homo.

Table 3 *HLA* types and representative genotypes in 6p21 of allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

ID	HLA-A		HLA-B		HLA-Cw		*rs2734583*	*rs3099844*	*rs9267445*	*rs9263726*	*rs3131643*	*rs1634776*
1	2402	3303	4002	**5801**	0302	0304	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
2	2402	3101	1501	5601	0303	0401	T/T	C/C	G/G	G/G	C/C	G/G
3	2402	3101	5201	**5801**	0302	1202	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
4	1101	1101	4801	**5801**	0302	0803	T/**C**	**A/A**	G/**C**	G/A	**T/T**	G/A
5	2402	2602	4006	5101	0801	1402	T/T	C/C	G/G	G/G	C/C	G/G
6	0201	1101	1518	3501	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
7	2402	3303	5201	**5801**	0302	1202	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
8	0201	2402	1527	4003	0304	0401	T/T	C/C	G/G	G/G	C/C	G/G
9	2402	2402	3501	5201	0303	1202	T/T	C/C	G/G	G/G	C/C	G/G
10	0210	1101	4002	4006	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
11	0207	2402	4601	5101	0102	1402	T/T	C/C	G/G	G/G	C/C	G/G
12	2402	3101	3901	4001	0304	0702	T/T	C/C	G/G	G/G	C/C	G/G
13	0207	3303	4601	**5801**	0102	0302	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
14	3101	3303	3901	**5801**	0302	0702	T/**C**	C/A	G/**C**	G/A	C/**T**	G/A
15	2402	3303	5101	**5801**	0302	1402	T/**C**	C/A	NA	G/A	**T/T**	NA
16	0201	3303	3802	**5801**	0302	0702	T/**C**	C/A	NA	G/A	**T/T**	NA
17	2402	3303	0702	**5801**	0302	0702	T/**C**	C/A	NA	G/A	C/**T**	NA
18	2402	3303	5101	**5801**	0302	0304	T/**C**	C/A	NA	G/A	**T/T**	NA

Abbreviations: HLA, human leukocyte antigen; NA, not available.
Single nucleotide polymorphisms data of rs2734583, rs3099844, rs9263726 and rs3131643 are from BeadChip analysis and TaqMan genotyping analysis. Single nucleotide polymorphisms data of rs9267445 and rs1634776 are from BeadChip analysis.
Underlines of HLA types mean that these types are in linkage disequilibrium. HLA-B*5801s are expressed by bold types.
Bold types of the nucleotide mean the variant allele.

Table 4a Association between *HLA-A* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-A allele	*Number of alleles detected (allele frequency)*		P	Odds ratio (95% CI)
	Case, n=36 (%)	*General population control (n=986)[a] (%)*		
0201	3 (8.3)	10.9	0.7895	
0206	0 (0)	10.4	0.0426	
0207	2 (5.6)	3.4	0.3650	
0210	1 (2.8)	0.1	0.0692	
1101	4 (11.1)	8.1	0.5299	
2402	13 (36.1)	35.6	1.000	1.02 (0.51–2.04)
2601	0 (0)	9.8	0.0417	
2602	1 (2.8)	2.2	0.5657	
3101	4 (11.1)	7.7	0.5195	
3303	8 (22.2)	7.9	0.0077	3.32 (1.46–7.54)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-A* types of which the allele frequencies in the Japanese population are more than 9% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

Table 4b Association between *HLA-B* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-B allele	*Number of alleles detected (allele frequency)*		P	Odds ratio (95% CI)
	Case, n=36 (%)	*General population control (n=986)[a] (%)*		
0702	1 (2.8)	5.2	1.000	
1501	1 (2.8)	7.2	0.5076	
1518	1 (2.8)	0.9	0.3025	
1527	1 (2.8)	0	0.0352	
3501	2 (5.6)	8.6	0.7621	
3802	1 (2.8)	0.3	0.1338	
3901	2 (5.6)	4.0	0.6520	
4001	1 (2.8)	5.1	1.0000	
4002	2 (5.6)	8.2	0.7620	
4003	1 (2.8)	1.1	0.3512	
4006	2 (5.6)	5.3	0.7150	
4403	0 (0)	6.9	0.1648	
4601	2 (5.6)	3.8	0.6441	
4801	1 (2.8)	2.7	1.0000	
5101	4 (11.1)	7.9	0.5244	
5201	3 (8.3)	13.7	0.4624	
5401	0 (0)	6.5	0.1620	
5601	1 (2.8)	1.0	0.3273	
5801	10 (27.8)	0.6	5.388×10^{-12}	62.8 (21.2–185.8)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-B* types of which the allele frequencies in the Japanese population are more than 6.5% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

*LD of HLA-B*5801 with SNPs on chromosome 6*

We compared the genotypic distributions of six SNPs, which were significantly associated with SJS/TEN (Table 2), with *HLA* types because these SNPs are located near the *HLA-B* gene. These 6 SNPs listed in Table 3 represent 21 SNPs in Table 2 because the other 15 SNPs are in absolute LD with 1 of the 6 SNPs. Representative six variants of the significant SNPs on chromosome 6 were found in all of the SJS/TEN patients who carried the *HLA-B*5801* (10 patients) (Table 3). Therefore, in order to evaluate LD in the Japanese

Table 4c Association between *HLA-Cw* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-Cw allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 234)[a] (%)*		
0102	2 (5.6)	17.0	0.0859	
0302	10 (27.8)	0	5.303×10^{-10}	
0303	2 (5.6)	7.8	1.000	
0304	4 (11.1)	11.3	1.000	
0401	4 (11.1)	6.5	0.2961	
0702	4 (11.1)	11.3	1.000	
0801	3 (8.3)	10.9	0.7777	
0803	1 (2.8)	2.6	1.000	
1202	3 (8.3)	10.4	1.000	
1402	3 (8.3)	5.7	0.4559	
1403	0 (0)	12.2	0.0192	

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-Cw* types of which the allele frequencies in the Japanese population are more than 10% or which were detected in this study.
[a]General population control data are cited from Tokunaga *et al.*[41]

Table 5 The linkage disequilibrium between *HLA* types and representative single nucleotide polymorphisms on 6p21 of 206 Japanese individuals

HLA	*rs3099844*	*rs3131643*	*rs2734583*	*rs9267445*	*rs9263726*	*rs1634776*
A	0.821	0.621	0.835	0.798	0.847	0.803
B	0.973	0.873	1.000	1.000	1.000	0.996
Cw	0.984	0.773	1.000	1.000	1.000	0.909

Abbreviation: HLA, human leukocyte antigen.
Data are expressed in *D′*.

Table 6 The linkage disequilibrium between representative single nucleotide polymorphisms on 6p21 and *HLA-B*5801* of 206 Japanese individuals

SNP	*D′*	r^2
rs3099844	0.930	0.866
rs3131643	0.929	0.674
rs2734583	1.000	0.931
rs9267445	1.000	0.896
rs9263726	1.000	1.000
rs1634776	1.000	0.905

Abbreviation: SNP, single nucleotide polymorphism.

population, LD coefficients (*D′*) were calculated between classical class 1 *HLA* types and six representative SNPs at 6p21, using the *HLA*-type and SNPs genotype data of 206 Japanese individuals, including 141 SJS/TEN cases and an additional 65 non-SJS/TEN Japanese subjects. As shown in Tables 5 and 6 representative SNPs on chromosome 6 showed LD for the *HLAs*. In particular, three SNPs (rs2734583, rs9267445 and rs9263726) showed a strong linkage with *HLA-B* and *Cw* alleles (Table 5). LD between six representative SNPs in 6p21 and *HLA-B*5801* are shown in Table 6. A novel observation was the absolute LD ($D' = 1$, $r^2 = 1$) between rs9263726 in *PSORS1C1* and the *HLA-B*5801* allele.

