position of K1, p.Leu486Pro, was reported in patients with EHK and severe palmoplantar hyperkeratosis (Fig 2, *C*) and digital contractures, and the affected individuals exhibited clinical features similar to our patient.[2] Therefore, our data suggest that a nonconservative amino acid change at codon 486 of K1 results in a severe form of generalized EHK.

The rod domains consist of four alfa-helical segments that possess a repeating heptad amino acid residue peptide motif *(a-b-c-d-e-f-g)n* that has the potential to form a two-chain coiled coil with a corresponding sequence (Fig 2, *D*).[3-5] The residues at position *a, d, e,* and *g* are considered to be highly sensitive to mutations.[6]

Our patient with generalized EHK had most severe palmoplantar hyperkeratosis compared to previously reported cases with mutations in *KRT1*. The leucine residue at codon 486 is located in the *a* position of the heptad repeat at the C-terminal end of the 2B helix, and the substitution of arginine for leucine seriously alters the character of amino acid. It is therefore reasonable to say that this mutation caused generalized EHK with severe palmoplantar hyperkeratosis, compared with that seen in patients harbouring mutations in the other residues.

Twenty-six EHK cases, including the present case with point mutations at the helix initiation motif (HIM) and HTM of *KRT1*, have been reported to date (Fig 2, *C*); Human Intermediate Filament Database [www.interfil.org/]). Only nine cases, including the present case, were diagnosed as generalized EHK with severe palmoplantar hyperkeratosis, and seven cases out of nine harbored missense mutations in the heptad repeat position *a, d, e,* and *g*. These facts indicate that the mutation site and the nature of amino acid alterations in K1 may determine the level of severity of palmoplantar hyperkeratosis.

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Scrotal elephantiasis secondary to hidradenitis suppurativa

To the Editor: We read with great interest the continuing medical education article by Alikhan et al[1] in the April 2009 issue of the *Journal* providing a comprehensive review of hidradenitis suppurativa (HS). We found the mention of scrotal lymphedema caused by HS to be of particular interest and would like to share our experience with a patient who developed the unusual complication of scrotal elephantiasis caused by longstanding HS.

A 58-year-old white man with no recent travel history who lived in social isolation presented with scrotal enlargement that began about a decade ago but had become notably worse over the past 3 years. He denied any history of sexually transmitted infections and recalled having had HS his entire adult life. The patient underwent several incision and drainage procedures in the emergency department over the years, had recently been treated briefly with oral antibiotics without improvement, and was finally referred for dermatologic evaluation. The physical examination revealed a massively enlarged, indurated scrotum that obscured his penis and had multiple sinus tracts draining clear, foul-smelling fluid (Fig 1). He had many violaceous nodules with open comedones in the intertriginous regions, including the left and right axillae, bilateral groins, bilateral inner thighs, and beneath the skin folds on his lower abdomen.

Punch biopsy specimens from the patient's left inner thigh and left lower abdomen revealed findings

Correspondence

Childhood subepidermal blistering disease with autoantibodies to type VII collagen and laminin-332

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MADAM, Autoimmune subepidermal blistering diseases include bullous pemphigoid, pemphigoid gestationis, linear IgA bullous dermatosis, mucous membrane pemphigoid (MMP), anti-p200 pemphigoid, epidermolysis bullosa acquisita (EBA) and bullous systemic lupus erythematosus.[1] Patients with EBA have IgG autoantibodies to type VII collagen while some patients with MMP have autoantibodies to laminin-332.[2,3] We describe a juvenile case of subepidermal blistering disease with autoantibodies to both type VII collagen and laminin-332. The present case is unique because of its childhood onset and successful remission following only topical steroid therapy.

A 12-year-old Japanese girl presented with pruritic eruptions on her scalp. A few weeks later, widespread pruritic vesicles gradually developed over her whole body. The vesicles were seen both on erythematous and normal skin (Fig. 1a, b). Blisters and erosions also appeared in her oral mucosa, but there was no involvement of genital or ocular mucous membranes (Fig. 1c). Neither nail changes nor alopecia were observed. She had no family history of any blistering disorders or autoimmune disease. There was no preceding illness or history of medication/vaccination that might have triggered her disease.

