previously, MIF is essential for T-cell activation and possibly contributes to maintaining Th1/Th2 imbalance [1]. Increased MIF expression has been reported in lesions from many immune/inflammatory diseases, including psoriasis, glomerulonephritis, transplant rejection, neuro-Behçet's disease, asthma, adult respiratory distress syndrome, and inflammatory eye diseases [3, 4, 7, 11–13, 16, 17, 20, 22, 24].

Atopic dermatitis (AD) frequently involves some ophthalmic features: blepharitis, chronic keratoconjunctivitis, keratoconus, early-onset cataract, and rarely, retinal detachment [9]. AD is a chronic inflammatory skin disorder and many reports have documented its pathogenesis in relation to genetic and immunological abnormalities as well as environmental factors [10]. Although abnormal populations of Th1 and Th2 subsets of helper T cells (Th1/Th2 imbalance) have been identified as a cause of the pathogenesis of AD [8, 13, 25], a decrease in delayed-type hypersensitivity (DTH) is considered to involve more than Th1/Th2 imbalance in AD [5]. MIF is essential for T-cell activation and possibly contributes to maintaining Th1/Th2 imbalance as described above [1]. Also, a prominent increase in systemic MIF levels was detected in patients with severe AD, and the levels decreased when the clinical symptoms improved following treatment with corticosteroid ointment [18, 19]. We hypothesized that a high concentration of MIF could exist in the regional fluid of the eye as well as in serum in cases of severe atopic dermatitis. Because the Scoring Atopic Dermatitis (SCORAD) index, which is determined on the basis of several criteria related to lesion spread and intensity as well as on subjective signs, is commonly used to evaluate AD [21], AD patients were classified into two groups, as moderate-to-severe or mild AD, according to SCORAD index in the present study. We measured the MIF levels in tears (lacrimal fluid) of patients with AD and compared them to those of patients with allergic conjunctivitis (AC) and healthy people.

Materials and methods

Patients

We studied 16 patients with AD and 9 subjects with AC who visited the Departments of Dermatology and Ophthalmology, Hokkaido University Hospital, Sapporo, Japan. Atopic dermatitis is a common inflammatory disorder characterized by a chronic and relapsing course. In order to evaluate the severity of the disease as objectively as possible, the European Task Force on Atopic Dermatitis developed a method allowing consistent assessment by means of a severity index, called the Scoring Atopic Dermatitis (SCORAD) index [21]. The index should be calculated as a sum of the following scores: (1) extent criteria (involved surface area), (2) intensity criteria (erythema, edema/papulation, oozing/crusting, excoriation, and lichenification), and (3) subjective symptoms (pruritus and insomnia) [21]. We classified cases of AD in this study as "moderate-to-severe" (SCORAD≥15) or "mild" (SCORAD<15) according to the SCORAD index. Each patient with moderate-to-severe AD had atopic manifestations on the facial skin. Allergic conjunctivitis (AC) was diagnosed by slit lamp examination according to the guidelines of diagnosis and treatment of conjunctivitis, reported elsewhere [23]. Although we collected tear samples out of pollen season (December, January, and February), five of ten AC patients stated that they had sensitivity to grass or birch pollen. Most of the AC patients were considered to be in the chronic phase of AC, and their conjunctival signs and symptoms were mild. Tear samples were collected from 9 patients with severe AD (mean age, 26.1 years; age range, 18–37 years), 7 patients with mild AD (mean age, 29.0 years; age range, 16–44 years), 10 patients with AC (mean age, 32.6 years; age range, 22–44 years), and 15 healthy volunteers (mean age, 34.6 years; age range, 23–45 years). All subjects were Japanese, and healthy volunteers with no history of AD were recruited from our colleagues as controls. Dermatologists and ophthalmologists also verified no manifestations of AD and AC in controls when their tear/serum samples were collected. Informed consent was obtained from every patient and control subject.

Collection of tears and sera

All of the experiments in this study followed the tenets of the Declaration of Helsinki. After informed consent was obtained, tear samples were collected from all subjects. To obtain unstimulated basal lacrimal fluid, the tear samples (10 μl) were collected with microcapillary tubes for microhematocrit (75 mm length, Funakoshi, Tokyo) at the lateral canthus of patients in the supine position without any anesthesia. After obtaining informed consent, serum samples were collected from two of the severe AD patients whose tear MIF levels were quite high (>27.2 ng/ml), exceeding one standard deviation (SD) from the group's median value. Also, two subjects were chosen randomly from the patients with mild AD and healthy controls to measure their serum MIF levels.

Tear samples were centrifuged immediately at 4°C to remove cells and transferred into new tubes. Tear and serum samples were stored at −80°C until further examination.

Measurement of MIF

Macrophage migration inhibitory factor levels were determined by a human MIF enzyme-linked immunosorbent assay (ELISA) (CosmoBio, Tokyo, Japan) as described previously [18]. The kit contains all reagents necessary for performing the assay. Statistical analysis was performed using the Mann-Whitney U test.

Table 1 Values and significance of MIF levels in tears

	MIF levels (Mean MIF±SE)	*P* values vs. normal
Normal controls	0.69±0.2	-
AD: moderate to severe	17.87±6.3	0.002**
AD: mild	0.93±0.08	0.07
Allergic conjunctivitis	2.76±0.86	0.008**

AD Atopic dermatitis
**$P<0.01$ (Mann-Whitney U test, two-tailed)

Results

The mean MIF level in lacrimal fluid collected from healthy control subjects was 0.69±0.2 ng/ml. The mean tear MIF levels were 17.87±6.3 ng/ml in cases of moderate-to-severe AD (SCORAD≥15), 0.93±0.08 ng/ml in cases of mild AD (SCORAD<15), and 2.76±0.86 ng/ml in cases of allergic conjunctivitis (AC). Tear MIF levels were significantly elevated in patients with moderate-to-severe AD compared to normal controls ($P=0.002$, Table 1). The tear MIF levels of patients with AC were also higher than those of healthy subjects ($P=0.008$, Table 1). We did not, however, detect any significant difference in tear MIF levels between patients with mild AD (SCORAD<15) and healthy control subjects ($P=0.07$, Table 1).

We then focused on two cases of severe AD in which the tear MIF levels were higher than 27.2 ng/ml, i.e., more than one standard deviation (SD) from the group's median value. Both had the severest skin manifestations of atopic dermatitis in this study when their tears were collected. After informed consent was obtained from these patients, serum samples were drawn and the serum MIF levels were measured. As shown in Table 2, their serum MIF levels were approximately equivalent to those in the lacrimal fluid of patients with severe AD. In contrast, although the serum MIF levels in cases of mild AD were still elevated compared to those of healthy controls, their MIF concentrations in tears were no higher than those of healthy controls (Table 2).

Table 2 MIF concentrations of tears and sera

Cases	Age/sex	Tear MIF (ng/ml)	Serum MIF (ng/ml)
1. AD: severe	20/M	63.4	79.7
2. AD: severe	35/M	30.1	42.0
3. AD: mild	44/F	0.9	17.5
4. AD: mild	16/M	0.7	12.2
5. Control	45/F	0.8	4.7
6. Control	24/F	1.0	3.2

AD Atopic dermatitis

Discussion

In the present study, we detected high levels of MIF in the lacrimal fluid of patients with severe AD. We previously reported an increase in serum MIF levels in patients with AD [18]. Although AD frequently involves some ophthalmic features (blepharitis, chronic keratoconjunctivitis, keratoconus, early-onset cataract, and retinal detachment), how MIF behaves in the ocular fluid of patients with AD remained unknown. We wished to determine how tear MIF levels of patients with severe AD compared with AC and healthy subjects.

MIF is expressed and secreted in many tissues: in the brain, eye (lens, corneal epithelial cells, iris, ciliary body, and retina), ear, immune cells (thymus, spleen, lymph nodes, blood, and bone marrow, by monocytes, macrophages, T cells, B cells, dendritic cells, eosinophils, basophils, neutrophils, and mast cells), lungs, breast, endocrine systems (pituitary gland, adrenal cortex, and pacreas), liver, testis, prostate, ovaries, gastrointestinal tract, kidney, fat tissue, skin (by keratinocytes, sebaceous glands, hair follicles, endothelial cells and fibroblasts), bone, and joints [4]. This study demonstrated that tear MIF concentration is significantly higher in patients with severe AD than in controls. Patients with AC also showed significantly higher levels of MIF than healthy controls; however, there were vast differences between the averages of AD and AC groups.

Since MIF levels in tears were elevated for both diseases involving the immune system, one possible source of tear MIF is the lymphocytes in conjunctival follicles. Another possible cause of the elevation of tear MIF levels in the eyes may be the lacrimal gland, but no study has been performed to determine if the lacrimal gland expresses or secretes MIF or not. A third possible source may be peripheral blood mononuclear cells (PBMCs) [14]. As previously reported, a substantial increase in systemic MIF levels was detected in patients with severe AD, and the levels decreased when the skin symptoms improved following treatment with corticosteroid ointment [18]. Furthermore, the elevated serum MIF was due to secretion from systemic PBMC [18, 19]. We found two patients with severe AD who showed extremely high levels (in excess of 30 ng/ml) of MIF in this study. To examine how blood PBMC contributes to MIF levels in lacrimal fluid, we collected serum samples from AD patients as well as tears. Because a very high serum MIF concentration was detected in both of these patients (Table 2) and the space of the oculi is limited, some proportion of MIF in tears may be attributable to a systemic increase in MIF. The secretion of MIF from PBMCs might contribute to the elevation of tear MIF levels more than regional inflammatory cells of the eyes in patients with severe AD. Since we did not collect blood samples from AC subjects, it is still unclear how much blood PBMC contributes to tear MIF levels in cases of AC. In the present study, AC patients did not have

obvious systemic inflammation, but local. Moreover, we detected higher tear MIF levels in the AC group than in the mild AD group, suggesting that MIF may be secreted in the eye to some extent. Further studies might be required in vernal or other etiologies of conjunctivitis, as well as for treated vs. untreated AC to clarify eye-derived MIF in tears.

This is the first report that MIF concentrations in tears are elevated in cases of severe AD in humans. In conclusion, MIF in regional ocular fluid may be involved in the induction or enhancement of ophthalmic features caused by severe AD.