Discussion

In order to explore new genetic biomarkers associated with the occurrence of allopurinol-related SJS/TEN Japanese patients, we conducted a GWAS using 890321 SNPs from patients with allopurinol-related SJS/TEN and an ethnically matched control group. The GWAS data indicated that most SNPs significantly associated with allopurinol-related SJS/TEN are located on or close to genes that overlap the 6p21 region, especially the genes neighboring *HLA-B*. There was no significantly associated SNP in any other region of the genome (Figures 1 and 2 and Table 2), indicating that the 6p21 region has the most important role in the progress of allopurinol-related SJS/TEN. We expected to find SJS/TEN-associated SNPs, which are unrelated to *HLA-B*5801* from this GWAS study because the association of *HLA-B*5801* with SJS/TEN is incomplete (10/18) in Japanese patients in contrast to Han Chinese[7] and Thai patients.[8] However, most

of significant SNPs were closely linked with *HLA-B*5801* (Table 6). Previous studies have indicated that a SNP (rs2395029) in the *HCP5*, which is on 6p21.3, is strongly associated with human immunodeficiency virus-1 set points,[28–30] abacavir-induced hypersensitivity[24–26] and flucloxacillin-induced liver injury.[31] This SNP is in strong LD with *HLA-B*5701* in Caucasians.[25] Another SNP in 6p21 in *PSORS1C1*, a psoriasis-susceptibility candidate gene, was related with psoriasis in Swedish and Canadian populations[17,18] and exhibits LD with *HLA-Cw*0602* in Canadian populations.[18] These reports suggest that SNPs located in 6p21 link with a specific type of classical class I *HLA* that could be an alternative biomarker for the physiological phenomenon. Therefore, we examined the LD between these SNPs, shown in Table 2, and *HLA-B*5801*, which has been regarded as a genetic biomarker of SJS/TEN not only in Han Chinese,[7] but also in Caucasians[9] and Japanese.[10] We found that all of the Japanese patients with the allopurinol-related SJS/TEN who had the *HLA-B*5801* (10 patients) also had variant SNPs of genes that are located in 6p21, including *BAT1*, *HCP5*, *PPIAP9*, *PSORS1C1* and *HLA-B* (Table 3). The analysis of the LD coefficients between SNPs located in 6p21 and *HLA* types in the Japanese population indicated that these SNPs are in strong LD with *HLA* types (Table 5), and an absolute LD between rs9263726 in *PSORS1C1* and *HLA-B*5801* was observed in the Japanese population (Table 6). These results mean that all subjects (14 individuals including 10 with allopurinol-related SJS/TEN) who carry *HLA-B*5801* are in complete accord with all subjects with minor A allele of rs9263726 in the Japanese population. Therefore, rs9263726 in *PSORS1C1* is an alternative biomarker for *HLA-B*5801* in the Japanese population. Conventional genotyping of rs9263726 based on allelic discrimination offers several advantages over *HLA-B* typing, which is determined by genotyping of several SNPs forming the *HLA-B*5801* haplotype. Various broadly used technologies (for example, TaqMan genotyping) allow the standardized identification of two distinct alleles in one reaction tube, limiting the risk of contamination and allowing high-throughput genotyping with high sensitivity and specificity. In addition, the test is largely independent of both the performance of and interpretation by laboratory personnel. SNP genotyping is also less time consuming and cheaper than sequence-based *HLA* typing, and it does not require specialized laboratories. Therefore, the easy detection of these SNPs has a practical and economical advantage in clinical application for predicting the onset of allopurinol-related SJS/TEN. Although the previous report revealed that three SNPs in *HLA* region strongly associated with allopurinol-related SCAR in Han Chinese,[7] the two SNPs analyzed by the Illumina Human 1M-DUO BeadChip showed only weak association in the Japanese. This ethnic difference might be due to the difference of LD.

The functional analysis of genes that carry these SNPs—including *HCP5*, *BAT1*, *PSORS1C1*, *CCHCR1*, *TCF19* and *POU5F1*—in the pathogenesis of allopurinol-related SJS/TEN might be useful for determining their relevance. *CCHCR1* is a regulator of keratinocyte proliferation or differentiation and is overexpressed in keratinocytes in psoriatic lesions.[20–23] *TCF19* is a potential trans-activating factor that could play an important role in the transcription of genes required for the later stages of cell cycle progression.[27] Possible psoriasis candidate genes near *HLA-B* include *PSORS1C1*,[17–19] *CCHCR1*,[22,23] and *POU5F1*.[32,33] Mutations in *BAT1* may be associated with rheumatoid arthritis.[34–36] *HCP5* encodes an endogenous retroviral element mainly that is expressed in immune cells and there is evidence that the SNP in this gene is protective against human immunodeficiency virus-1 infection.[37–39] The functions and relevance of these genes suggest that the pathogenesis of allopurinol-related SJS/TEN might involve not only an immune system disorder, but also processes of cell proliferation and differentiation.

In conclusion, the results of this GWAS of allopurinol-related SJS/TEN in Japanese patients show that SNPs in genes located in 6p21, which are in LD with *HLA-B*5801*, are strongly associated with the cutaneous adverse reaction. Therefore, these SNPs, especially rs9263726, prove to be predictors for allopurinol-related SJS/TEN in Japanese, and their genes might be involved in the pathogenesis of allopurinol-related SJS/TEN. The OR of rs9263726 is extremely high from this case–control study and the typing cost of SNP is much cheaper than that of *HLA* typing. Moreover, the SJS/TEN has a very severe adverse reaction of allopurinol, which is high mortality. Therefore, we believe that the screening of rs9263726 genotype before allopurinol administration is necessary to prevent SJS/TEN in allopurinol-treated Japanese patients, although its allele frequency is very low in the Japanese. Association analyses of other ethnic populations are needed for confirming and comparing the results obtained in this study. *In vitro* functional studies of these genes are also necessary for identification of the physiological and molecular pathways leading to allopurinol-related SJS/TEN.

Conflict of interest

The authors declare no conflict of interest except one member of JPDSC, Mitsubishi Tanabe Pharma, which is a distributor of allopurinol in Japan.

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Appendix

Japan Pharmacogenomics Data Science Consortium (JPDSC)

The Japan Pharmacogenomics Data Science Consortium is composed of Astellas Pharma, Otsuka Pharmaceutical, Daiichi Sankyo, Taisho Pharmaceutical, Takeda Pharmaceutical and Mitsubishi Tanabe Pharma, and is chaired by Ichiro Nakaoka (Takeda Pharmaceutical).

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Note

Development of a Rapid and Inexpensive Assay for Detecting a Surrogate Genetic Polymorphism of *HLA-B*58:01*: A Partially Predictive but Useful Biomarker for Allopurinol-related Stevens-Johnson Syndrome/toxic Epidermal Necrolysis in Japanese

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Allopurinol-induced Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) is strongly associated with *HLA-B*58:01* in various populations including Japanese. We demonstrated that several single nucleotide polymorphisms (SNPs) around the *HLA* region on chromosome 6 were strongly linked with *HLA-B*58:01* in a previous study using Japanese allopurinol-related SJS/TEN patients. Their very strong linkage suggests that these SNPs could be used as surrogate biomarkers to find carriers of *HLA-B*58:01* to avoid these serious adverse effects. In the present study, to expedite the application of this pharmacogenomic information to the proper usage of allopurinol in a clinical situation, we developed a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay for the genotyping of rs9263726 in the *psoriasis susceptibility 1 candidate 1* (*PSORS1C1*) gene, which is in absolute linkage disequilibrium ($r^2 = 1$, $D' = 1$) with *HLA-B*58:01*. The developed PCR-RFLP assay using FokI restriction enzyme was able to detect three different genotypes, GG, GA, and AA of rs9263726 robustly, and thus to find *HLA-B*58:01* carriers. This robust and inexpensive assay would be useful for pre-screening the subjects with *HLA-B*58:01*, a genetically high risk factor for allopurinol-induced SJS/TEN.

Keywords: allopurinol; PCR-RFLP; screening test; Stevens-Johnson syndrome; toxic epidermal necrolysis

Introduction

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe cutaneous adverse reactions (SCARs).[1] SJS and TEN, considered variants of the same skin disorder, are characterized by the development of limited (in SJS) or widespread (in TEN) detachment and blistering of the skin epidermis and mucous epithelium, often with organ involvement.[1,2] The incidence of SJS/TEN is very rare, estimated to occur at about 2 patients per million individuals per year in Caucasians,[3] but these SCARs require intensive care due to the high mortality rates (1–5% for SJS and 20–30% for TEN) and long-term treatments for subsequent complications, especially ocular pathologies.[1] SJS/TEN are idiosyncratic SCARs that have been considered, for a long time, to be difficult to predict, but human lymphocyte antigen (HLA) types have recently been reported to be associated with the onset of SJS/TEN in a drug-specific manner.[1,4]

Allopurinol is a widely-prescribed urate-lowering drug and has known to be the most common causative drug for SJS/TEN in Japan.[4,5] In 2005, Hung *et al.* reported that an *HLA* allele *B* variant, *HLA-B*58:01*, is strongly associated with allopurinol-induced SCARs consisting of SJS, TEN and

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hypersensitivity syndrome in a Han Chinese population.[6] They found that 100% (51/51 patients) of the case patients had this *HLA* type, while only 15% (20/135 patients) of the tolerant control had, giving an odds ratio (OR) of 580.3 (sensitivity = 100%, specificity = 85%). This association was later confirmed in Thai (SJS/TEN patients, OR = 348.3, sensitivity = 100%, specificity = 87%),[7] Korean (SJS/TEN/drug-induced hypersensitivity syndrome patients, OR = 97.8, sensitivity = 92%, specificity = 89%),[8] European (SJS/TEN patients, OR = 80, sensitivity = 56%)[9] and Japanese (SJS/TEN patients, OR = 62.8, sensitivity = 56%)[10,11] populations. Although the associations have been partial, especially in Europeans and Japanese, *HLA-B**58:01* is thought to be a useful biomarker for allopurinol-induced SJS/TEN.