General laboratory examinations revealed no apparent abnormalities except for an increased serum IgE level (668·8 IU mL^{-1}; normal < 100 for age 7–14 years). A skin biopsy was taken from the edge of one blister on her right forearm. Light microscopy showed a subepidermal blister with an inflammatory cell infiltrate consisting of mainly neutrophils in the upper dermis (Fig. 2a). Direct immunofluorescence of the patient's lesional skin showed *in vivo* linear deposits of IgG and C3 at the epidermal basement membrane zone (Fig. 2b). On the blistered area, deposition of IgG and C3 was demonstrated on the dermal side of the separated skin (arrows, Fig. 2b). Indirect immunofluorescence with the patient's serum on 1 mol L^{-1} NaCl-split normal human skin showed IgG antibodies bound to the dermal side of the blister (Fig. 2c). Immunoblot analysis revealed that the patient's serum reacted with a 290-kDa protein in dermal extracts, and further with purified laminin-332 α3 protein (145, 165 kDa) (Fig. 2d, e). Laminin-332 was obtained from human keratinocytes and was purified using an antilaminin-332 affinity column as

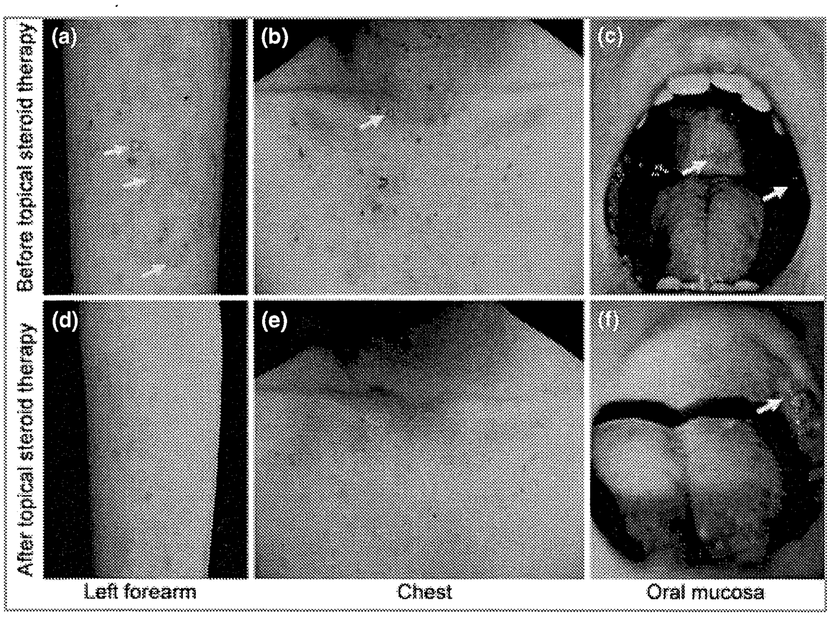

Fig 1. Clinical manifestations of the skin and oral mucosa. (a–c) Before topical steroid therapy. Erythema and tense vesicles on the left forearm and chest (a and b, arrows). Blisters and erosions over the oral mucosa (c, arrows). (d–f) After topical steroid therapy. Skin lesions healed within 9 days of the beginning of treatment, leaving residual pigmentation, scars and milia (d and e). Blisters and erosions on the oral mucosa subsided (f, arrow).