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Polymorphisms in the Muscarinic Receptor 1 Gene Confer Susceptibility to Asthma in Japanese Subjects

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Rationale: The human cholinergic receptor muscarinic-1 (CHRM1) is widely distributed in the lungs. In patients with asthma, CHRM1 may be involved in airway constriction, airway epithelial cell proliferation, and airway inflammation. The *CHRM1* gene is located on chromosome 11q13, which is one of the candidate loci for asthma and atopy.

Objectives: To determine the role of the *CHRM1* gene polymorphisms in asthma.

Methods: We studied nine single-nucleotide polymorphisms (−18379G > A, −9697C > T, −6965T > C, −4953A > G, +267A > C, +1353C > T, +3970C > G, +5418C > G, and +5455G > T) in a case-control study using 326 patients with asthma and 333 healthy control subjects. We also examined functional consequences of the −9697C > T and −4953A > G polymorphisms at the regulatory region using an mRNA reporter assay.

Measurements and Main Results: Two common single-nucleotide polymorphisms (−9697C > T and −4953A > G) were associated with asthma. The odds ratio for the TT homozygotes at the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% confidence interval, 0.12–0.73; p = 0.008), and the odds ratio for the GG homozygotes at the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% confidence interval, 1.04–3.34; p = 0.038). Haplotype analysis showed that the −9697T/−6965T/−4953A haplotype was associated with a lower risk of asthma (p = 0.00055) and the −9697C/−6965T/−4953G haplotype was associated with an increased risk of asthma (p = 0.020). The −9697T/−4953A haplotype was also associated with lower luciferase activity *in vitro* compared with the −9697C/−4953G haplotype.

Conclusions: This study, together with an *in vitro* functional study, suggests that the *CHRM1* gene is an important susceptibility locus for asthma on chromosome11q13.

Keywords: case-control studies; IgE; muscarinic cholinergic receptor-1; single-nucleotide polymorphism

The cholinergic nerves are the dominant neural bronchoconstrictor pathway in humans (1). They release acetylcholine onto muscarinic receptors causing cholinergic bronchoconstriction (2), mucous hypersecretion, and edema in the airways. Increases in cholinergic nerve activity and cholinergic hypersensitivity are associated with asthma, and patients with asthma are hypersensitive to the bronchoconstricting actions of muscarinic agonists (3). The human cholinergic receptor muscarinic 1 (CHRM1; Online Mendelian Inheritance of Man database no. 118510) is widely localized in the human lung, including the alveolar walls, bronchial epithelial cells, parasympathetic ganglia, neuromuscular junction, and submucosal glands (4). Studies using pirenzepine, a muscarinic antagonist selective for M1 receptors, have shown that M1 muscarinic receptors are involved in vagally induced bronchoconstriction (5–7). M1 receptor–deficient mice showed increased bronchoconstriction in response to 10^{-8} M muscarine in peripheral airways (8), suggesting the existence of an M1 receptor–dependent pathway counteracting cholinergic bronchoconstriction. M1 receptors also play a role in mast cell function (9), epithelial cell proliferation in the trachea (10), release of neutrophil and monocyte chemotactic activity from epithelial cells (11), acetylcholine-induced relaxation of the human pulmonary veins (12), and regulation of water and electrolyte secretion on submucosal glands (13). Taken together, CHRM1 is critically involved in the pathophysiology of asthma.

The gene encoding CHRM1 exists on chromosome 11q13, which has been linked to asthma and asthma-related phenotypes in several genomewide searches (14–17). Given the important role of muscarinic cholinergic mechanisms in asthma, the *CHRM1* gene is biologically an excellent candidate for asthma susceptibility in the region of chromosome 11q13. Thus, in the current study, we examined whether genetic variations in the *CHRM1* gene are associated with asthma. To gain insight into the possible molecular basis of the disease association, we also examined functional consequences of single-nucleotide polymorphisms (SNPs) at the regulatory region of the *CHRM1* gene.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Genetic studies repeatedly have linked asthma and asthma-related phenotypes to chromosome 11q13, on which several biological candidate genes are located.

What This Study Adds to the Field

Gene coding the human cholinergic receptor muscarinic-1 (CHRM1) is an important susceptibility locus for asthma at chromosome 11q13.

METHODS

See online supplement for additional details.

Study Subjects

A total of 659 unrelated Japanese adults were enrolled in the study (Table 1). Asthma was defined on the basis of recurrent episodes of at least two of three symptoms (cough, wheeze, and dyspnea) that are associated with demonstrable reversible airflow limitation (15% variability in FEV_1 or in peak expiratory flow rate either spontaneously or with an inhaled, short-acting β_2-agonist), or increased airway responsiveness to methacholine, or both, as described elsewhere (18).

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TABLE 1. BASIC CHARACTERISTICS OF 659 JAPANESE SUBJECTS

	Healthy Control Subjects (*n* = 333)	Subjects with Asthma (*n* = 326)	p Value*
Age, yr, median (range)	41 (18–72)	45 (16–79)	< 0.0001
Sex, n (male/female)	208/125	148/178	< 0.0001
Smoking, n (never/ex-/current)	226/12/95	190/69/67	< 0.0001
Atopy, n (%)	170 (51)	237 (73)	< 0.0001
Total serum IgE, log IU/ml, mean (SD)	1.84 (0.627)	2.40 (0.622)	< 0.0001
FEV_1, % predicted, mean (SD)	—	69.2 (13.5)	
% Reversibility in FEV_1, median (range)	—	16.9 (0–211)	

* One-way analysis of variance or χ^2 test was used where appropriate.

Identification of Polymorphisms

Genomic DNA from Japanese subjects was genotyped for +267A > C (rs2067477) and +1353C > T (rs2067480), because an association of these polymorphisms at the muscarinic M1 receptor gene with cognitive function in schizophrenic patients has been reported (19). We selected an additional seven SNPs for genotyping based on the frequency and location of SNPs and the linkage disequilibrium (LD) structure in and around the *CHRM1* gene. We initially obtained genotyping data of 26 HapMap SNPs (spanning 31.6 kb around the gene) from 45 unrelated Japanese subjects at the International HapMap Project (available online at http://hapmap.org/). To select tagSNPs in this region, we used the multimarker predictor method implemented in the Tagger program (20). Tag set was generated (using a threshold r^2 of 0.8) using 14 common SNPs with a minor allele frequency of more than 0.05 in the Japanese population.

As the +267A > C and +1353C > T polymorphisms were in a complete LD, we genotyped a total of eight SNPs (−18379G > A [rs1938677], −9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499], +1353C > T [rs2067480], 3970C > G [rs4963323], 5418C > G [rs11601597], and 5455G > T [rs11605665]) for all individuals (n = 659). These SNPs were typed using the assay that combines kinetic (real-time quantitative) polymerase chain reaction (PCR) with allele-specific amplification, as described elsewhere (18). The PCR products were detected using the ABI 7700 Sequence Detection System with the dsDNA-specific fluorescent dye SYBR Green I (Applied Biosystems, Foster City, CA). The −4953A > G polymorphism was typed using TaqMan assay (Applied Biosystems).

Statistical Analysis

The association of the *CHRM1* gene polymorphism was measured by odds ratio (OR) with 95% confidence intervals (CI) as estimates of relative risk for the development of asthma. We used the Hardy-Weinberg equilibrium (HWE) program (21) to compare observed numbers of genotypes with the numbers of genotypes expected under HWE. For haplotype analyses, we used the Haplo.score program, which adjusts for covariates and calculates simulation p values for each haplotype (22).

Luciferase Reporter Gene Assay

We constructed two promoter reporter plasmids by placing two haplotypes (−9697C/−4953G and −9697T/−4953A) into the pGL3-Basic vector. Human neuroblastoma IMR32 cells (1×10^6) were transiently transfected with 9.5 μg of one of the two constructs and 0.5 μg of the pRL-TK vector, an internal control for transfection efficiency. After 24 h, we measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan). Results were expressed as means ± SEM and were compared by paired *t* test.

Electrophoretic Mobility Shift Assay

Transcription factor (nuclear factor [NF]-κB or upstream stimulating factor [USF]-1)-DNA binding activity was analyzed using the electrophoretic mobility shift assay (EMSA) kit (Panomics, Redwood, CA), according to the manufacturer's instructions.

RESULTS

Characteristics of the 333 healthy control subjects and 326 subjects with asthma are shown in Table 1. The median age of subjects with asthma was significantly higher than in healthy control subjects ($p < 0.0001$). There were significantly more females in the asthma group than in the control group ($p < 0.0001$). Subjects with asthma were more likely to be atopic and had higher levels of total serum IgE than did healthy control subjects (χ^2 test or analysis of variance, $p < 0.0001$). More than 50% of the control subjects were atopic, which is consistent with

TABLE 2. COMPARISONS OF ALLELE AND GENOTYPE FREQUENCIES OF EIGHT *CHRM1* SINGLE-NUCLEOTIDE POLYMORPHISMS BETWEEN PATIENTS WITH ASTHMA AND CONTROL SUBJECTS

SNP	Allele/Genotype	HC *n* (%)	BA *n* (%)	p Value*
−18379 (rs1938677)	G	361 (54.5)	335 (48.1)	0.33
	A	301 (45.5)	311 (51.9)	
	GG	107 (32.3)	92 (28.5)	0.56
	GA	146 (44.1)	151 (46.7)	
	AA	78 (23.6)	80 (24.8)	
−9697 (rs2075748)	C	508 (76.3)	533 (81.7)	0.015
	T	158 (23.7)	119 (18.3)	
	CC	195 (58.6)	216 (66.3)	0.039
	CT	118 (35.4)	101 (31.0)	
	TT	20 (6.0)	9 (2.7)	
−6965 (rs542269)	T	491 (73.9)	472 (73.1)	0.72
	C	173 (26.1)	174 (26.9)	
	TT	184 (55.4)	175 (54.2)	0.94
	TC	123 (37.1)	122 (37.8)	
	CC	25 (7.5)	26 (8.0)	
−4953 (rs1942499)	A	477 (71.6)	434 (66.6)	0.047
	G	189 (28.4)	218 (33.4)	
	AA	174 (52.3)	147 (45.1)	0.15
	AG	129 (38.7)	140 (42.9)	
	GG	30 (9.0)	39 (12.0)	
+1353 (rs2067480)	C	615 (92.3)	608 (93.3)	0.52
	T	51 (7.7)	44 (6.7)	
	CC	284 (85.3)	287 (88.0)	0.19
	CT	47 (14.1)	34 (10.5)	
	TT	2 (0.6)	5 (1.5)	
+3970 (rs4963323)	C	533 (80.3)	524 (80.9)	0.78
	G	131 (19.7)	124 (19.1)	
	CC	216 (65.1)	211 (65.1)	0.75
	CG	101 (30.4)	102 (31.5)	
	GG	15 (4.5)	11 (3.4)	
+5418 (rs11601597)	C	414 (62.3)	385 (59.6)	0.31
	G	250 (37.7)	261 (40.4)	
	CC	132 (39.7)	113 (35.0)	0.44
	CG	150 (45.2)	159 (49.2)	
	GG	50 (15.1)	51 (15.8)	
+5455 (rs11605665)	G	538 (81.5)	496 (77.5)	0.074
	T	122 (18.5)	144 (22.5)	
	GG	218 (66.1)	192 (60.0)	0.19
	GT	102 (30.9)	112 (35.0)	
	TT	10 (3.0)	16 (5.0)	