A recent report showed that based on the very strong association of the *HLA-B**15:02* allele with SJS/TEN in the Han Chinese population (sensitivity = 98%, specificity = 96%),[12] prospective testing for *HLA-B**15:02* and subsequent avoidance of carbamazepine therapy resulted in zero occurrence of SJS/TEN in Taiwan.[13] Based on this result and the severity of these adverse reactions, a pre-screening test is now mandatory and covered by the National Health Insurance in Taiwan, although its positive predictive value could be estimated at around 3% using the values of their study. Thus, examining *HLA-B**58:01* prior to allopurinol administration may be also valuable to avoid allopurinol-induced SJS/TEN. However, testing *HLA* types is relatively laborious, time-consuming and expensive. Very recently, we found that several single nucleotide polymorphisms (SNPs) around the *HLA* region on chromosome 6 were strongly linked with *HLA-B**58:01* in a group of SJS/TEN patients.[11] In general, a single SNP can be easily genotyped and inexpensively compared to the *HLA* type. Thus, the linked SNPs could be used as alternatives to testing for *HLA-B**58:01* when deciding on the application of drug therapies involving allopurinol. To expedite the application of this pharmacogenomic information for the proper usage of allopurinol in clinical settings, we developed a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method that can genotype SNPs easily without high skills and inexpensively.

Materials and Methods

Patients: Japanese SJS/TEN patients from unrelated families were recruited from July 2006 through April 2010 at participating institutes of the Japan Severe Adverse Reactions (JSAR) research group.[11] In addition, SJS/TEN patients were recruited through a nationwide blood-sampling network system in Japan for severe drug adverse reactions operated by the National Institute of Health Sciences under the auspices of the Ministry of Wealth, Labour and Welfare and the Federation of Pharmaceutical Manufacturers' Associations of Japan. Genomic DNA was extracted from blood leukocytes as described previously.[10] DNA samples extracted from the cord blood of healthy Chinese-Americans were purchased from AllCells (Emeryville, CA, USA). The ethics committees of the National Institute of Health Sciences and each participating institute of the JSAR research group approved this study. Written informed consent was obtained from all cases and healthy Chinese-American subjects.

Genotyping of single nucleotide polymorphism by TaqMan assay and *HLA* types: *HLA-B* types were determined by the sequencing-based method as reported in a previous paper.[11] Of the several SNPs linked with *HLA-B**58:01*, we selected rs9263726 (110G>A, Arg37His) in *psoriasis susceptibility 1 candidate 1* (*PSORS1C1*) as a surrogate marker for *HLA-B**58:01*, because this SNP was in absolute linkage disequilibrium ($r^2 = 1$, $D' = 1$) with *HLA-B**58:01* and associated with SJS/TEN with an odds ratio of 61.2 ($p = 3.64 \times 10^{-8}$) in the dominant genotype mode.[11] This variation was located *ca.* 215 kb away from the *HLA-B* gene, detected at a minor allele frequency of 0.006 (12/1982 alleles), which was the same as that of the reported Japanese frequency of *HLA-B**58:01* (0.006),[14] and in Hardy-Weinberg equilibrium ($p = 0.847$).[11] In allopurinol-related SJS/TEN patients, the minor allele frequency of *HLA-B**58:01* and rs9263726 was 0.278.[11] rs9263726 was genotyped using TaqMan SNP Genotyping Assays (C_30352071_10, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions using 5 ng of genomic DNA from Japanese SJS/TEN patients or healthy Chinese-Americans. Hardy-Weinberg equilibrium was analyzed by Fisher's exact test using SNPAlyze ver. 3.1 software (Dynacom, Chiba, Japan).

Genotyping of rs9263726 by PCR-RFLP: PCR primers (forward: 5′-AAGCTCCATCCACCCCTGGT-3′ and reverse: 5′-ACACATTGGGTGGGGGACAT-3′) were designed to amplify a *PSORS1C1* genomic fragment containing the rs9263726 SNP locus. PCR was performed using *Ex*-Taq (0.625 units) (Takara Bio Inc., Shiga, Japan) with a pair of primers (0.2 μM) and genomic DNA (50 ng). The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 7 min. Aliquots of PCR products (5 μl) were then digested by the addition of 0.4 units of FokI restriction endonuclease (New England Biolabs, Beverly, MA, U.S.A.) in the presence of 1 × Buffer 4 (New England Biolabs) at 37°C for 2 h. Restriction mixtures were incubated at 65°C for 20 min to inactivate FokI, and then electrophoresed through a 15–25% gradient acrylamide gel (MULTIGEL II Mini, Cosmo Bio Co., Ltd., Tokyo, Japan). Following electrophoresis, the gels were stained with ethidium bromide, and DNA was visualized by placing the gel on a UV transilluminator.

Results and Discussion

First, we compared the results of genotyping rs9263726 in *PSORS1C1* with the PCR-RFLP and TaqMan SNP Geno-

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typing assays (C_30352071_10). DNA from Chinese-Americans was used since the frequency of *HLA-B*58:01* in this population is reportedly higher than in the Japanese population.[6,10] Preliminary, experiments using the TaqMan assay showed that the 200 DNA samples from Chinese-Americans contained 161 homozygotes of the major allele (GG), 36 heterozygotes (GA), and 3 homozygotes of the minor allele (AA) of rs9263726 (data not shown), which distribution was in Hardy-Weinberg equilibrium ($p = 0.550$). In addition, we confirmed that the 3 subjects with homozygous AA surely had homozygous *HLA-B*58:01* (data not shown). From the DNA from Chinese-Americans genotyped by TaqMan assay, 5 samples with GG, 4 with GA, and 2 with AA of rs9263726 were selected to establish the PCR-RFLP method. In the developed assay, the 260 bp PCR products derived from the A allele of rs9263726 were digested with Fok I produced two bands (141 bp and 119 bp), while those derived from the G allele remained as the parent single band (260 bp) (**Supplementary Fig. 1A**). Genotypes of these samples by PCR-RFLP assay were 100% in concordance with those from the TaqMan SNP assay, indicating that this is a robust method of genotyping rs9263726.

Next, in order to validate this PCR-RFLP assay, the rs9263726 locus was genotyped for the DNA samples with or without *HLA-B*58:01* of 27 Japanese SJS/TEN patients for whom *HLA-B* types had been previously determined.[10,11] The following SJS/TEN samples were selected: 5 *HLA-B*58:01* heterozygous carriers and 22 other *HLA-B* allele carriers. The other *HLA* types were selected based on an allele frequency ≥ 0.01 in Japanese control populations,[14,15] although a *HLA-B*44:02* sample (allele frequency = 0.01) was not available. As shown in **Table 1** and **Supplementary Fig. 1B**, the 5 patients with heterozygous *HLA-B*58:01* were also heterozygotes for rs9263726 (GA), and the remaining 22 patients with the other *HLA-B* types were major homozygotes for this SNP (GG). Thus, our developed PCR-RFLP assay can robustly predict the *HLA-B*58:01* status of SJS/TEN patients.

Very recently, Kostenko *et al.* generated a monoclonal antibody to recombinant HLA-B*57:01 protein and developed a flow cytometric assay for the detection of HLA-B57-positive peripheral blood mononuclear cells.[16] This antibody can cross-react with HLA-B58 proteins and thus could be used to pre-screen for *HLA-B*58:01* carriers. However, this assay method cannot discriminate *HLA-B*57:01* from *B*58:01* and uses blood cells, making it laborious and expensive (*i.e.*, a flow cytometer is necessary). In contrast, our PCR-RFLP method does not require a high skill set, and can be done at a low cost without use of specific machines, although a DNA extraction step is necessary.