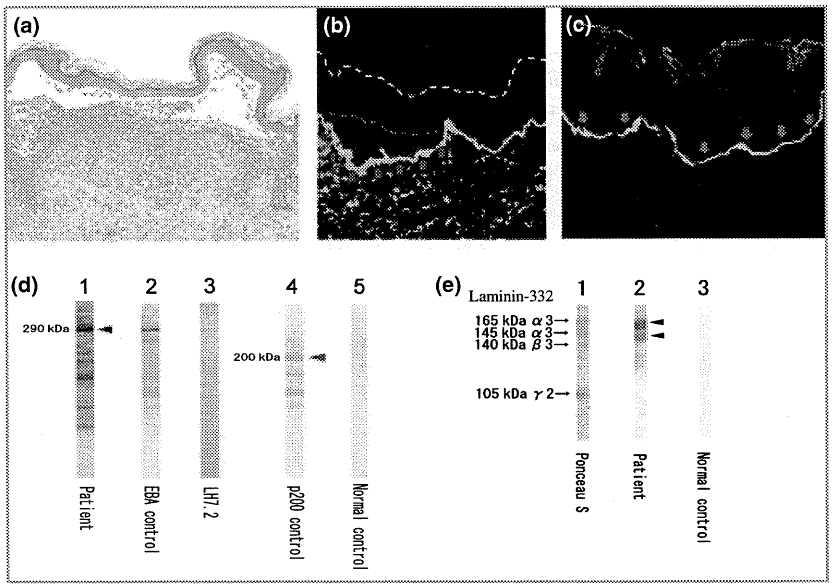

Fig 2. Histopathological findings, immunofluorescence staining and immunoblot analyses. (a) A subepidermal blister with an inflammatory cell infiltrate composed of mainly neutrophils in the upper dermis (haematoxylin and eosin; original magnification × 40). (b) Direct immunofluorescence showed *in vivo* linear deposits of IgG along the basement membrane zone. On the blister area, deposition of IgG was shown to be towards the dermal side of separated skin (arrows) (original magnification × 40; white dotted line is the skin surface and red dotted line is the roof side of separated skin). (c) Indirect immunofluorescence with the patient's serum on 1 mol L^{-1} NaCl-split normal human skin showed IgG antibodies bound to the dermal side (arrows) (original magnification × 40). (d) Immunoblot analysis revealed that the patient's serum (lane 1), like both serum from a reference patient with epidermolysis bullosa acquisita (EBA, lane 2) and monoclonal antibody LH7.2 to type VII collagen (lane 3), reacted with a 290-kDa protein in dermal extracts (arrowhead). Control anti-p200 serum did not react with the 290-kDa but with a 200-kDa protein (red arrowhead) (lane 4). Normal control serum (lane 5) showed reactivity with neither. (e) In immunoblotting of purified laminin-332, lane 1 shows Ponceau S stain (protein staining using amido black). Reactivity with 145-kDa and 165-kDa purified laminin-332 α3 protein (arrowheads) was indicated in the patient's serum (lane 2), but not in the normal control serum (lane 3).

previously described.[4,5] Purified laminin-332 was a generous gift from Dr S. Amano, Shiseido Life Science Research Centre, Yokohama, Japan. The patient was diagnosed as having an autoimmune subepidermal blistering disease with circulating autoantibodies to type VII collagen and laminin-332.

Treatment was initiated with 0·05% clobetasol propionate ointment 20 g daily to skin lesions, which healed within 9 days after the beginning of treatment, leaving residual pigmentation, scars and milia (Fig. 1d, e). Blisters and erosions on the oral mucosa subsided without any topical therapy (Fig. 1f). The dose of topical corticosteroids was progressively decreased, and no recurrence of skin lesions was observed. The titre of antibasement membrane zone antibodies in indirect immunofluorescence studies decreased from 1 : 320 to 1 : 40 over 2 months. We performed further immunoblot analyses on five serial serum samples obtained from the patient after her antibasement membrane zone antibodies decreased. All five samples showed similar reaction bands to both 290-kDa protein in dermal extracts and purified laminin-332 α3 protein (145, 165 kDa) (data not shown). Hence it is difficult to speculate the major target antigen in this patient from these results. No local or systemic side-effects of topical corticosteroids were noticed during the entire treatment duration.

EBA and MMP are distinct autoimmune bullous diseases that are both characterized by autoantibodies to dermoepidermal junction components.[1] Detection of autoantibodies to either type VII collagen or laminin-332 differentiates these two diseases.[1] Interestingly, besides antitype VII collagen antibodies, circulating antilaminin-332 α3 antibodies were also found in our patient's serum. According to our survey of the literature, three other previous cases of subepidermal blistering disease with circulating antibodies to both type VII collagen and antilaminin-332 have been reported (Table 1).[6–8] All of the reported cases are of adult onset, thus our report is the first juvenile case. Similar to our patient, these reported patients all presented with mucosal involvement.