Definition of abbreviations: BA = bronchial asthma; HC = healthy controls.
* χ^2 Test.

recent findings that the prevalence of atopy (as indicated by specific IgE against common inhaled allergens) among Japanese subjects is increasing (23, 24). Prebronchodilator baseline FEV_1 at an initial visit to our hospital was examined for 293 subjects with asthma (89.9%), and improvement of FEV_1 after bronchodilator therapy (400 μg salbutamol) or after a course of standard asthma medications (inhaled corticosteroids, long-acting β_2-agonists, theophylline, or leukotriene modulators) was recorded for 214 (65.6%) subjects with asthma (Table 1).

All eight of the SNPs investigated were within the HWE in the control group ($p > 0.05$). The overall success rate for genotyping was 99.6%. Of the eight SNPs, two common SNPs (−9697C > T [rs2075748] and −4953A > G [rs1942499]) in the regulatory region of the *CHRM1* gene had a significant association with asthma (Table 2). Both of these SNPs were significantly associated with asthma when the analysis was adjusted for age, sex, smoking status, and atopic status (Table 3). The OR for the TT homozygotes of the −9697C > T polymorphism was 0.29 compared with the CC homozygotes (95% CI, 0.12–0.73; $p = 0.008$), and the OR for the GG homozygotes of the −4953A > G polymorphism was 1.86 compared with the AA homozygotes (95% CI, 1.04–3.34; $p = 0.038$).

We analyzed data from the eight SNPs with the Haploview program (25) and identified two haplotype blocks (Figure 1) in our case-control population. Haplotype block I comprised three SNPs in the regulatory region (−9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499]), and haplotype block II comprised three SNPs (+1353C > T [rs2067480], +3970C > G [rs4963323], +5418C > G [rs11601597]) in the coding exon and the 3′-UTR. Haplotype analyses were performed in both blocks I and II. The frequency of *CHRM1* haplotypes is shown in Table 4. In block I, the −9697T/−6965T/−4953A haplotype was associated with a significantly lower risk of asthma ($p = 0.00055$) and the −9697C/−6965T/−4953G haplotype was associated with a significantly increased risk of asthma ($p = 0.020$). Inspection of specific haplotypes revealed that this association is most likely due to −9697C > T and −4953A > G, because the same allele for −6965T > C is part of both risk and protective haplotypes. In contrast, none of the haplotypes in block II was associated with asthma.

In the case-only study, associations between asthma-related phenotypes, such as total serum IgE levels and atopy, and the polymorphisms of *CHRM1* were also investigated. We could not find any significant association between the genotypes of the eight SNPs and total serum IgE levels or atopy (*see* Tables E1 and E2 in the online supplement).

The transcriptional activity of the *CHRM1* SNPs at the regulatory region was compared between the −9697C/−4953G haplotype and the −9697T/−4953A haplotype transiently transfected into human neuroblastoma IMR32 cells. Luciferase activity in cell extracts was assessed 24 h after transfection, and was expressed as fold increase in the activity of the *CHRM1* reporter constructs compared with the pRL-TK vector. Figure 2 shows that the reporter plasmid carrying the −9697T/−4953A promoter displayed 37% lower transcriptional activity compared with the plasmid carrying the −9697C/−4953G promoter ($p = 0.019$).

TABLE 3. GENETIC IMPACT ON ASTHMA OF EIGHT SINGLE-NUCLEOTIDE POLYMORPHISMS IN AND AROUND THE CHRM1 *GENE*

		OR (*95% CI*)	
SNP	Genotype	Adjustments (−)	Adjustments (+)*
−18379G > A (rs1938677)	GG	1 (Reference)	1 (Reference)
	GA	1.20 (0.84–1.72)	1.14 (0.77–1.71)
	AA	1.19 (0.79–1.81)	1.33 (0.84–2.12)
−9697C > T (rs2075748)	CC	1 (Reference)	1 (Reference)
	CT	0.77 (0.56–1.07)	0.73 (0.51–1.06)
	TT	0.41 (0.18–0.91)†	0.29 (0.12–0.73)‡
−6965T > C (rs542269)	TT	1 (Reference)	1 (Reference)
	TC	1.04 (0.75–1.44)	1.00 (0.70–1.44)
	CC	1.09 (0.61–1.97)	1.04 (0.54–2.02)
−4953A > G (rs1942499)	AA	1 (Reference)	1 (Reference)
	AG	1.28 (0.93–1.78)	1.38 (0.96–1.98)
	GG	1.54 (0.91–2.60)	1.86 (1.04–3.34)†
+1353C > T (rs2067480)	CC	1 (Reference)	1 (Reference)
	CT	0.72 (0.45–1.15)	0.61 (0.37–1.02)
	TT	2.47 (0.48–12.9)	1.96 (0.31–12.5)
+3970C > G (rs4963323)	CC	1 (Reference)	1 (Reference)
	CG	1.03 (0.74–1.44)	1.12 (0.78–1.62)
	GG	0.75 (0.34–1.67)	0.78 (0.33–1.86)
+5418C > G (rs11601597)	CC	1 (Reference)	1 (Reference)
	CG	1.24 (0.88–1.73)	1.29 (0.89–1.88)
	GG	1.19 (0.75–1.89)	1.16 (0.70–1.93)
+5455G > T (rs11605665)	GG	1 (Reference)	1 (Reference)
	GT	1.25 (0.90–1.74)	1.28 (0.89–1.85)
	TT	1.82 (0.81–4.1)	1.73 (0.73–4.09)

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

* Adjustment for matching factors and potential confounding factors, including sex, age, smoking status, and atopic status, was performed by unconditional logistic regression analysis.

† $p < 0.05$.

‡ $p < 0.01$.

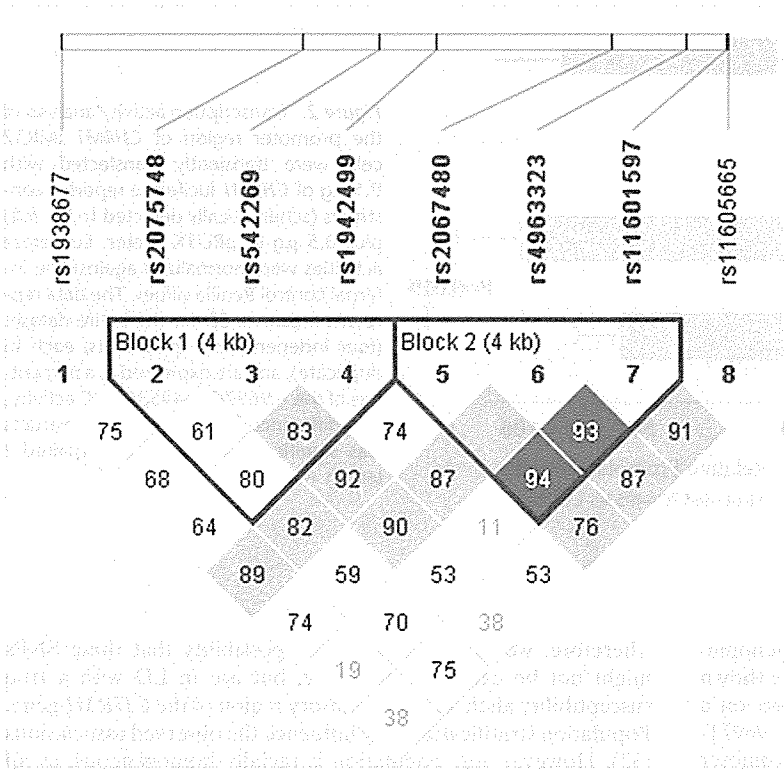

Figure 1. Locations and linkage disequilibrium (LD) map structure of single-nucleotide polymorphisms (SNPs) in and around the *CHRM1* Haploview plot shows pairwise LD (D′ values) for 8 SNPs based on genotypes of 659 individuals of the case-control study. The eight SNPs include −18379G > A [rs1938677], −9697C > T [rs2075748], −6965T > C [rs542269], −4953A > G [rs1942499], +1353C > T [rs2067480], +3970C > G [rs4963323], +5418C > G [rs11601597], and +5455G > T [rs11605665]. LD blocks are framed in *black* and were classified according to the "solid spine" option (25). Each *square* plots the level of LD (D′ values) between a pair of SNPs.

This difference in transcriptional activity was consistent in eight independent experiments.

EMSA failed to show a robust difference in binding affinity of NF-κB to the −9697T or the −9697C allele, or in binding affinity of USF-1 to the −4953A or the −4953G allele (data not shown).