Although the testing of rs9263726 or *HLA-B*58:01* cannot perfectly predict allopurinol-induced SJS/TEN, it may be better for the *HLA-B*58:01*-positive patients to avoid the administration of allopurinol, as do the *HLA-B*15:02*-positive patients for carbamazepine in Taiwan. Because allopuirol is a xantine oxidase inhibitor, febuxostat, having the same pharmacological effect through a different structure, might be an alternative drug for the *HLA-B*58:01*-positive patients, although further studies are clearly necessary to prove that SJS/TEN induced by febuxostat is surely not to be associated with *HLA-B*58:01*.

Table 1. *HLA* and rs9263726 genotypes in Japanese SJS/TEN patients

ID	*HLA-B**		rs9263726
1	58:01	46:01	G/A
2	58:01	51:01	G/A
3	58:01	51:01	G/A
4	58:01	38:02	G/A
5	58:01	07:02	G/A
6	07:02	51:01	G/G
7	13:01	35:01	G/G
8	15:01	40:02	G/G
9	15:11	40:02	G/G
10	15:18	38:02	G/G
11	35:01	40:02	G/G
12	37:01	40:06	G/G
13	39:01	51:01	G/G
14	40:01	40:01	G/G
15	48:01	51:02	G/G
16	40:02	40:06	G/G
17	40:06	52:01	G/G
18	44:03	44:03	G/G
19	46:01	35:01	G/G
20	51:01	35:01	G/G
21	52:01	52:01	G/G
22	54:01	54:01	G/G
23	55:02	51:01	G/G
24	56:01	46:01	G/G
25	59:01	35:01	G/G
26	67:01	39:01	G/G
27	40:03	54:01	G/G

In conclusion, we have developed a robust PCR-RFLP genotyping assay for rs9263726 in *PSORS1C1*, which is in absolute linkage disequilibrium with *HLA-B*58:01*, a partially predictive but useful biomarker for allopurinol-related SJS/TEN in Japanese. The genotyping of rs9263726 by this easy and inexpensive method makes it useful for the prospective screening of patients with *HLA-B*58:01* in the future.

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ORIGINAL ARTICLE

Dexamethasone-mediated transcriptional regulation of rat carboxylesterase 2 gene

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Abstract

1. Rat carboxylesterase 2 (rCES2), which was previously identified as a methylprednisolone 21-hemisuccinate hydrolase, is highly inducible by dexamethasone in the liver. In the present study, we investigated the molecular mechanisms by which this induction occurs.
2. Injection of dexamethasone (1 mg/kg weight) into rats resulted in increases in the expression of rCES2 mRNA in a time-dependent manner with a peak at 12 h after injection. In primary rat hepatocytes, the expression level of rCES2 mRNA was increased by treatment with 100 nM dexamethasone, and the increase was completely blocked in the presence of 10 μM mifepristone (RU-486), a potent inhibitor of glucocorticoid receptor (GR), or 10 μg/mL cycloheximide, a translation inhibitor. Luciferase assays revealed that 100 nM dexamethasone increased *rCES2* promoter activities, although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. The increased activities were completely inhibited by treatment of the hepatocytes with 10 μM RU-486.
3. Based on these results, it is concluded that dexamethasone enhances transcription of the *rCES2* gene via GR in the rat liver and that the dexamethasone-mediated induction of rCES2 mRNA may be dependent on *de novo* protein synthesis. Our results provide clues to understanding what compounds induce rCES2.

Keywords: rCES2, induction, glucocorticoid receptor

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Introduction

Carboxylesterases (CESs, EC 3.1.1.1) belong to the α,β-hydrolase-fold family (Bencharit et al., 2003). CESs catalyse hydrolytic reactions in a variety of xenobiotic and endobiotic substrates because of their ability to hydrolyse a broad spectrum of ester, amide, thioester, and carbamate compounds (Sanghani et al., 2004). CESs are classified into five families (CES1-5) (Satoh and Hosokawa, 2006). The major two CES families, CES1 and CES2, are well characterised compared with others. The two families have different substrate specificities. CES1 mainly hydrolyses a substrate that can be converted to a small alcohol moiety and a large acyl moiety. This is in contrast to CES2, which mainly hydrolyses a substrate that can be converted to a large alcohol moiety and a small acyl moiety. A number of chemical compounds are known to induce expression of CESs, yet there is little information about the molecular mechanisms of induction of CESs.

CESs are prominently involved in many pharmaceutical agents. Many esterified drugs such as cocaine and meperidine are metabolised by CESs into inactive products (Potter and Wadkins, 2006). On the other hand, a number of ester-containing prodrugs, whose pharmacological activities are generally masked, are hydrolysed by CESs and then exert their activities. Examples of prodrugs are the anticancer drug CPT-11 (irinotecan), which is metabolised by CES1 and CES2 to the active metabolite SN-38 (Humerickhouse et al., 2000), and the anti-influenza drug

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oseltamivir, which is metabolised by CES1 to Ro 64-0802 (Shi et al., 2006). Thus, CESs play an important role in determining the metabolic fate of many drugs.

Dexamethasone is a synthetic glucocorticoid and is used to treat many different conditions such as skin diseases, asthma, and rheumatoid arthritis. Dexamethasone alters expression levels of a large number of genes including tyrosine aminotransferase (Schmid et al., 1987), glutamine synthetase (Gaunitz et al., 2002) and Na^{+}-K^{+}-ATPase (Celsi et al., 1991) and also many drug-metabolizing enzymes such as CYP2C9 (Gerbal-Chaloin et al., 2002), CYP3A4 (Pascussi et al., 2001) and human CES1 (Zhu et al., 2000). With respect to gene regulation by dexamethasone, some molecular mechanisms are known. In many cases, homodimers consisting of glucocorticoid receptors (GRs) activated by ligands such as dexamethasone directly bind to glucocorticoid response elements (GREs) and regulate gene expression. At high concentrations (more than 10 μM), dexamethasone can activate pregnane X receptor as well as GR, leading to alteration of gene expression (Huss and Kasper, 2000; Pascussi et al., 2001). In addition, GR activated by dexamethasone contributes to induction of the *CYP2A6* gene by interacting with hepatocyte nuclear factor-4α (Onica et al., 2008). Increased or decreased drug-metabolizing enzymes can change drug potency *in vivo* and influence the incidence of adverse effects. Therefore, the molecular mechanisms by which dexamethasone regulates *CES* gene expression need to be understood to accurately predict the potency of drugs metabolised by CESs.

The expression levels of several rat *CES* genes are altered by dexamethasone treatment. Zhu et al. (2000) reported that the expression of rat CES1 (hydrolase A, B, and S) in the liver was suppressed after intraperitoneal injection of dexamethasone. GR is involved in the molecular mechanisms of the suppression (Shi et al., 2008). In contrast, rat CES2 (rCES2) (GenBank ID, AB191005), previously called CES RL4, is markedly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms underlying the induction of rCES2 have remained unknown. Dexamethasone-mediated induction of rCES2 is known to cause a drug interaction. Since rCES2 hydrolyses methylprednisolone 21-hemisuccinate (MPHS), which is prescribed for various conditions including systemic lupus erythematosus, hemorrhagic shock and rejection episodes in renal transplant recipients, to the active metabolite methylprednisolone, treatment with dexamethasone increases MPHS hydrolase activity in the rat liver. Drug-mediated induction or repression of rat *CES* gene expression should be noted as long as rats continue to be used commonly in non-clinical studies.

The goal of this study was to determine the molecular mechanisms underlying dexamethasone-mediated induction of rCES2. In the present study, treatment of primary rat hepatocytes with mifepristone (RU-486), a potent antagonist of GR, resulted in inhibition of both increase in rCES2 mRNA and elevation of *rCES2* promoter activity caused by dexamethasone. These results suggested that GR plays a critical role in dexamethasone-mediated transcriptional activation of the *rCES2* gene. Moreover, results obtained by using cycloheximide indicated the possibility that *de novo* protein synthesis is necessary for the induction of rCES2 mRNA by dexamethasone.