Our case is unique in its course and prognosis as well as age at onset. All of the previously reported patients needed systemic corticosteroids or immunosuppressant agents for proper disease control. In the studies by Jonkman *et al.*[6] and Umemoto *et al.*,[7] the bullous lesions of the patients relapsed after systemic prednisolone was tapered. The skin lesions of the patient reported by Baican *et al.*[8] were refractory to systemic prednisolone, azathioprine and dapsone. However, our juvenile case was successfully treated with only topical steroids, and no recurrence was observed in the following 6 months. Our case suggests that the treatment outcome and prognosis of juvenile cases are better than those of adult-onset cases. Further accumulation of similar juvenile cases is needed to confirm this hypothesis. The differ-

Table 1 Comparison of four reported patients with circulating antitype VII collagen and antilaminin-332 antibodies

Patient	Age (years)/sex	Skin lesion	Mucosal involvement	Treatment	Outcome	Immunoblot analysis	Reference
1	64/F	Blisters and erythema on the hands and feet	Oral/genital	Oral prednisolone 80 mg daily	Lesions resolved without scars/milia. Mild relapse occurred when tapering to oral prednisolone 5 mg daily	Type VII collagen, laminin-332 α3	Jonkman et al.[6]
2	46/M	Erythematous plaque, blisters, erosions and crusts on the trunk and extensor aspects of extremities	Oral	Oral colchicine 1·5 mg daily (refractory to prednisolone, azathioprine and dapsone)	Previous lesions healed with milia and scars. Free of new blisters but erythematous plaque persisted with erosions and crusts	Type VII collagen, laminin-332 α3, γ2	Baican et al.[8]
3	35/F	Vesicular lesions on the face, neck and upper back	Oral/genital	Oral prednisolone 40 mg daily	Lesions resolved without scars/milia. Mild relapse occurred when tapering to oral prednisolone 25 mg daily	Type VII collagen, laminin-332 α3, β3	Umemoto et al.[7]
4	12/F	Blisters, erosions and erythema on the face, trunk, hands and feet	Oral	Topical clobetasol propionate ointment 20 g daily	Lesions resolved with scars and milia. No recurrence was found	Type VII collagen, laminin-332 α3	Our patient

ences between childhood-onset and adult-onset cases seem to mirror those of EBA at different ages. Compared with adult cases, childhood EBA cases respond relatively better to treatment, and usually low-dose oral prednisolone and dapsone are effective and sufficient.[1]

In conclusion, we report the first juvenile case with autoantibodies to both type VII collagen and laminin-332, successfully treated with only topical steroid therapy. Our case suggests that juvenile cases have different characteristics from those of adult-onset cases in their course, including treatment outcome and prognosis. As topical steroid therapy has several advantages over systemic corticosteroids due to less severe complications, we consider topical steroids as preferable to systemic steroids for childhood-onset autoimmune subepidermal bullous disease.

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STUDY

Clinical Correlations With Dermatomyositis-Specific Autoantibodies in Adult Japanese Patients With Dermatomyositis

A Multicenter Cross-sectional Study

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Objective: To clarify the association of clinical and prognostic features with dermatomyositis (DM)-specific autoantibodies (Abs) in adult Japanese patients with DM.

Design: Retrospective study.

Setting: Kanazawa University Graduate School of Medical Science Department of Dermatology and collaborating medical centers.

Patients: A total of 376 consecutive adult Japanese patients with DM who visited our hospital or collaborating medical centers between 2003 and 2008.

Main Outcome Measures: Clinical and laboratory characteristics of adult Japanese patients with DM and DM-specific Abs that include Abs against Mi-2, 155/140, and CADM-140.