DISCUSSION

Given a high *a priori* biological plausibility for asthma, we tested the hypothesis that the allelic variants at the regulatory region of the *CHRM1* confer susceptibility to asthma by conducting a case-control study in a relatively large population of unrelated Japanese subjects. In accordance with our primary hypothesis, we found that the presence of the −9697CC genotype, −4953GG genotype, or the −9697C/−4953G haplotype at the regulatory region was significantly associated with a diagnosis of asthma. Our genetic association study had several strengths: first, muscarinic receptors, including M1, have been biologically implicated in the pathogenesis of asthma; second, the gene encoding the CHRM1 is located on chromosome 11q13, a genomic region

TABLE 4. ESTIMATED HAPLOTYPE FREQUENCIES OF THE *CHRM1* GENE POLYMORPHISMS

				Haplotype Frequency			
				Control (*n* = 333)	Asthma (*n* = 326)	Haplotype-specific Score	p Value* (*Empirical*)
Block I							
Haplotype	−9697/−6965/−4953						
1	T	T	A	0.207	0.158	−3.03	0.00055
2	C	C	A	0.239	0.234	−0.23	0.827
3	C	T	A	0.250	0.254	0.057	0.959
4	C	T	G	0.268	0.303	2.15	0.020
Block II							
Haplotype	+1353/+3970/+5418						
1	T	C	C	0.371	0.391	0.120	0.131
2	C	C	C	0.353	0.349	0.808	0.814
3	C	G	C	0.192	0.179	0.926	0.918
4	C	C	G	0.371	0.391	0.454	0.481

Haplotype frequencies were estimated using the Haplo.Stats program. In Block I (regulatory region of the gene), haplotype analyses showed that the −9697T/−6965T/−4953A haplotype was associated with a significantly lower risk of asthma (p = 0.00055) and the −9697C/−6965T/−4953G haplotype was associated with a significantly increased risk of asthma (p = 0.020). In contrast, in Block II, none of haplotypes showed a significant association with asthma. Note that haplotype-specific scores give effect estimates; negative haplotype-specific scores are associated with a protective effect, and positive haplotype-specific scores are associated with an increased risk. Haplotypes with frequencies less than 0.05 were excluded from the analyses.

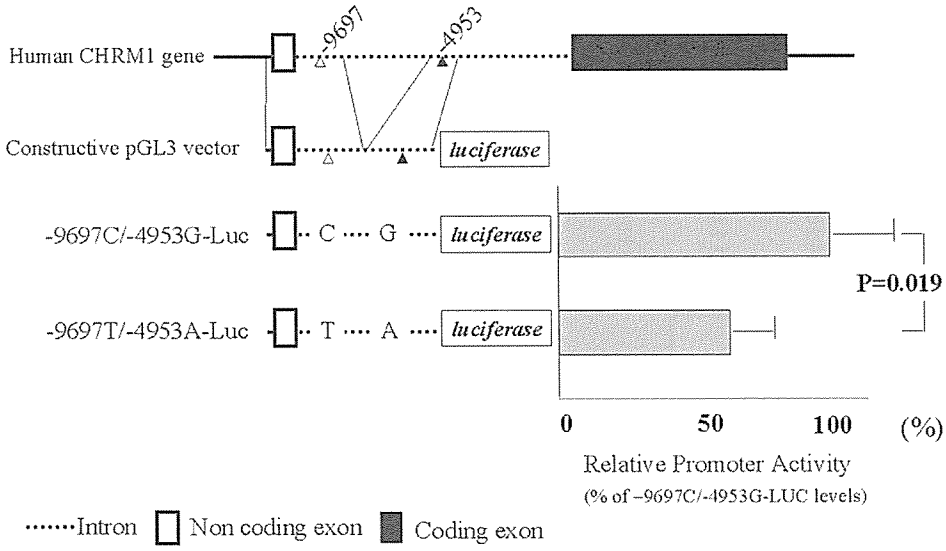

Figure 2. Transcription activity analysis of the promoter region of *CHRM1* IMR32 cells were transiently transfected with 9.5 μg of *CHRM1* luciferase reporter constructs (schematically depicted to the *left*) plus 0.5 μg of pRL-TK vector. Luciferase activities were normalized against the internal control Renilla values. The data represent means ± SD for the entire dataset (four independent experiments, each in duplicate), and are expressed as a percentage of the −9697C/−4953G LUC activity. The difference between these constructs was significant at $p = 0.019$ (paired *t* test; $n = 8$).

linked to the diagnosis of asthma and atopy in several genome-wide scans (14–17); third, *in vitro* functional analyses have shown that the haplotype at the regulatory region has an effect on a basal promoter function in IMR32 cells, with the −9697T/−4953A haplotype associated with 37% decreased promoter activity compared with the −9697C/−4953G haplotype. Accordingly, our data suggest that the −9697/−4953 haplotype may influence the affinity of a particular nuclear protein to the regulatory region of the *CHRM1* gene, resulting in altered transcriptional activity and ultimately leading to a higher or lower risk of asthma.

Although the exact mechanisms underlying the involvement of the *CHRM1* gene in the pathogenesis of asthma remain to be identified, several reports indicate that the cholinergic pathway has an important role in the pathogenesis of asthma, in particular in the regulation of bronchoconstriction, airway inflammation, and airway remodeling. An M1 receptor–dependent pathway counteracts cholinergic bronchoconstriction, possibly via the release of a relaxing agent (8); both respiratory epithelia and sympathetic nerve terminals within bronchial smooth muscle are equipped with M1 receptors (7, 26) and releasable bronchodilating agents, such as nitric oxide and prostaglandin E_2 (27). Studies with the M1 receptor–preferring antagonist, pirenzepine, have also suggested the existence of pulmonary M1 receptors modulating airway diameter (28). Furthermore, Jones and colleagues (29) demonstrated that stable expression in RBL-2H3 mast cells of the M1 muscarinic acetylcholine receptor leads to carbachol-stimulated mast cell degranulation. An animal model of asthma showed that anticholinergic agents protect against allergen-induced airway remodeling (30). Together with these *in vivo* and *in vitro* findings, our findings support the contention that CHRM1 plays an important role in the pathogenesis of asthma. Our findings may be of considerable relevance to asthma treatment, providing an important basis for identification of individuals for whom the cholinergic pathway could be targeted.

Sequence analysis indicated that the T allele at the −9697C > T polymorphism creates a potential NF-κB binding site and that the A allele at the −4953A > G polymorphism creates a potential USF-1 transcription factor binding site by reference to the MatInspector or TFSEARCH database (31). We, however, failed to see any difference in binding intensities of these nuclear factors to the −9697C > T or −4953A > G polymorphism. Therefore, we cannot exclude the possibility that these SNPs might not be causative in nature, but are in LD with a true susceptibility allele in the regulatory region of the *CHRM1* gene. Population stratification may influence the observed associations (32). However, our population is racially homogeneous, as all subjects recruited in the study were from the Japanese population, which is considered monoracial; thus, our subjects had a relatively low risk of population stratification effects. Furthermore, we recruited all participants in the current study from a single institute to minimize the chance of mixing populations with inherently diverse allele frequencies of a susceptibility gene. In addition, all SNPs were in HWE in a set of unrelated healthy subjects. Therefore, we believe that the usual problems associated with population stratification may be of limited importance in the present study. Nevertheless, we acknowledge that population stratification may have influenced the present findings, and that the findings of the current study are preliminary and do not, by themselves, conclusively confirm an etiologic relationship. A more comprehensive approach that examines the functional consequences of the *CHRM1* promoter polymorphisms and identifies the possible promoter-dependent mechanism for an association between *CHRM1* and asthma is required.

In conclusion, given the important role of muscarinic cholinergic mechanisms in pulmonary disease, this case–control study, together with an *in vitro* functional analysis, suggests that the *CHRM1* gene is an important susceptibility locus for asthma at chromosome 11q13. The −9697/−4953 haplotype at the regulatory region of the gene may contribute to the development of asthma by altering the human lung muscarinic receptor system in ways that could account for the increased *in vivo* lung cholinergic hyperresponsiveness found in patients with asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Bacterial infection-induced generalized Hailey–Hailey disease successfully treated by etretinate

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Summary

Hailey–Hailey disease (HHD; familial benign chronic pemphigus) is a hereditary blistering disorder characterized by episodic maceration and erosions mainly in intertriginous areas, and generalized eruptions are rarely seen. We report here a 51-year-old woman with generalized HHD who was successfully treated with oral etretinate. The present case suggests that oral etretinate is effective against the generalized eruptions even in cases in which bacterial infection has triggered the generalization of HHD.

Report

A 51-year-old woman was referred to our hospital for the treatment of generalized erythema. The patient had had erythematous plaques on her axillae and groin for about 20 years (Fig. 1a,b). Her eruptions tended to become exacerbated in summer and remitted in winter. She was treated with corticosteroid ointments for 8 years without any complete improvement of the eruption. Skin-biopsy specimens from the erythema on her left axilla showed parakeratosis, hyperkeratosis and suprabasal acantholysis (Fig. 2a). From these clinical and histopathological findings, she was diagnosed as having Hailey–Hailey disease (HHD). Two months later, the skin lesions suddenly extended to her trunk, arms and thighs (Fig. 1c,d).

Physical examination revealed severe erythema, milia-sized pustules, crusts and scales on her whole body. In addition to the widespread rash, she had malodorous plaques with fissures in her axillae and groin. She did not show any signs of fever, although her C-reactive protein was slightly elevated at 1.97 mg/L (normal < 0.24). Renal and liver functions and her peripheral blood haematological parameters were all within normal limits. Culture of the exudate from a skin lesion in her groin revealed a growth of *Pseudomonas aeruginosa* and *Staphylococcus* species. The skin-biopsy specimen from a pustule on her thigh revealed an inflammatory cell infiltration in the superficial dermis and microabscesses in the epidermis (Fig. 2b). Suprabasal blister formation with acantholysis was also seen. In the biopsy from the erythematous lesion on her thigh, suprabasal acantholysis and slight inflammatory infiltration were observed (Fig. 2c). She had been taking the same medication for several years, and there was no notable or suspected drug history to trigger the drug eruption. Drug lymphocyte-stimulation tests and patch tests were negative for all the medications she had been taking. From these findings, the extensive rash was diagnosed as generalized HHD presumably induced by cutaneous bacterial infection. The patient was treated with oral etretinate (60 mg daily) and topical corticosteroids. Only washing with a shower was performed for the superficial infection in the intertriginous areas. The lesions were cleared within approximately 2 weeks, while the erythema on the intertriginous areas remained.