Materials and methods

Materials

Dexamethasone-water soluble, triamcinolone acetonide, cycloheximide, RU-486, MPHS, and methylprednisolone were purchased from Sigma-Aldrich (St. Louis, MO). Dexamethasone (used with corn oil), corn oil, collagenase, a Ligation-Convenience kit, and prednisolone were purchased from Wako Pure Chemical Industries (Osaka, Japan). Trypsin inhibitor from soybean (>7000 BAEE units/mg), Williams' medium E without phenol red, Opti-MEM I, DNase I, and a Zero Blunt TOPO PCR Cloning kit were purchased from Invitrogen Life Technologies (Carlsbad, CA). Pentobarbital sodium (somnopentyl) was purchased from Schering-Plough Corp. (Kenilworth, NJ). Human recombinant insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals Inc. (Princeton, NJ). A nylon mesh filter was purchased from Sefar Inc. (Heiden, Switzerland). AteloCell (native collagen bovine dermis) was purchased from Koken Co. Ltd. (Tokyo, Japan). FuGENE HD transfection reagent was purchased from Roche Diagnostics Corp. (Indianapolis, IN). BD Matrigel (phenol red-free) was purchased from BD Biosciences (Bedford, MA). ISOGEN was purchased from Nippon Gene (Toyama, Japan). A ReverTra Ace qPCR RT kit, THUNDERBIRD Probe qPCR Mix, KOD -plus- DNA Polymerase, and *Kpn* I were purchased from Toyobo (Osaka, Japan). *Xho* I was purchased from Takara Shuzo (Kyoto, Japan). TaqMan Gene Expression Assays (probe and primer sets) for rCES2 (AssayID: Rn00592205_m1) (FAM) and rat tyrosine aminotransferase (TAT) (Rn01431532_m1) (FAM) and Pre-Developed TaqMan Assay Reagents for eukaryotic 18S rRNA (VIC) were purchased from Applied Biosystems (Foster, CA). Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). *Dpn* I was purchased from New England BioLabs (Hitchin, Hertfordshire, UK).

Intraperitoneal injection of dexamethasone

Male Sprague–Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) of 5–6 weeks (150–200 g) of age were used in this experiment. Dexamethasone in corn oil (0.25 mg/mL) was intraperitoneally injected into rats at a dose of 1 mg/kg body weight, or only corn oil was injected as a control in the same way. Rats were put under anesthesia with diethyl ether at 3, 6, 12, and 24 h after injection of dexamethasone or at 24 h after injection of only corn oil for the control, and the livers were removed for relative quantification of mRNA expression and protein expression. One

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rat per experiment was used at each time point and the experiment was repeated thrice ($n=3$/time point).

Isolation of hepatocytes

Rat hepatocytes were isolated using a collagenase two-step perfusion method described by Seglen (1976) with some modifications. Rats were anesthetised by intraperitoneal injections of pentobarbital (100 mg/kg body weight) and the abdominal cavity was incised. An indwelling needle consisting of an inner needle and a flexible cover needle was inserted into the portal vein, and the inner needle was removed from the indwelling needle. The flexible cover needle and portal vein were immediately bound using a clamp, and the cover needle was connected to a tube with a pump. A pre-perfusate (137 mM NaCl, 5.37 mM KCl, 1.05 mM $MgCl_2 \cdot 6H_2O$, 0.832 mM $MgSO_4 \cdot 7H_2O$, 0.500 mM $NaH_2PO_4 \cdot 2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 0.500 mM EGTA, 4.17 mM $NaHCO_3$, and 5.00 mM D-glucose; pH 7.2 and 37°C) was flowed through the portal vein at a flow rate of ~24 mL/min. The inferior vena cava was immediately cut to make an exit site for the pre-perfusate. About 6 min later, the pre-perfusate was replaced by flowing a collagenase solution (137 mM NaCl, 5.37 mM KCl, 5.05 mM $CaCl_2$, 0.500 mM $NaH_2PO_4 \cdot 2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 4.17 mM $NaHCO_3$, 1 g/L collagenase, and 100 mg/L trypsin inhibitor; pH 7.5 and 37°C). The collagenase solution was flowed for ~6 min at the same flow rate. After perfusion of the collagenase solution, the digested liver was resected and washed briefly with ice-cold Hanks' balanced salt solution, HBSS (137 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.336 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 5.55 mM D-glucose, and 4.17 mM $NaHCO_3$; pH ~7.3). Cells were dispersed from the digested liver in 50 mL of ice-cold HBSS and the cellular suspension was filtrated through a nylon mesh filter (pore size, 150 μm). The filtrated cells were centrifuged for 2 min at 4°C and the supernatant was aspirated. For removal of non-parenchymal hepatocytes, the cells containing hepatocytes were suspended in 15 mL of cold HBSS and centrifuged for 2 min at 4°C, and the supernatant was removed (This series of steps for removal was performed twice.). Finally, the obtained hepatocytes were suspended in 20 mL of an ice-cold standard culture medium (Williams' medium E without phenol red, containing 0.25 U/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin) and the viability of hepatocytes was assessed by 0.4% trypan blue exclusion. Hepatocytes for which viability exceeded 85% were used in the present study.

Primary cultures of rat hepatocytes for mRNA expression analysis

Primary cultures of rat hepatocytes were performed using the method previously described by Kocarek and Reddy (1996) with some modifications to extract total RNA and perform the ensuing real-time polymerase chain reaction (PCR). Hepatocytes were isolated from SD rats of 5–7 weeks (~160–250 g) of age by the method described above. The cells were suspended in the standard culture medium and were seeded at 7.5×10^5 cells/mL × 2 mL/well onto 6-well plates coated with 0.5 mg/well (for example, 3.57 mg/mL × 140 μL/well) of matrigel. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Twenty-four hours after seeding, the culture medium in the plates was replaced with 2 mL fresh medium. Dexamethasone (water-soluble) was dissolved in Milli-Q (MQ) water to make a 100-μM dexamethasone solution as a stock solution. RU-486 or cycloheximide was dissolved in ethanol to make a 10-mM RU-486 solution or 1-mM and 10-mg/mL cycloheximide solutions as stock solutions. The culture medium in plates was again replaced with 2 mL fresh medium 48 h after seeding, and reagents (drugs and vehicles) were added to the culture medium. The drug solutions or solvents were added at 0.1% (v/v). Extraction of total RNA from hepatocytes in each well was performed 24 h after treatment with reagents.

Relative quantification of mRNA expression

Relative quantification of target transcripts was performed essentially as described previously (Hori and Hosokawa, 2010). Total RNA was extracted from rat liver pieces and primary hepatocytes using an ISOGEN and treated with DNase I to prevent contamination by DNA. The treated RNA and a ReverTra Ace qPCR RT kit were used to synthesise first-strand cDNA. The expression level of rCES2 mRNA was analysed using cDNA (1 μg/sample), a THUNDERBIRD Probe qPCR Mix, gene-specific TaqMan probe and primer sets, and an Applied Biosystems 7500 Real-Time PCR System. Rat CES2 mRNA expression was normalised with 18S rRNA expression. The $\Delta\Delta C_t$ method was used for analysis of data on rCES2 mRNA expression. The conditions of real-time PCR were as follows: 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of rat TAT mRNA was analysed in the same way as that for rCES2 mRNA.

Western blot analysis

The expression level of rCES2 protein in the rat liver was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot essentially as previously described (Furihata et al., 2005). Rat liver pieces obtained from rats that were injected with or without dexamethasone were homogenised with 1.15% KCl. The 20% (w/v) homogenates were centrifuged at 9000*g* for 20 min, and the supernatants were centrifuged at 105,000*g* for 1 h. After replacement of the supernatants with fresh KCl buffer, the samples were again centrifuged at 105,000*g* for 1 h. The microsomal pellets were suspended in SET buffer (0.25 M sucrose, 1 mM EDTA·2Na, and 10 mM Tris-HCl; pH 7.4). SDS-PAGE was performed using 10% polyacrylamide gels and 10 μg/well of microsomal protein, and Western blot was performed using polyclonal anti-rCES2 antibodies, which were previously prepared (Derbel et al., 1996). The density of bands was measured

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using ImageJ 1.44o software (National Institutes of Health, Bethesda, MD).

MPHS hydrolase activity assay

MPHS hydrolase activity was determined essentially according to the method described previously (Furihata et al., 2005). Rat liver microsomes were preincubated in citrate-phosphate buffer (pH 5.5) for 5 min at 37°C. The reaction was initiated by adding MPHS dissolved in 1% dimethylformamide water to the mixture. The mixture (50 mM citrate-phosphate buffer, 0.04–0.3 mg/mL microsomal protein, and 400 μM MPHS) was incubated for 15 min at 37°C. The reaction was terminated by adding 100 μL of acetonitrile containing 40 μM prednisolone, which was used as an internal standard, to 100 μL of the mixture. After the removal of protein, the amount of methylprednisolone formed from MPHS was determined by HPLC. Methylprednisolone was detected at a wavelength of 254 nm. HPLC system consisted of a LC-20AD pump unit (Shimadzu Corp., Kyoto, Japan), a SIL-20A autosampler (Shimadzu), a CTO-10AS VP column oven (Shimadzu), a SPD-20A UV/VIS detector (Shimadzu), a SCL-10A VP system controller (Shimadzu), and a Mightysil RP-18 GP 150 mm × 4.6 mm column (Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (pH 7.0)/acetonitrile (65:35, v/v) and was delivered at a flow rate of 0.9 mL/min.