Results: In patients with DM, anti–Mi-2, anti–155/140, and anti–CADM-140 were detected in 9 (2%), 25 (7%), and 43 (11%), respectively. These DM-specific Abs were mutually exclusive and were detected in none of 34 patients with polymyositis, 326 with systemic sclerosis, and 97 with systemic lupus erythematosus. Anti–Mi-2 was associated with classical DM without interstitial lung disease or malignancy, whereas anti–155/140 was associated with malignancy. Patients with anti–CADM-140 frequently had clinically amyopathic DM and rapidly progressive interstitial lung disease. Cumulative survival rates were more favorable in patients with anti–Mi-2 compared with those with anti–155/140 or anti–CADM-140 ($P<.01$ for both comparisons). Nearly all deaths occurred within 1 year after diagnosis in patients with anti–CADM-140.

Conclusion: Dermatomyositis-specific Abs define clinically distinct subsets and are useful for predicting clinical outcomes in patients with DM.

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Polymyositis (PM) and dermatomyositis (DM) represent a group of chronic inflammatory disorders characterized by myogenic changes, skin eruptions, or both. Clinical features are heterogeneous, with various degrees of skin manifestations, myositis, and pulmonary involvement, which considerably determine the severity and prognosis.[1] Although the causes of these disorders remain unclear, autoimmunity is considered to have a critical role because the presence of diagnostic autoantibodies (Abs), known as myositis-related Abs, is a prominent feature.[2] A variety of serum Abs are detected in patients with PM/DM, including Abs reactive with aminoacyltransfer RNA synthetase (ARS),[3] signal recognition particle,[4] and Mi-2.[5] These Abs are associated with clinically distinct subsets of PM/DM, that is, anti-ARS with interstitial lung disease (ILD), arthritis, Raynaud phenomenon, and mechanic hand[6,7]; anti–signal recognition particle with acute-onset severe refractory PM[8-10]; and anti–Mi-2 with typical DM with a lower risk of ILD and internal malignancy and good response to treatment.[11-13] In addition, anti–PM-Scl, anti-Ku, and anti-U1RNP Abs are associated with myositis overlap syndrome.[14] Therefore, identification of myositis-related Abs is use-

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ful in defining clinically homogenous patient subsets, in predicting prognosis, and in clarifying the pathogenesis. Recently, 2 myositis-related Abs, anti–155/140[15,16] and anti–CADM-140,[17] have been reported. Subsequent studies have revealed corresponding autoantigens: transcriptional intermediary factor 1-γ for anti–155/140[18] and melanoma differentiation–associated gene 5 for anti–CADM-140.[19] Anti–155/140 Abs are reported to represent malignancy-associated or juvenile DM,[15,20] and anti–CADM-140 Abs are reported to be associated with amyopathic DM and rapidly progressive ILD (RP-ILD).[17,19] Although precise clinical details of these 2 Abs need to be clarified further, anti–155/140, anti–CADM-140, and anti–Mi-2 Abs are considered to be highly specific for DM.

To our knowledge, this is the first large comprehensive study that includes all currently available myositis-related Abs intended for a variety of adult Japanese patients with DM. We focused particularly on anti–Mi-2, anti–155/140, and anti–CADM-140 and attempted to investigate a correlation between these 3 DM-specific Abs and clinical features and prognosis in detail.

METHODS

PATIENTS AND SERUM SAMPLES

Serum samples were obtained from 376 adult Japanese patients with DM who were observed in the Department of Dermatology, Kanazawa University, Kanazawa, Japan, and collaborating medical centers between January 1, 2003, and December 31, 2008. Of the 376 patients with DM, 325 fulfilled the criteria of Bohan and Peter.[21,22] The remaining 51 patients fulfilled the criteria of Sontheimer[23] because of the absence of clinical muscle symptoms and the presence of subsistent clinical DM skin eruptions. Clinically amyopathic DM included patients with amyopathic DM and patients with hypomyopathic DM. Patients with hypomyopathic DM had DM rash and subclinical evidence of myositis on electrophysiologic, radiologic, or laboratory evaluation.[24] Thirteen patients (5 with anti–155/140 Abs and 8 with anti–CADM-140 Abs) who were observed at Nagasaki University[25] were included in this study. As controls, serum samples from 34 patients with PM, 326 with systemic sclerosis, and 97 with systemic lupus erythematosus who were observed during the same period were also assessed. The diagnosis of PM was based on the criteria of Bohan and Peter.[21,22] All the patients with systemic lupus erythematosus or systemic sclerosis fulfilled the American Rheumatism Association criteria.[26,27] A PM/DM overlap was diagnosed by the coexistence of systemic lupus erythematosus or systemic sclerosis in addition to PM or DM.