HHD is a blistering disorder showing autosomal dominant inheritance, first described in 1939.[1] The causative genetic defect was identified as a mutation in *ATP2C1*, the gene on chromosome 3q21 coding an ATP-driven transmembrane calcium pump.[2] Generalized

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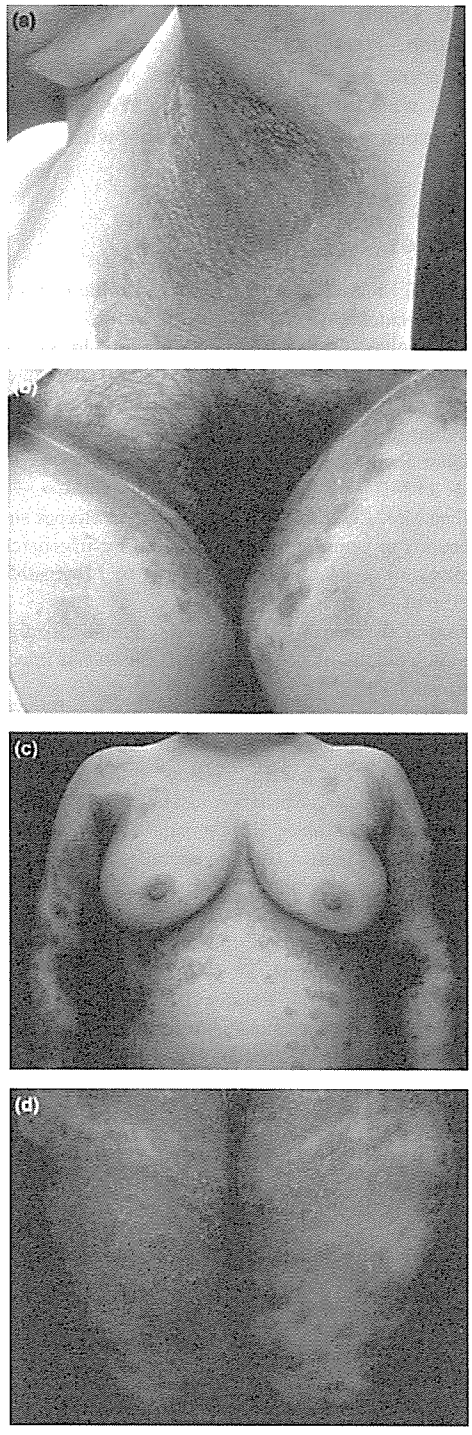

Figure 1 The clinical features of the original localized lesions (a, b) and the generalized eruptions (c, d). Before the disease became more generalized, erythema was seen in the axillae (a) and in the groin (b). After the generalization, erythematous lesions were seen in the arms and the trunk (c) and in the thigh (d).

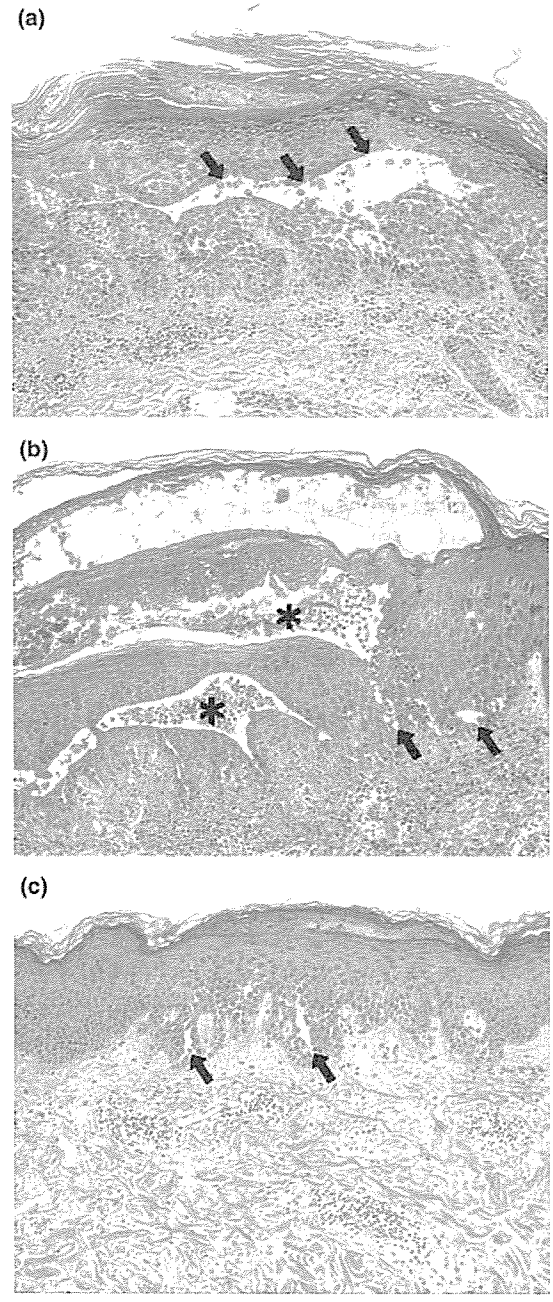

Figure 2 (a) Parakeratosis, hyperkeratosis and suprabasal acantholysis (arrows) were observed in the skin biopsy specimen from a typical HHD lesion from the patient's left axilla before the start of the generalized disease. (b) After the eruptions had spread to most of her body, biopsy specimens from a pustule on her thigh revealed a microabscess in the epidermis (*) and a suprabasal blister formation with acantholysis (arrows). (c) In a generalized erythematous area from her thigh, suprabasal acantholysis (arrows) is apparent and a slight infiltration of lymphocytes can be seen in the subcorneal area (haemotoxylin and eosin; original magnification × 50).

forms of HHD are remarkably rare events. The exact pathogenetic mechanisms of generalized HHD have not yet been clarified, although bacterial and herpes simplex virus infections, arthropod infestation, and drug eruptions are thought to be possible triggers.[3–8] *P. aeruginosa* and *Staphylococcus* species were detected in cultures from the intertriginous plaques of our patient and the superficial infection was thought to act as a trigger for the formation of the generalized HHD lesion. As described in the previous reports,[5,8] superficial cutaneous infection could initiate the exacerbation of HHD through a Koebner-like response.

As for the treatment, a variety of systemic and topical therapeutic agents including systemic steroids and antibiotics have been reported as effective for generalized HHD.[3–8] In a few reported cases of bacterial infection-induced generalized HHD, most of the patients were treated with systemic antibiotics with or without systemic steroids. The use of retinoids is controversial in HHD.[9] As far as we know, etretinate has not been tried as a treatment for generalized HHD in the literature, except for a case with vesiculobullous HHD treated with low-dose etretinate.[10] However, we thought as the generalized eruptions exhibited a similar nature to that of the original localized HHD lesions, retinoids might be effective against both types of lesion, even if the eruptions had been induced by bacterial infection. Thus, we successfully treated these generalized eruptions in the present case with oral etretinate. Etretinate is the only available retinoid in Japan, except for all-*trans* retinoic acid, which is a treatment for acute promyelocytic leukaemia. Anti-bacterial treatment including systemic and local antibiotics was not needed.

The present case suggests that oral etretinate is effective as a treatment for generalized HHD, even in cases in which bacterial infection has triggered the excerbation. Etretinate was withdrawn from sale in Europe some years ago, and the active metabolite, acitretin, was introduced instead. We can expect a similar response to acitretin in patients with generalized HHD.

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Childhood epidermolysis bullosa acquisita with autoantibodies against the noncollagenous 1 and 2 domains of type VII collagen: case report and review of the literature

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Conflicts of interest
None declared.

Summary

Epidermolysis bullosa acquisita (EBA) is an acquired subepidermal bullous disease characterized by IgG autoantibodies to type VII collagen, a major component of anchoring fibrils. Most patients with EBA are adult and develop autoantibodies to the noncollagenous (NC) 1 domain of type VII collagen. We describe a 4-year-old Japanese boy presenting pruritic vesicles and tense blisters over his whole body. Immunofluorescence studies revealed linear IgG/C3 deposits along the dermal–epidermal junction of the patient's skin, and circulating IgG autoantibodies mapping to the dermal side of 1 M NaCl-split skin. By immunoblotting analysis using dermal extracts as a substrate, the patient's IgG antibodies labelled a 290-kDa protein corresponding to type VII collagen. Immunoblotting studies using recombinant proteins demonstrated that the patient's circulating autoantibodies recognized not only the NC1 but also the NC2 domain of type VII procollagen. Review of the previously reported cases and the present case suggested that patients with EBA with autoantibodies to regions other than the NC1 domain are all children younger than 10 years of age with clinical features of an inflammatory phenotype.

Epidermolysis bullosa acquisita (EBA) is an autoimmune blistering skin disease with circulating IgG autoantibodies to type VII collagen, a major component of anchoring fibrils,[1] composed of three identical alpha chains. Each chain consists of a long central triple helical collagenous domain flanked by a large amino-terminal 145-kDa noncollagenous (NC) 1 domain and a smaller carboxyl-terminal 20-kDa NC2 domain. Type VII procollagen molecules form antiparallel dimers that are stabilized by disulphide bonding at the carboxyl terminus, and a portion of the NC2 domain is removed by specific proteolytic cleavage to yield the mature type VII collagen. Several dimers aggregate laterally to form the unique cross-banded structure, i.e. anchoring fibrils, which comprise antiparallel dimers and contain NC1 domains at both ends, locating in the lamina densa and forming semicircular loops visible by the electron microscope.[2]

Two distinct phenotypes of EBA have been described: the classical noninflammatory type and the inflammatory type. In a large number of adults with the clinically classical type of EBA it has previously been recognized that autoantibodies are directed against epitopes within the NC1 domain, whereas no immunoreactivity with the NC2 or the triple helical domain was detected.[3] However, novel variants with reactivity to the NC2 and/or the triple helical domains have recently been reported in five children with the inflammatory subtype of EBA.[4–6] These findings indicated that some distinct immunopathological differences between childhood and adult EBA could be present.

We describe a 4-year-old Japanese boy with EBA and review previously reported cases of childhood EBA.

Case report

A 4-year-old Japanese boy, generally in good health, developed painful oral blisters and erosions. A few weeks later, widespread pruritic vesicles and tense blisters appeared over almost his entire body, but especially on his scrotum and buccal mucosa. The blisters were seen both on an erythematous base and on normal skin, and showed an annular arrangement in some regions (Fig. 1). His nails were not affected. He had no family history of any blistering disorders.