Reporter constructs

The 5′-flanking region from −2957 to +51 (−2957/+51), when the transcription start site of the *rCES2* gene is +1, was amplified by PCR with KOD -plus- DNA Polymerase, rat genomic DNA as a template, and primers listed in Table 1. The primers were designed on the basis of the sequence obtained from a search by basic local alignment search tool (BLAST) with the sequence of AB191005. The amplified fragment was cloned into pCR -Blunt II-TOPO vector provided in the Zero Blunt TOPO PCR Cloning kit following the manufacturer's instructions, and this plasmid was designated pCR-rCES2 −2957/+51. After sequencing, pCR-rCES2 −2957/+51 was digested by the two restriction enzymes, *Kpn* I and *Xho* I. After purification, the digested fragment containing the sequence of −2957/+51 was ligated using a Ligation-Convenience kit to pGL3-Basic vector, a luciferase reporter vector, digested by the same restriction enzymes. After amplification using competent *E. coli* JM109 and the ensuing purification, this plasmid was designated pGL3-rCES2 −2957/+51 for luciferase assays. A variety of deletion plasmids (pGL3-rCES2 −1955/+51, −1569/+51, −991/+51, −662/+51, −195/+51, and −73/+51) were made in the same manner as that described above, except that these deletion plasmids were made using primers listed in Table 2 and pCR-rCES2 −2957/+51 as a template. The plasmid of pGL3-rCES2 −6/+51 was made by a site-directed mutagenesis method described below. First, PCR was performed using pGL3-rCES2 −195/+51 as a template and primers that were designed to anneal to the same sequence on opposite strands and to carry a *Kpn* I site (Table 3). The PCR product, plasmid, was electrophoresed and the target plasmid was extracted. The plasmid was treated with *Dpn* I to digest template plasmid, pGL3-rCES2 −195/+51. Following transformation of JM109 and purification of the plasmid, sequencing was performed to confirm the existence of the *Kpn* I site in the plasmid. Then the plasmid was digested by *Kpn* I and the fragment containing the sequence of −6/+51 was self-ligated using a Ligation-Convenience kit. In this way, pGL3-rCES2 −6/+51 plasmid was made.

Table 1. Specific primers in the 5′-flanking region of the *rCES2* gene for genomic cloning and luciferase assays.

Position	Sequence
Forward primer	
−2957/−2933	5′-GATGGCTGCGTGATACTTCTTCTGG-3′
Reverse primer	
+23/+51	5′-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3′

Table 2. Specific primers in the 5′-flanking region of the *rCES2* gene for luciferase assays.

Position	Sequence
Forward primer	
−1955/−1936	5′-ATCTTGGTGCCTTCTAACTG-3′
−1569/−1549	5′-TCTTTGACTAGCGAAATGGTG-3′
−991/−968	5′-TTTTTTTTTCTGGTGATGGATTCG-3′
−662/−642	5′-CCTCTGGAGACACTTCAGACA-3′
−195/−173	5′-AGTCCACACTGTGCCTTTCCAGG-3′
−73/−51	5′-TTCACCCACGACATCATGTTCCC-3′
Reverse primer	
+23/+51	5′-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3′

Table 3. Mutant primers for site-directed mutagenesis to make *Kpn* I site (small letter).

Position	Sequence
Forward primer	
−25/+10	5′-CCTGCCTGGGCAAggtaccCGGTTTATTCTTCCTG-3′
Reverse primer	
−25/+10	5′-CAGGAAGAATAAACCGggtaccTTGCCCAGGCAGG-3′

Transient transfection and luciferase assay

Transient transfection into primary rat hepatocytes was performed using the method previously described by Runge-Morris et al. (1999) with some modifications. Hepatocytes were isolated from SD rats of 5–7 weeks (~150–250 g) of age by the method described above. Hepatocytes were suspended in a cold standard culture medium which was supplemented with 100 nM triamcinolone acetonide (TA). The cells were seeded at 3×10^5 cells/mL × 0.5 mL/well onto 24-well plates coated thinly with collagen. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Following ~6 h of seeding, the medium was replaced with 0.5 mL of Opti-MEM I, and plasmids were transfected to the cells as follows: 500 ng/well pGL3 plasmid (12.5 ng/μL), 50 ng/well phRL-TK

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plasmid (12.5 ng/μL), and 1.4 μL/well FuGENE HD. Five hours after transfection, the medium was replaced with 0.5 mL of the standard culture medium without TA, and hepatocytes were overlaid with 0.05 mg/well (for example, 3.57 mg/mL × 14 μL/well) of matrigel. Thirty hours after addition of matrigel, hepatocytes were treated with either 100 nM dexamethasone or MQ water in 0.5 mL of fresh medium without TA and again overlaid with 0.05 mg/well of matrigel. Simultaneously with dexamethasone or MQ water, in an experiment for which results are shown in Figure 5C, hepatocytes were exposed to RU-486 or ethanol. Twenty-four hours later, hepatocytes were rinsed once with PBS (-), and dual-luciferase reporter assays were performed according to the manufacturer's instructions.

Statistical Analysis

Multiple groups were compared by one-way analysis of variance (one-way ANOVA) followed by Dunnett's (Figure 1A and 1C) or Tukey's (Figures 2, 3, and 5C) multiple comparison test. Two groups were compared by Student's *t*-test (Figures 4 and 5A). These statistical analyses were performed using the free software R version 2.13.0 (R Development Core Team, 2011). A value of $P < 0.05$ was considered statistically significant.

Results

Dexamethasone-mediated increases in TAT and rCES2 mRNA expression and alterations of rCES2 protein expression in the rat liver

Dexamethasone was injected into rats to investigate temporal changes in TAT and rCES2 mRNA expression. The expression levels of rCES2 mRNA peaked at 12 h after injection of dexamethasone and the maximum level was ~400-fold higher than that of the control (dexamethasone 0 h) (Figure 1A). The level of TAT mRNA, which is well known to be induced by dexamethasone, was also increased by dexamethasone. The increased level of TAT mRNA was highest at 6 h after injection, and this change was in agreement with the results of a previous study (Shi et al., 2008). Significant increases of rCES2 protein and MPHS hydrolase activity were observed at 12 and 24 h after injection (Figure 1B and 1C).

Comparison between rat primary hepatocytes and livers in expression of TAT and rCES2 mRNA

The expression levels of TAT or rCES2 mRNA in rat hepatocytes and livers were compared. Hepatocytes as a control were treated with both water and ethanol. When based on the average ΔC_t value obtained from