Clinical information was collected retrospectively from all the patients by reviewing their clinical medical records. Initial symptoms were defined as clinical presentation at the first clinic visit. Muscle involvement in an initial symptom included clinical signs of muscle disease or abnormality evaluated using electrophysiologic, radiologic, or laboratory tests. The patients were diagnosed as having ILD according to the results of chest radiography, chest computed tomography, and pulmonary function tests, which included the percentage predicted values for forced vital capacity and diffusing capacity for carbon monoxide. A subset of patients with RP-ILD was defined as those with progressive dyspnea and progressive hypoxemia and a worsening of interstitial changes on the chest radiograph within 1 month from the onset of respiratory symptoms. Malignancy that included internal and hematologic malignancy in patients with DM was defined using criteria described previously.[11] No patient with DM had a history of malignant disease. The protocol was approved by the Kanazawa University Graduate School of Medical Science and Kanazawa University Hospital.

IMMUNOPRECIPITATION

Immunoprecipitation (IP) assays were performed using extracts of the leukemia cell line K562.[17] A total of 10 μL of the patient's serum was bound to 2 mg of protein A–Sepharose beads (Amersham Biosciences, Piscataway, New Jersey) in 500 μL of IP buffer (10mM Tris hydrochloride, pH 8.0; 50mM sodium chloride; and 0.1% Nonidet P-40 [Caledon Laboratories Ltd, Georgetown, Ontario, Canada]) and was incubated for 2 hours at 4°C and then washed 5 times with IP buffer. Autoantibody-coated Sepharose beads were mixed with 100 μL of ^{35}S-methionine–labeled K562 cell extracts derived from 10^6 cells and rotated at 4°C for 2 hours. After 5 washes, the beads were resuspended in sodium dodecyl sulfate sample buffer, and the polypeptides were fractionated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography. Anti–Mi-2 immunoprecipitated polypeptides of 200 to 240, 150, and 65 to 75 kDa, and anti–155/140 immunoprecipitated 155- and 140-kDa proteins. Anti–Mi-2, anti–155/140, and anti–CADM-140 were considered positive if serum samples produced precipitin lines with immunologic identity to reference sera.[13,15,17]

IDENTIFICATION OF ANTI–CADM-140

The presence of anti–CADM-140 was confirmed in serum samples that immunoprecipitated a protein with a molecular weight of 140 kDa by IP assay by immunoblots and enzyme-linked immunosorbent assay using recombinant melanoma differentiation–associated gene 5 as an antigen.[19] This procedure aimed to exclude several other Abs, such as anti–NXP-2 (previously termed *MJ*),[28] that target a protein of approximately 140 kDa.

STATISTICAL ANALYSIS

The Fisher exact probability test was used for comparison of frequencies, and 1-factor analysis of variance was used for multiple comparisons. $P < .05$ was considered statistically significant. All data are reported as mean (SD).

RESULTS

DISEASE SPECIFICITY OF THE MYOSITIS-RELATED Abs

Figure 1 shows representative results of an IP assay. A total of 47 serum samples from patients with DM immunoprecipitated a protein with a molecular weight of approximately 140 kDa. Of these samples, 43 (91%) were reactive with melanoma differentiation–associated gene 5 by immunoblots and enzyme-linked immunosorbent assay, confirming the presence of anti–CADM-140. The frequencies of myositis-related Abs in patients with PM, DM, systemic sclerosis, and systemic lupus erythematosus are summarized in **Table 1**. Anti–Mi-2 antibodies were found in 2% of serum samples from patients with DM, anti–155/140 in 7%, and anti–CADM-140 in 11%, but none of these 3 DM-related Abs was detected in patients with PM or other connective tissue diseases. In addition, they did not coexist. These 3 Abs accounted for 21% of all patients with DM.