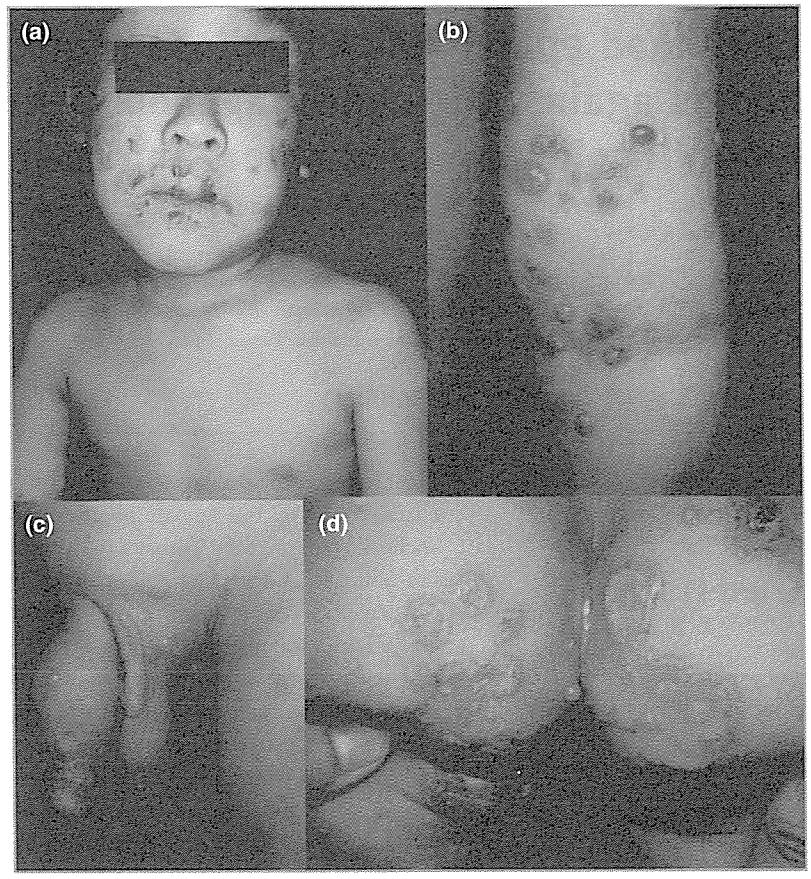

Fig 1. Clinical features. Disseminated, tense vesicles, bullae and erosions with crusts were seen over erythematous plaques on the face (a), arm (b), genital area (c) and buttocks (d).

General laboratory examinations including full blood count, liver and kidney function tests, urinalysis and C-reactive protein were within normal limits except for an increased number of eosinophils (13·5%) in his peripheral blood. Antinuclear antibodies were negative, and antiherpes simplex virus antibodies were not detected. Viral culture of vesicular fluid and a Tzanck smear test on a vesicular base were both negative.

Histopathology of a skin biopsy from the dorsal side of his foot revealed subepidermal blisters with an inflammatory infiltrate of lymphocytes, neutrophils and eosinophils in the papillary and superficial reticular dermis. In particular, neutrophils and nuclear dust were seen at the tips of the oedematous dermal papillae (Fig. 2).

On direct immunofluorescence (IF), linear deposits of IgG and C3, but not IgA, were seen along the dermal–epidermal junction of the patient's skin biopsy specimen. Indirect IF studies using 1M NaCl-split normal human skin as a substrate showed IgG deposits on the dermal side of the artificial split at a titre of 1 : 160 (Fig. 3). Circulating autoantibodies to the NC16a domain of the 180-kDa bullous pemphigoid (BP) antigen (BP180) were not detected using a BP180 enzyme-linked immunosorbent assay kit (MBL, Naka-ku, Nagoya, Japan). Immunoblot analysis demonstrated that the patient's serum reacted both with a 290-kDa protein in dermal extracts and with recombinant type VII collagen (Fig. 4a,b). In addition to the

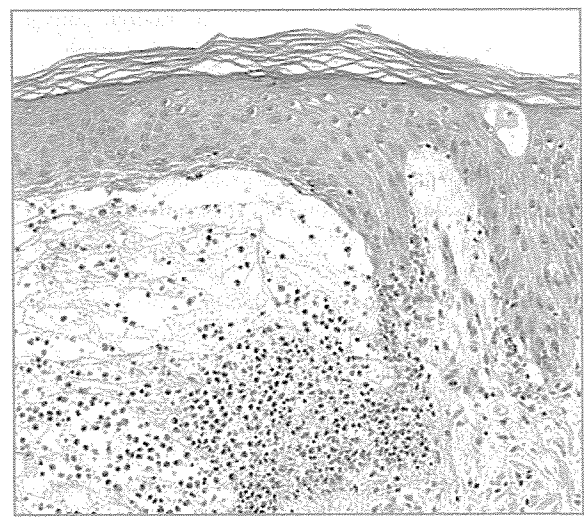

Fig 2. Histopathology. A subepidermal blister with an inflammatory infiltrate of lymphocytes together with eosinophils and neutrophils in the papillary dermis (haematoxylin and eosin; original magnification × 125).

290-kDa band, many additional bands were seen in the immunoblot analysis with patient serum using dermal extracts as a substrate. These additional bands were thought to reflect

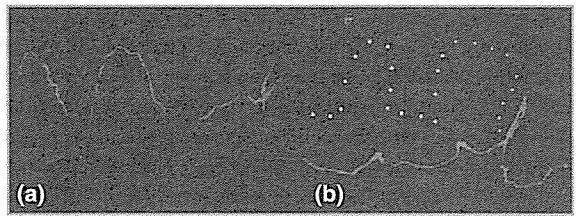

Fig 3. (a) By direct immunofluorescence (IF), linear IgG deposits were seen along the dermal–epidermal junction in the patient's skin. (b) Indirect IF using 1M NaCl-split human skin as a substrate revealed linear IgG deposits along the dermal side of the artificial split. Dots show roof side of the split skin.

reactivity of the patient's serum to background degradation products because, in this immunoblot analysis, we used high-concentration sera in order not to overlook the 200-kDa band in p200 pemphigoid. Epitope analyses with type VII procollagen recombinant fragments revealed that the patient's serum recognized the NC1 and the NC2 domains (Fig. 4c).

The boy was treated with oral prednisolone (1·1 mg kg^{-1} daily) for 2 weeks, which inhibited new blister formation. When the dose of prednisolone was reduced to 0·4 mg kg^{-1} daily, new blister formation was observed. Oral dapsone 1·5 mg kg^{-1} daily was added, but on the next day, erythematous macules appeared on his upper trunk. Thus, dapsone was discontinued and the dose of prednisolone was increased to 0·7 mg kg^{-1} daily. During the next 2 months, the patient kept taking oral prednisolone 0·5 mg kg^{-1} daily, and the blisters steadily healed, leaving hyperpigmented and hypopigmented macules and milia.

Discussion

EBA is a relatively uncommon disease with an incidence about 10 times less than that of BP. EBA was first defined with the following characteristic features: trauma-induced bullae that heal with milia and scars mainly over the joints of the hands, elbows, feet and knees; nail dystrophy; typically adult onset; a negative family history of epidermolysis bullosa; and exclusion of other bullous diseases on the basis of clinical and laboratory evidence. The definitive features now include histological and immunopathological findings, such as subepidermal blisters; circulating autoantibodies against skin basement membrane zone antigens, binding to the dermal side of NaCl-split skin; IgG/C3 deposits in the anchoring fibril zone; and the presence of antitype VII collagen antibodies that are identified as a 290-kDa band by immunoblot analysis on dermal extracts.[1] Among patients with clinically diagnosed EBA, antibodies can be detected by immunoblotting with dermal extracts in approximately 30% of cases,[7] and by indirect IF using NaCl-split skin as a substrate in approximately 50% of cases.[8]

Two distinct phenotypes of EBA have been described, i.e. the classical noninflammatory type and the inflammatory type. The classical type presents with marked skin fragility, blisters and erosions at sites of trauma, and heals with scarring and milia. The inflammatory type can mimic almost all other chronic bullous diseases, and its clinical differentiation from BP, cicatricial pemphigoid and linear IgA bullous dermatosis may be difficult.[4–6,9–26] In some patients, characteristics of both classical and inflammatory phenotypes of EBA have been observed,[9] but most patients with EBA in adulthood appear to have the classical type of EBA.

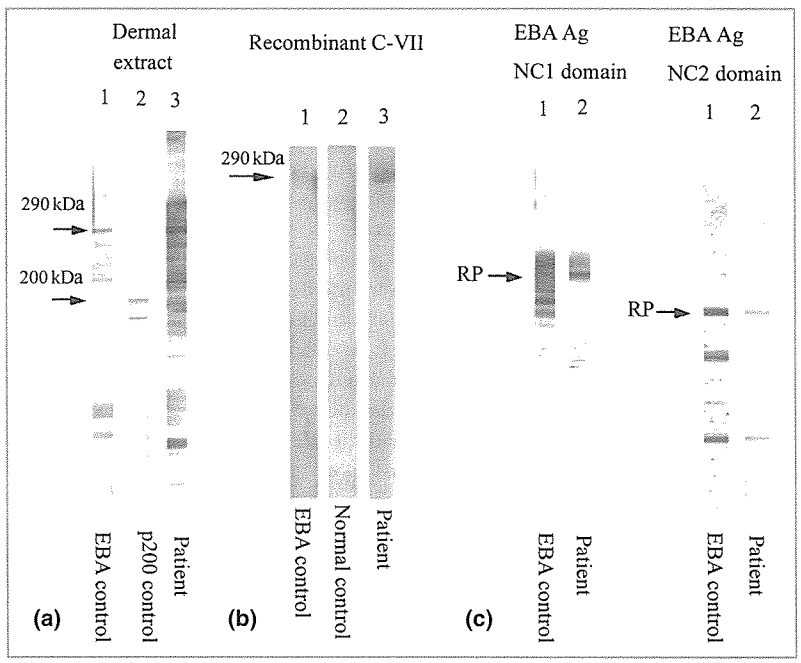

Fig 4. (a) Immunoblot analysis of patient's circulating autoantibodies on normal human dermal extracts. Arrows indicate the 290-kDa epidermolysis bullosa acquisita (EBA) antigen and the 200-kDa anti-p200 pemphigoid antigen. Control EBA serum (lane 1) and patient's serum (lane 3) reacted with EBA antigen, whereas control anti-p200 serum (lane 2) showed no 290-kDa band. (b) Immunoblot analysis of patient's circulating autoantibodies on recombinant type VII collagen. Arrow indicates the 290-kDa EBA antigen. Control EBA serum (lane 1) and patient's serum (lane 3) reacted with EBA antigen, whereas normal control serum (lane 2) showed no reactivity. (c) Immunoblot analyses using recombinant fusion proteins of noncollagenous (NC) 1 and NC2 domains of type VII collagen. Arrows indicate respective positions of fusion proteins. Both control EBA serum (lane 1) and patient's serum (lane 2) reacted with NC1 and with NC2.