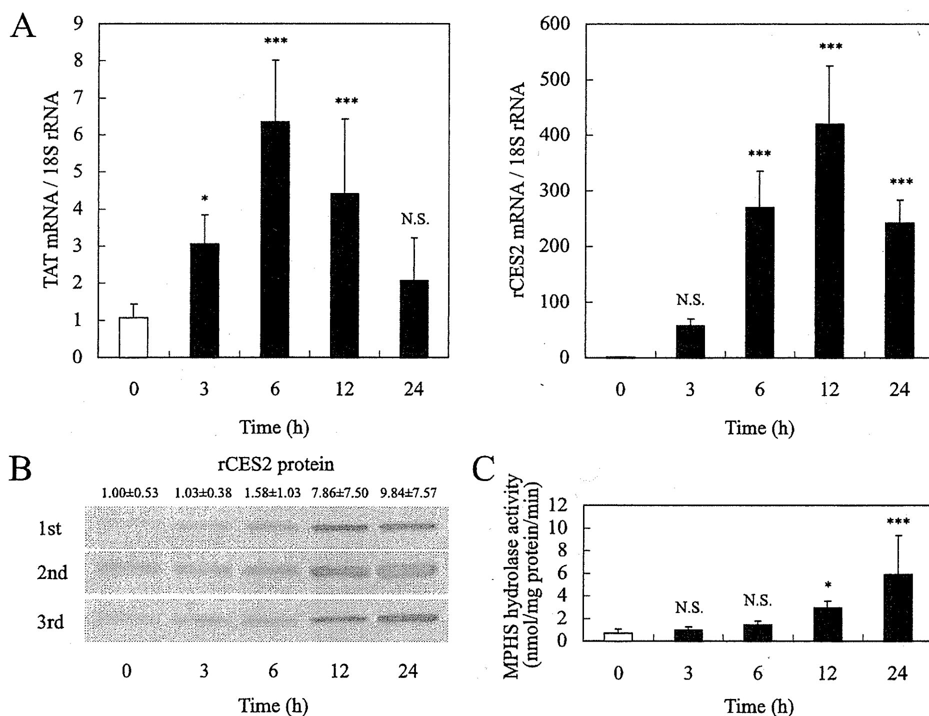

Figure 1. (A) Time course of increases in TAT and rCES2 mRNA by dexamethasone. Rats were sacrificed at 3, 6, 12, and 24 h after injection of dexamethasone, and liver pieces were used to determine the expression levels of TAT and rCES2 mRNA by real-time PCR. Each value is shown as the mean ± standard deviation (SD) of three independent experiments ($n = 3$/group), which were performed in triplicate. In analysis of TAT mRNA, the C_t (threshold cycles) values (mean ± SD) of a control (dexamethasone 0 h) were 23.1 ± 1.04 (TAT) and 12.6 ± 0.813 (18S rRNA). In analysis of rCES2 mRNA, the C_t values of the control were 27.8 ± 0.905 (rCES2) and 12.5 ± 0.535 (18S rRNA). N.S. indicates not statistically significant. Statistically significant difference; $*P < 0.05$ and $***P < 0.001$. (B) Alterations of rCES2 protein expression by dexamethasone. Relative expression levels of rCES2 protein (mean ± SD of three independent experiments) were estimated using a standard curve that generated by a microsomal sample obtained form a rat injected with dexamethasone. (C) Temporal changes in MPHS hydrolase activity in liver microsomes after injection of dexamethasone. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Statistically significant differences; $*P < 0.05$ and $***P < 0.001$.

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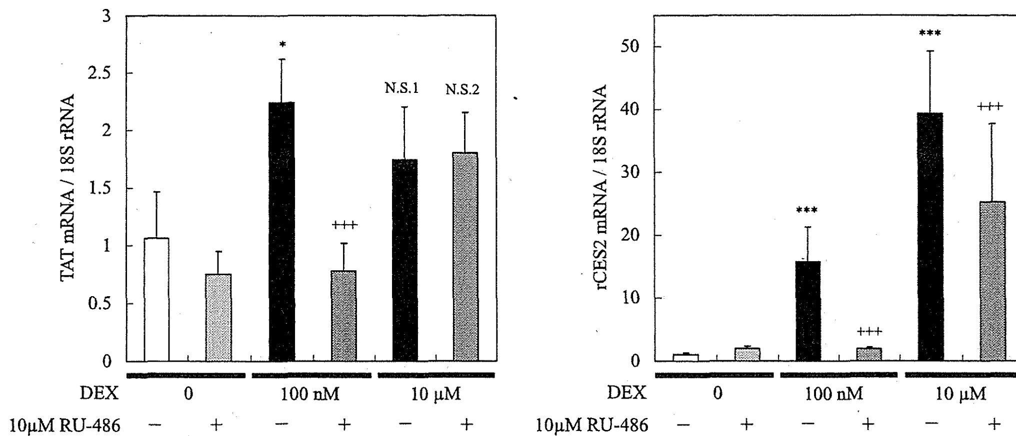

Figure 2. Effects of dexamethasone and RU-486 on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone or water in the presence of RU-486 or ethanol. The expression levels of TAT and rCES2 mRNA were analysed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Hepatocytes as a control (white bars) were treated with both water and ethanol. In analysis of TAT mRNA, the average C_t values (mean ± SD) of the control were 21.9 ± 0.410 (TAT) and 11.5 ± 0.189 (18S rRNA). In analysis of rCES2 mRNA, the average C_t values of the control were 23.9 ± 0.433 (rCES2) and 11.7 ± 0.247 (18S rRNA). DEX indicates dexamethasone. N.S.1 indicates not statistically significant (control versus DEX treatment). N.S.2 indicates not statistically significant (DEX treatment versus DEX+RU-486 treatment). Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$ and $^{***}P < 0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+++}P < 0.001$.

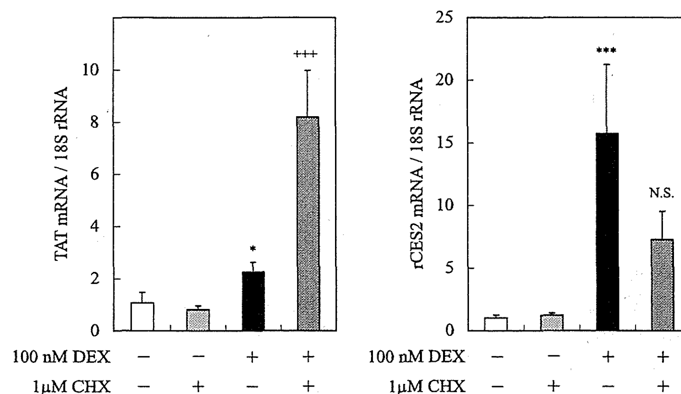

Figure 3. Effect of 1 μM cycloheximide on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone (100 nM) or water in the presence of cycloheximide (1 μM) or ethanol. The expression levels of TAT and rCES2 mRNA were analysed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Data analysis was performed in combination with data used to make Figure 2. DEX and CHX indicate dexamethasone and cycloheximide, respectively. N.S. indicates not statistically significant (DEX treatment versus DEX+CHX treatment) and we retain the null hypothesis because P value is 0.0748. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$ and $^{***}P < 0.001$. Statistically significant differences (DEX treatment versus DEX+CHX treatment); $^{+++}P < 0.001$.

the control hepatocytes, rCES2 mRNA expression ratio (rCES2 mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.02 ± 0.235 and 0.126 ± 0.0491, respectively. Likewise, TAT mRNA expression ratio (TAT mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.06 ± 0.400 and 0.946 ± 0.328, respectively.

Alterations of TAT and rCES2 mRNA expression by dexamethasone, RU-486, and cycloheximide in primary rat hepatocytes

The effects of dexamethasone, RU-486, and cycloheximide on TAT and rCES2 mRNA expression were investigated. Dexamethasone (100 nM and 10 μM) dose-dependently caused an elevation of rCES2 mRNA in primary rat hepatocytes (Figure 2). The increased rCES2 mRNA by 100 nM dexamethasone was completely inhibited by treatment with 10 μM RU-486 (Figure 2). Treatment with 1 μM cycloheximide for 24 h resulted in repression, in part, of the increase in rCES2 mRNA by 100 nM dexamethasone (Figure 3), while the elevation of rCES2 mRNA was completely inhibited by treatment with 10 μg/mL (~36 μM) cycloheximide for 24 h (Figure 4). In the case of TAT mRNA, 100 nM dexamethasone increased the expression, and the effect of 10 μM dexamethasone was weaker than that of 100 nM dexamethasone (Figure 2). Note that the expression of 18S rRNA was decreased by only ~2- to 3-fold at 10

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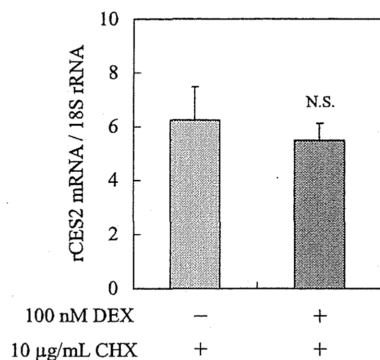

Figure 4. Effect of 10 μg/mL cycloheximide on the expression of rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24h with medium containing dexamethasone (100nM) or water in the presence of cycloheximide (10 μg/mL) or ethanol. The expression levels of rCES2 mRNA were analysed by real-time PCR. The vertical axis indicates the ratio (rCES2 mRNA/18S rRNA) based on the same control as shown in Figures 2 and 3. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate. The average C_t values (mean ± SD) of the samples obtained from the hepatocytes treated with 10 μg/mL cycloheximide without dexamethasone were 22.7±0.422 (rCES2) and 13.1±0.227 (18S rRNA). N.S. indicates not statistically significant.

μg/mL cycloheximide and that the values of the ratio (rCES2 mRNA/18S rRNA) were seemingly increased by treatment with 10 μg/mL cycloheximide. Therefore, we separated data obtained from samples with 10 μg/mL cycloheximide from the other data on mRNA expression and performed analysis as described in Statistical Analysis and the legend to Figure 4.