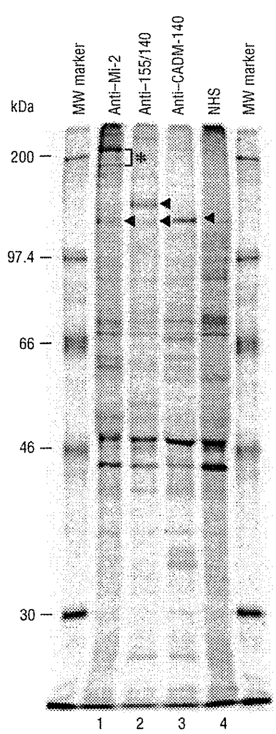

Figure 1. Immunoprecipitation assay of autoantibodies related to dermatomyositis. Immunoprecipitation of ^{35}S-methionine–labeled K562 cell extracts was performed on serum samples from patients with dermatomyositis (lanes 1-3) and on normal human serum (NHS) (lane 4), separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and analyzed by autoradiography. The molecular weight (MW) marker lane includes protein bands corresponding to 200, 97.4, 66, 46, and 30 kDa. Arrowheads indicate Mi-2 (lane 1), 155/140 (lane 2), and CADM-140 (lane 3) proteins. *Two hundred– to 240-kDa proteins of Mi-2.

Table 1. Frequency of Myositis-Related Autoantibodies in Patients With Connective Tissue Diseases

	Patients, No. (%)				
Autoantibodies	**DM (n=376)**	**PM (n=34)**	**PM/DM Overlap (n=21)**	**SSc (n=326)**	**SLE (n=97)**
Anti-Jo-1	21 (6)	4 (12)	0	1 (0.3)	0
Anti-ARS excluding anti-Jo-1	49 (13)	6 (18)	0	7 (2)	1 (1)
Anti-SRP	7 (2)	2 (6)	0	1 (0.3)	0
Anti-Mi-2	9 (2)	0	0	0	0
Anti-155/140	25 (7)	0	0	0	0
Anti-CADM-140	43 (11)	0	0	0	0
Anti-U1RNP	11 (3)	1 (3)	8 (38)	20 (6)	19 (20)
Anti-PM-Scl	0	0	0	0	0
Anti-Ku	2 (0.5)	3 (9)	6 (29)	3 (1)	0

Abbreviations: anti-ARS, anti–aminoacyltransfer RNA synthetase; anti-SRP, anti–signal recognition particle; DM, dermatomyositis; PM, polymyositis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

Table 2. Malignancy and Interstitial Lung Disease in Patients With DM Without Autoantibodies to Mi-2, 155/140, or CADM-140 and Patients With DM With These 3 Autoantibodies[a]

	Patients, No. (%)			
Variable	**Anti–Mi-2 Positive (n=9)**	**Anti–155/140 Positive (n=25)**	**Anti–CADM-140 Positive (n=43)**	**Others (n=299)**
Interstitial lung disease	1 (11)	3 (12)	40 (93)	105 (35)
Malignancy	0	17 (68)	4 (9)	18 (6)

Abbreviation: DM, dermatomyositis.
[a] $P<.001$ for all.

COMPARISON OF CLINICAL FEATURES AMONG PATIENTS WITH DM WITH DM-SPECIFIC Abs

First, we compared the rates of malignancy and ILD in the 77 patients with the 3 DM-specific Abs and the 299 patients with DM who did not have any of the 3 DM-specific Abs (**Table 2**). Interstitial lung disease was seen most frequently in patients with anti–CADM-140 Abs, and the incidence of malignancy was highest in patients with anti–155/140 Abs ($P<.001$ for all comparisons).