Table 1 Patients with childhood epidermolysis bullosa acquisita in whom epitopes for autoantibodies were detected by immunoblot analysis

Patient	Age (years)/ race/sex	DIF (BMZ)	IgG deposits in NaCl split skin IIF	Immunoblot on dermal extracts (kDa)	Immunoblot on epidermal extracts (kDa)	Recombinant protein immunoblot analysis					Therapy	Reference
						NC1	TH	NC2	rBP180	NC16a ELISA		
1	8/Japanese/F	IgG, IgA, C3	Dermal side	290 (+)	230 (+)	–	+	–	ND	–	Prednisolone + dapsone	4
2	1/Japanese/M	IgG, C3	Dermal side	290 (+)	No reactivity	–	+	+	ND	+	Methylprednisolone + dapsone	4
3	2/Japanese/F	IgG, C3	Dermal side	290 (+)	No reactivity	–	+	+	ND	–	Prednisolone	4
4	4/European/F	IgG, C3	Dermal side	290 (+)	No reactivity	+	+	+	–	ND	Prednisolone + dapsone	5
5	5/Japanese/M	IgG, C3	Dermal side	290 (+)	No reactivity	+	+	+	ND	–	Betamethasone	6
6	4/Japanese/M	IgG, C3	Dermal side	290 (+)	ND	+	ND	+	ND	–	Prednisolone	Present case

DIF, direct immunofluorescence; BMZ, basement membrane zone; IIF, indirect immunofluorescence; NC, noncollagenous domain; TH, triple helical collagenous domain; rBP180, recombinant 180-kDa bullous pemphigoid antigen; NC16a, NC16a domain of BP180; ELISA, enzyme-linked immunosorbent assay; ND, not done.

In contrast, patients with childhood EBA (16 years old or younger) are very rare. To our knowledge, there are 33 cases of childhood EBA including the present case reported in the literature.[4–6,9–26] Among them, six patients were Japanese and the others were non-Japanese including at least five caucasians and five Africans. These patients presented mainly with the inflammatory subtype. Some differences exist between EBA in adults and in children. In adults, mucosal involvement was seen in approximately 50% of cases, while in children, mucosal lesions were seen in the vast majority of the reported cases.[9,18] The adult form of EBA is known to be difficult to treat, requiring high doses of prednisolone, dapsone, immunosuppressive agents, plasmapheresis etc. In contrast, dapsone and low-dose prednisolone are usually effective in treating childhood EBA. The prognosis in childhood EBA seems to be much better than in adult EBA.[4–6,9–26]

The major epitopes of circulating autoantibodies in adult patients with the classical type of EBA are known to be located within the NC1 domain, but neither in the NC2 nor in the triple helical domains.[3] However, in five cases of childhood EBA (four Japanese children and one European child) reported in the literature, autoantibodies have been found to recognize epitopes on the NC2 and/or the triple helical domain over the last years (Table 1).[4–6] These five patients and the present patient were all children under 10 years of age, who all showed similar clinical features of the inflammatory subtype of EBA, and complete clearing or a relatively good response to treatment. Interestingly, two of these children also demonstrated autoantibody reactivity towards the 230-kDa BP antigen and the NC16a domain of BP180.[4]

Schmidt *et al.*[5] mentioned this phenomenon and termed it 'epitope spreading'. Epitope spreading sometimes occurs in other adult human autoimmune skin disorders, and has been observed in a few cases of EBA, specifically in young children.[5] The molecular events in epitope spreading remain to be elucidated, but it is supposed that the skin damage from autoimmune or inflammatory processes could subsequently induce autoimmunity to a sequestered or closely related antigen or epitope.

Five of the six patients with EBA with autoantibodies to the NC2 and/or the triple helical domains are Japanese children. Tanaka *et al.*[4] suggested that the phenomenon may be unique to a particular ethnic group. To confirm this hypothesis, detailed epitope mapping in bullous dermatosis cases with similar clinical and pathological features will be required, especially in children from different ethnic groups.

Further studies on autoantibodies in larger series of patients with EBA are needed to clarify the correlation between the presence of certain autoantibodies against specific subdomains of type VII collagen and their clinical features, such as classical or inflammatory phenotype, the patient's age at onset, clinical course and prognosis.

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ARTICLE

Colocalization of Multiple Laminin Isoforms Predominantly beneath Hemidesmosomes in the Upper Lamina Densa of the Epidermal Basement Membrane

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SUMMARY Multiple laminin isoforms including laminins 5 (α3 β3 γ2), 6 (α3 β1 γ1), 10 (α5 β1 γ1), and possibly laminins 7 (α3 β2 γ1) and 11 (α5 β2 γ1) are present in the epidermal basement membrane. However, only the precise epidermal ultrastructural localization of laminin 5 (α3 β3 γ2) has been elucidated. We therefore determined the precise expression and ultrastructural localization of the α5, β1, β2, and γ1 chains in the epidermis. The expression of laminin chains in skin samples was analyzed from patients with epidermolysis bullosa (EB, n=15) that harbor defects in specific hemidesmosome (HD)-associated components. The expression of the α5, β1, and γ1 chains (present in laminins 10/11) and β2 chain (laminins 7/11) was unaffected in all intact (unseparated) skin of EB patients including Herlitz junctional EB with laminin-5 defects (n=6). In the basement membrane of human epidermis, the α5, β1, β2, and γ1 chains were expressed but also localized to the dermal vessels. Immunogold electron microscopy of normal human epidermis localized the α5, β1, β2, and γ1 chains to the upper lamina densa, with between 84% and 92% of labeling restricted to beneath the HDs, similar to laminin 5 (n≥200 gold particles per sample, sample number n=4) but distinct from collagen IV labeling (with only 63% labeling beneath HDs, p<0.001). Taken together, the majority of the α5β1/β2γ1 laminin chains are located beneath HDs. This suggests that laminin-10-associated chains have specific functions or molecular interactions beneath HDs in the epidermal basement membrane.

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KEY WORDS
anchoring filament
epidermal basement membrane
hemidesmosome
immunoelectron microscopy
laminin 5
laminin 10

IN SKIN, laminins are present in the epidermal basement membrane, around blood vessels, nerves, and adnexal structures. It is generally thought that laminin 5 (α3 β3 γ2), possibly laminin 6 (α3 β1 γ1), and laminin 10 (α5 β1 γ1) are expressed in the human epidermal basement membrane (Aumailley and Rousselle 1999) (see Figure 1). The expression of laminins 7 (α3 β2 γ1) and 11 (α5 β2 γ1) has yet to be confirmed in the adult human epidermis (Aumailley and Rousselle 1999). Of these, laminin 5 (α3 β3 γ2) is the most well-studied epidermal isoform (Nishiyama et al. 2000; Mercurio et al. 2001; Geuijen and Sonnenberg 2002; McMillan et al. 2003b). Laminin 5 is thought to be crucial for the correct assembly and adhesion of hemidesmosomes (HDs) via the receptor, the α6β4 integrin (Niessen et al. 1994).

The distinct roles of laminin isoforms in the processes of cutaneous morphogenesis are poorly understood. Laminin 10 (α5 β1 γ1), however, has recently been implicated in several functions including hair follicle development (Li et al. 2003). In an α5 chain (laminin 10/11) knockout mouse model, the addition of exogenous laminin 10 was used to correct follicular development (Li et al. 2003). Laminin 10 is therefore implicated in hair follicle cell growth and adhesion (Gu et al. 2001; Pouliot et al. 2002; Li et al. 2003). Cell adhesion assays have demonstrated that multiple laminins (including laminins 5, 10, and 11) can act as adhesive substrates for keratinocytes and that this adhesion is mediated by the integrins α3β1 and α6β4

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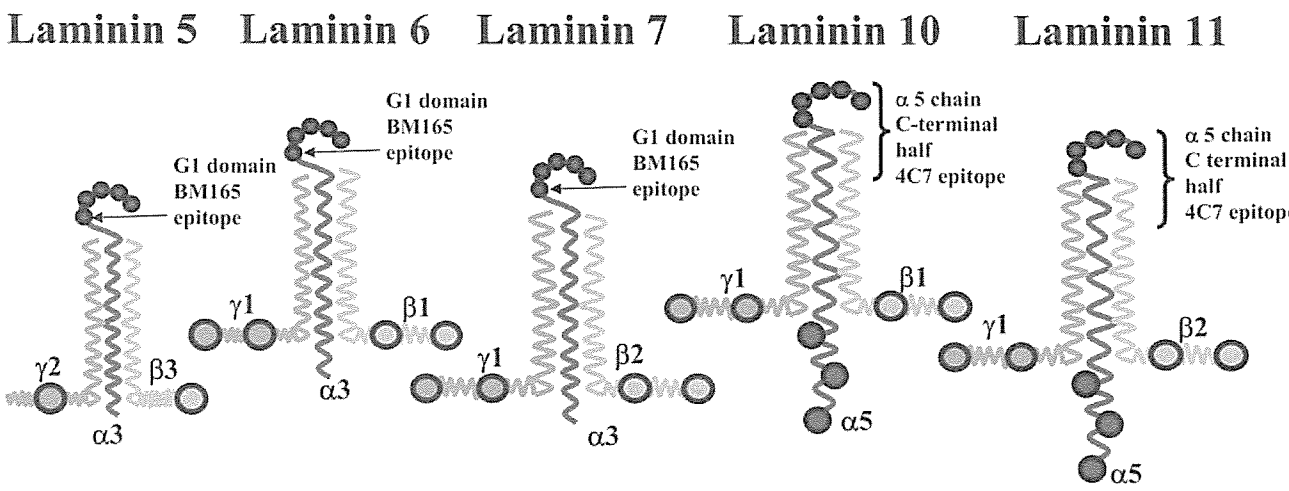

Figure 1 Schematic diagram showing the structure of laminin 5 and 10 chains and the position of two antibody binding sites in the α3 chain G1 domain (BM165) and the carboxyl terminal half of the α5 chain (4C7). This schematic diagram is not drawn to scale and does not include any α3 chain splice variants of laminin 5 (α3 β3 γ2). The antibody BM165 binds to the first globular (G1) domain of the α3 chain of laminins 5/6 (McMillan et al. 2003b), whereas the antibody 4C7 binds to the carboxyl terminal half end of the α5 chain (present in both laminins 10 and 11).