Activation of *rCES2* promoters by dexamethasone and inhibition of the activation by RU-486

Nucleotide sequences necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were investigated using transient transfections of deletion plasmids consisting of a variety of lengths of the *rCES2* promoter and dual-luciferase assays. Dexamethasone treatment, however, tended to decrease values of *Renilla* luciferase activity (Figure 5A and C). The decreased levels appeared to vary among cell populations that have pGL3 plasmids harbouring different *rCES2* promoter regions, implying that phRL-TK plasmid is unsuitable for an internal control in the present study. Therefore, to determine the effect of dexamethasone on *rCES2* promoter activity, we analysed data on *Firefly* luciferase activity instead of data on relative luciferase activity (*Firefly*/*Renilla*). Basal transcriptional activities were almost the same among the plasmids including the region of –195/+51 (Figure 5A). The basal transcriptional activity of the plasmid including the region of –195/+51 was decreased by more than half by deletion of the sequence from –195 to –74 and was abolished by additional deletion of the sequence from –73 to –7. Dexamethasone treatment resulted in an ~2- to 3-fold increase in promoter activity in the region of –2957/+51 (Figure 5A and C). Ten micromolars of RU-486 repressed the elevation of the promoter activation by 100nM dexamethasone in the region of –2957/+51 (Figure 5C). The *rCES2* promoter activity tended to be increased by dexamethasone associating with the sequences of –73/–7 and –991/–663 (Figure 5B). The dexamethasone-mediated promoter activation was abolished by RU-486 treatment in the region of –73/+51 as well as in the region of –2957/+51 (Figure 5C).

Discussion

MPHS is hydrolysed to methylprednisolone in rat hepatic microsomes (Hattori et al., 1981). MPHS hydrolase activity is increased following each injection of various glucocorticoids including dexamethasone and also methylprednisolone (Hattori et al., 1992a; Hattori et al., 1992b). We previously identified an MPHS hydrolase as rCES2 that is strongly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms by which the induction occurs have remained unknown. In the present study, we demonstrated that GR contributes to dexamethasone-mediated transcriptional activation of the *rCES2* gene.

First, we confirmed an increase in rCES2 mRNA at a lower concentration of dexamethasone than that employed in our previous study. Our previous study using reverse transcription-PCR showed that the expression of rCES2 mRNA was markedly increased when rats were injected with dexamethasone at a dose of 5mg/kg body weight for 4 consecutive days (Furihata et al., 2005). Consistent with this observation, when rats were injected with dexamethasone at a single does of 1mg/kg body weight in the present study, more than 200-fold increases in rCES2 mRNA were observed in the liver from 6 to 24h after injection (Figure 1A). Hattori et al. (1992b) previously showed that when a suspension of dexamethasone in sesame oil was intraperitoneally administered to rats as a single dose of 60 μmol/kg (~23.5mg/kg), MPHS hydrolase activity in rat microsomes rapidly increased and plateaued between 20 and 40h after a 4-h lag period. Consistent with their results for hydrolase activity, the expression level of rCES2 mRNA increased with time until 12h after injection of dexamethasone and the increase of rCES2 mRNA was followed by strong increases of rCES2 protein and MPHS hydrolase activity (Figure 1B and C). Hansen et al. (1999) reported that the mean of the maximum concentrations (C_{max}) in plasma was 682ng/mL (~1.7 μM) after a single subcutaneous injection of 0.8mg/kg dexamethasone into pregnant rats. Thus, our results indicate that treatment with ~2 μM of dexamethasone for 6 to 24h is probably sufficient for a significant increase in rCES2 mRNA in the liver.

In rat primary hepatocytes, the level of rCES2 mRNA was increased by treatment with 100nM dexamethasone (Figure 2). This result implies that the responsiveness of the primary hepatocytes used in the present study to dexamethasone was restored and that the elevation of rCES2 mRNA occurred in hepatic parenchymal cells. Dexamethasone-mediated increase in TAT mRNA,

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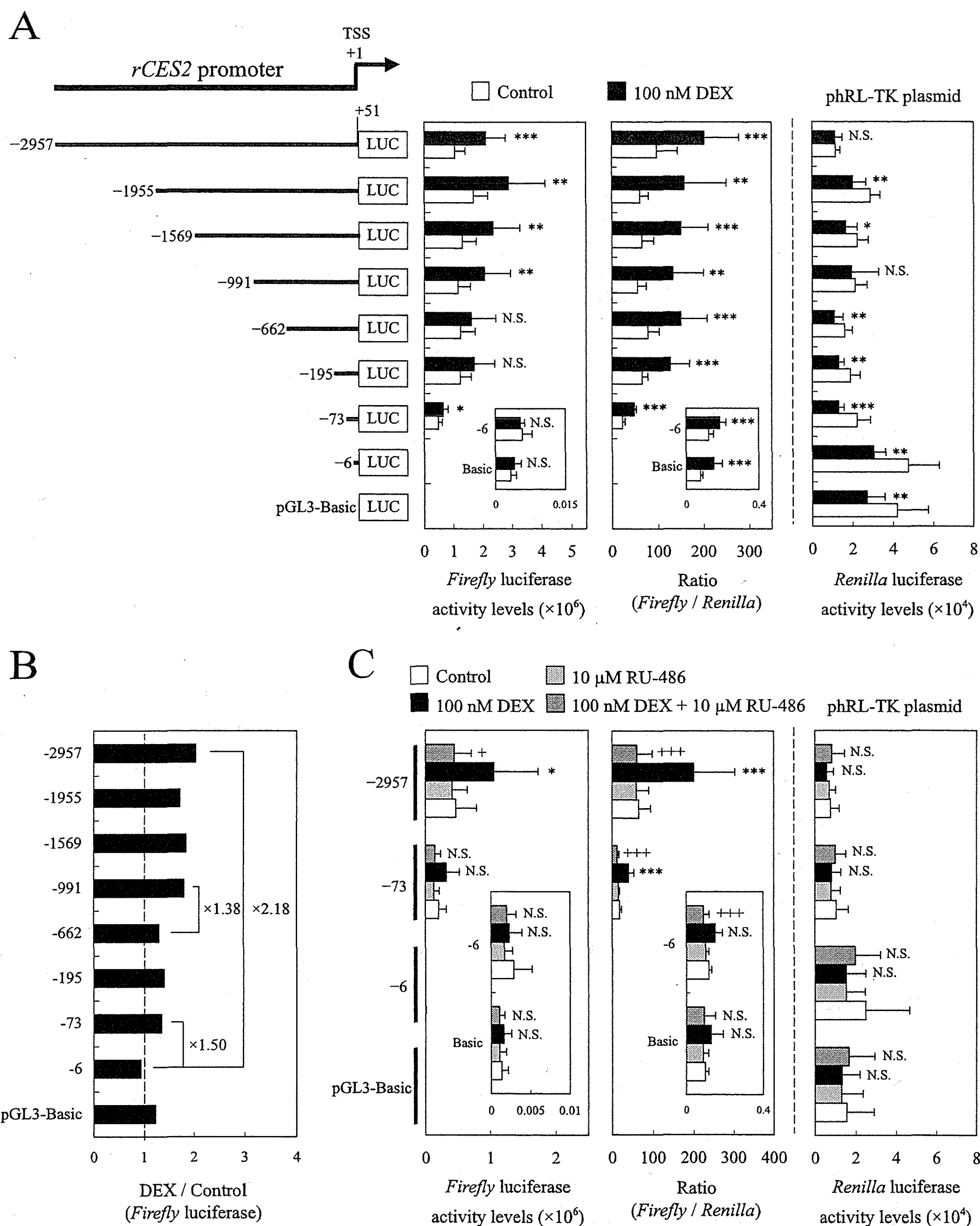

Figure 5. Analysis of the 5′-flanking region of the *rCES2* gene. (A) Deletion analysis of the 5′-flanking region of the *rCES2* gene. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid (*Firefly* luciferase) and 50 ng/well phRL-TK plasmid (*Renilla* luciferase) were treated for 24 h with dexamethasone (100 nM) or water, and dual-luciferase assays were performed. TSS indicates the transcription start site of the *rCES2* gene. Hepatocytes as a control were treated with water (white bars). DEX indicates dexamethasone. Statistically significant differences (control versus DEX treatment within each corresponding group); *$P<0.05$, **$P<0.01$, and ***$P<0.001$. N.S. indicates not statistically significant. Four independent experiments were performed in triplicate. (B) Promoter regions necessary for response to dexamethasone. The ratios (DEX/control) were based on the results of *Firefly* luciferase activity levels shown in Figure 5A. (C) Effect of RU-486 on dexamethasone-mediated activation of *rCES2* promoters. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid and 50 ng/well phRL-TK plasmid were treated for 24 h with dexamethasone (100 nM) or water in the presence of RU-486 (10 μM) or ethanol, and dual-luciferase assays were performed. Three independent experiments were performed in triplicate. Statistically significant differences (control versus DEX treatment within each corresponding group); *$P<0.05$ and ***$P<0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+}P<0.05$ and $^{+++}P<0.001$.

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