(Pouliot et al. 2002). However, in certain non-epithelial cells, the integrins α3β1, α6β1, and α6β4 and α dystroglycan are expressed and have been identified as possible laminin 10/11 receptors (Kikkawa et al. 1998, 2000; Yu and Talts 2003).

Antibodies are now available that recognize specific laminin chains and provide new tools to investigate the structure of the epidermal basement membrane. These antibodies include 4C7 (Engvall et al. 1990; Tiger et al. 1997) that recognizes a carboxyl terminal domain of the human α5 chain of laminins 10 (α5 β1 γ1, see Figure 1) and 11 (α5 β2 γ1). This antibody blocks the epitope involved in neurite cell adhesion to the laminin α5 chain (Engvall et al. 1986; Makino et al. 2002). Further laminin-specific antibodies to β and γ chains include 2E8 (recognizing the β1 chain (Engvall et al. 1986), D18 (γ1) (Sanes et al. 1990), and C4 (β2) (Hunter et al. 1989).

To better understand the position and possible functions of epidermal molecules, we examined the precise localization of the α5, β1, β2, and γ1 laminin chains and collagen IV in the interfollicular and follicular epidermal basement membrane. In addition, the expression of laminin chains was also assessed in a range of epidermolysis bullosa (EB) patients' skin harboring defects in several basement membrane components, including laminin 5. We have quantitatively analyzed and compared the localizations of laminin α5, β1, β2, and γ1 chains with that of laminin 5 (α3 β3 γ2) from our previously published data (McMillan et al. 2003b) and collagen IV. Comparison of α5, β1, β2, and γ1 chain expression with laminin 5, a well-studied HD-associated isoform, will determine a more precise localization for these laminin isoforms. Our data support the hypothesis that multiple laminin isoforms colocalize beneath HDs in normal and diseased epidermal basement membranes.

Materials and Methods

Skin Samples

Samples of adult and neonatal control skin from non-specialized sites (abdomen, arm, thigh, $n=8$; and scalp skin, $n=2$) were obtained from routine surgical procedures. Skin samples were frozen for cryostat sectioning or processed for post-embedding immunogold electron microscopy (IEM) as described below. In all cases, the biopsies were performed with the patient's or guardian's informed consent, with the relevant institutional approval for experiments handling human material, and in accordance with the Helsinki Declaration.

Skin samples from patients affected with a group of rare genodermatoses, EB, were included in this study ($n=15$, see Table 1). Details of the number of patients for each EB disease subtype, their age at biopsy, details of any identified mutations, or significant results of diagnostic antibody staining are listed, in addition to the results of their laminin antibody staining findings (see Table 1). Four Herlitz junctional (HJ) EB patients harbored laminin-5 chain mutations that were reported in the literature (Takizawa et al. 1998a–d). In one EB simplex associated with muscular dystrophy (EBS-MD) patient, genetic defects have been reported (Pulkkinen et al. 1996).

Confocal Immunofluorescence Microscopy

Indirect immunofluorescence was performed as previously described (Kennedy et al. 1985) using cryostat skin sections. Laminin chain expression was confirmed in control skin using the following antibodies: 4C7 recognizing the human α5 chain (see Figure 1) present in laminins 10 and 11 (dilution

Table 1 Comparison of laminin 5 and laminin 10 expression in patients with various forms of epidermolysis bullosa

EB disease subtype	Deficient protein	Patients' sex, age, mutation, and staining details	Laminin-5 γ3 chain GB3 expression	Laminin-10 α5 chain 4C7 expression
Control (5)	Normal	M/31 years, 33 years, 41 years, 45 years, F/15 years	+++	+++
HJEB (6)	Laminin 5	M/1 month, W610X/Q166X LAMB3	−	+/++
		F/9 months, 1997-2A>C (homozygous, LAMB3)		
		F/1 month, Q936X LAMB3		
		M/1 month, 1929delCA/W610X LAMB3		
		F/2 months, M/1 month, laminin-5 negative (GB3 moAb)		
NHJEB (2)	Laminin 5	M/12 years, M/5 years, laminin-5 reduced (GB3 moAb)	+	+++
NHJEB (2)	Collagen XVII	M/21 years, F/35 years, G252X collagen XVII negative (233, 1A8C moAb)	+++	+++
SRDEB (2)	Collagen VII	M/7 years, M/1 year, collagen VII negative (LH7:2 moAb)	+++	+++
JEB-PA/PA-EBS (1)	Integrin α6β4	F/1 month, α6β4 integrin negative (3E1,GOH3 moAb)	+++	+++
EBS-MD (2)	Plectin	M/27 years, M/9 years, plectin negative (HD1-121 moAb)	+++	+++

EB, epidermolysis bullosa; HJEB, Herlitz junction epidermolysis bullosa; NHJEB, non-Herlitz junctional epidermolysis bullosa; SRDEB, severe recessive dystrophic epidermolysis bullosa; JEB-PA, junctional epidermolysis bullosa associated with pyloric atresia; which is also known as EBS-PA, epidermolysis bullosa associated with pyloric atresia. +++, normal, bright staining pattern along dermal–epidermal junction; ++, reduced dermal–epidermal junction staining compared to controls; +, severely reduced dermal–epidermal junction staining compared to controls; −, absent dermal–epidermal junction staining compared to controls.

1:25; Chemicon International, Temecula, CA) (Engvall et al. 1990; Tiger et al. 1997). The monoclonal antibody 2E8 recognizing the β1 chain (neat) (Engvall et al. 1986), the monoclonal antibody D18 that recognizes the γ1 chain (see Figure 1) (neat) (Sanes et al. 1990), and an antibody C4 to the β2 chain (see Figure 1) (used neat) (Hunter et al. 1989) were also included. The antibodies 2E8, D18, and C4 were obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). The mouse monoclonal M3F7 recognizing the helical domain of the α1 and α2 chains of collagen IV (used neat) (Foellmer et al. 1983) was also obtained from the Developmental Studies Hybridoma Bank. Laminin-5 antibodies included the mouse monoclonal BM165 directed against the laminin-5 α3 chain terminal first globular (G1) domain (see Figure 1) (diluted 1:50) (Marinkovich MP, unpublished data) (McMillan et al. 2003b); K140 directed against the laminin-5 β3 chain adjacent to domain IV; GB3 directed against the laminin-5 γ2 chain (Harlan Sera Lab; Loughborough, UK); and a rabbit polyclonal serum directed against the entire laminin-5 molecule (1:200) (McMillan et al. 2003b). The melanocyte marker antibody TMH-1 recognized the b-locus protein (rat antibody, 1:10 dilution) and was previously described by Masunaga et al. (1996).

Epidermal sections were fixed in cold acetone (−20C) for 10 min and incubated with 5% normal rabbit sera in 0.1 M Dulbecco's PBS for 5 min at 37C. Sections were incubated with primary antibodies and subsequently with secondary antibodies conjugated to fluorescein isothiocyanate or Texas Red (FITC; rabbit anti-mouse IgG or goat anti-rabbit IgG, 1:200; DAKO, Tokyo, Japan; Texas Red conjugated donkey anti-rabbit; Amersham, UK). To label TMH-1, a preabsorbed cyanine (CY5)-conjugated goat anti-rat antibody was used (Jackson ImmunoResearch; West Grove, PA). All secondary antibodies were diluted in 3% BSA in 0.1 M PBS for 30 min at 37C in a darkened, humidified chamber. Sections were then labeled with a ToPro-3 nuclear counterstain (diluted 1:20,000, blue channel; Jackson ImmunoResearch) if appropriate. The sections were then mounted in Permafluor (Thermo Shandon; Pittsburgh, PA) and examined with a confocal microscope (Fluoview FV300; Olympus, Tokyo, Japan) using an inverted microscope (IX70; Olympus). Controls included normal skin cryostat sections with the primary antibody substituted by PBS, myeloma supernatant, or an irrelevant immunoglobulin isotype, as a negative control. All experiments were performed at least in duplicate.

Immunogold Electron Microscopy

Four samples of human skin were cryofixed and processed for postembedding IEM according to the previously described methods (Shimizu et al. 1989,1990). Samples were washed in PBS and cryoprotected in 20% glycerol (in PBS) for up to 1 hr at 4C. Subsequently, cryofixation was performed in liquid propane at −190C using a freeze plunge apparatus (Leica CPC; Cambridge, UK) followed by freeze substitution over 3 days at −80C in methanol using an automated freeze substitution system (AFS; Leica). Specimens were embedded in Lowicryl K11M (Ladd Research Industries; Burlington, VT) resin over 4 days at −60C. The temperature was gradually raised and the resin was polymerized under UV light and liquid nitrogen vapor at 10C. Ultrathin sections were then cut and collected on pioloform-coated nickel grids. Sections were stained with uranyl acetate only (15 min) and observed with a transmission electron microscope (Hitachi H-7100; Tokyo, Japan) at 75 kV. Blocks showing good ultrastructure were selected for immunolabeling experiments. Sections were preincubated in buffer containing PBS with 5% normal goat serum (NGS), 1% BSA, and 0.1% gelatin. Primary antibodies or human antisera were all diluted in PBS buffer containing 1% NGS, 1% BSA, and 0.1% gelatin and incubated at 37C for 2 hr. The sections were then washed in a drop of PBS buffer four times (5 min each) and placed on a drop of secondary linker antibody, again diluted in PBS buffer (for 2 hr at 37C). The secondary antiserum, rabbit anti-mouse IgG (DAKO; Ely, UK) was diluted 1:500. Sections were then incubated with a final antibody layer using 5-nm gold-conjugated labeled goat anti-rabbit or goat anti-mouse antibodies (Biocell; Cardiff, UK) diluted 1:500 in Tris-buffered saline (TBS) for 2 hr at 37C. For double labeling on K11M sections, the α5 chain of laminins 10/11 (4C7 and a 5-nm