Next, we compared demographic, clinical, and laboratory data in each DM-specific Ab-based subgroup (**Table 3**). Patients with anti–CADM-140 had the lowest prevalence of DM but the highest prevalence of clinically amyopathic DM ($P<.001$ and $P<.001$, respectively, for all comparisons). Regarding initial symptoms, although muscle or muscle and skin involvement is less common in clinically amyopathic DM, the addition of ILD led to a higher prevalence of combined muscle, skin, and lung disease in the anti–CADM-140 subset ($P<.04$ for all comparisons). For clinical features, fever and arthritis were most frequently seen in patients with anti–CADM-140 ($P<.001$ and $P=.02$, respectively, for all comparisons). Patients with anti–CADM-140 had ILD at the highest rates ($P<.001$ for all comparisons), whereas malignancy was most frequently seen in patients with anti–155/140 ($P<.001$ for all comparisons). Malignancies were observed in 17 of 25 patients with anti–155/140 Abs, and 3 of those had double malignancy: 7 patients with lung cancer, 3 with breast cancer, 2 with colon cancer, 2 with gastric cancer, and a single case of prostate, biliary tract, pancreas, ovarian, and nasopharyngeal cancer and non-Hodgkin lymphoma. Ten of 17 patients with malignancy simultaneously developed DM, and 6 of 17 had malignancy before the development of DM.

Regarding skin eruptions, punctate hemorrhages on the perionychium were most frequently seen in patients with anti–Mi-2 ($P=.04$ for all comparisons). In patients with anti–155/140, punctate hemorrhages on the perionychium were more frequently seen in those without malignancy than in those with malignancy ($P=.007$) (**Table 4**). The frequency of truncal erythema in patients with anti–CADM-140 was lowest among the 3 subgroups ($P=.001$ for all comparisons). On the other hand, patients with anti–CADM-140 had skin ulcers most frequently among the 3 subgroups ($P<.008$ for all comparisons). However, the presence of skin ulcers was not a prognostic marker in patients with anti–CADM-140 (**Table 5**).

Table 3. Demographic, Clinical, and Laboratory Features in 77 Japanese Patients With DM According to the Presence of 3 DM-Specific Autoantibodies

Variable	Anti–Mi-2 Positive (n=9)	Anti–155/140 Positive (n=25)	Anti–CADM-140 Positive (n=43)	*P* Value
Age at onset, median (range), y	45 (16-66)	62 (31-79)	53 (15-76)	.005
Sex, M/F, No.	6/3	11/14	9/34	.01
Diagnosis, %				
Classical DM	100	72	23	<.001
Clinically amyopathic DM	0	28	77	<.001
Initial symptom, %				
Skin alone	44	40	35	.80
Muscle alone	11	4	0	.14
Lung alone	0	0	5	.44
Skin and muscle	44	44	12	.005
Skin and lung	0	4	21	.80
Muscle and lung	0	0	0	...
Skin, muscle, and lung	0	8	28	.04
Clinical features, %				
Fever	22	24	74	<.001
Raynaud phenomenon	0	12	28	.12
Muscle weakness	100	72	23	.02
Arthritis	11	8	42	.02
Interstitial lung disease	11	12	93	<.001
Malignancy	0	68	9	<.001
Skin eruptions, %				
Heliotrope rash	67	72	56	.37
Facial erythema other than heliotrope rash	56	88	51	.12
Gottron sign (hand)	89	96	86	.41
Gottron sign (elbow or knee)	56	76	77	.32
Periungual erythema	89	76	72	.67
Punctate hemorrhages on the perionychium	89	44	37	.04
Truncal erythema	89	88	35	<.001
Skin ulcers	0	4	30	.008
Cutaneous calcification	11	0	2	.29
Laboratory findings, mean (SD)				
CK, U/L	5283 (3649)	1364 (2263)	425 (1667)	<.001
LDH, U/L	814 (439)	564 (726)	547 (241)	.38
KL-6, U/mL	378 (1553)	476 (527)	2122 (1923)	<.001

Abbreviations: CK, creatine kinase; DM, dermatomyositis; ellipsis, not applicable; LDH, lactate dehydrogenase.
SI conversion factors: To convert CK and LDH to microkatals per liter, multiply by 0.0167.

Table 4. Clinical and Laboratory Features in 25 Anti–155/140 Antibody–Positive Patients With Dermatomyositis by the Presence or Absence of Malignancy

Variable	Malignancy Present (n=17)	Malignancy Absent (n=8)	*P* Value
Age at onset, median (range), y	68 (54-79)	49 (31-63)	<.001
Follow-up, mean, y	1.8	3.5	.06
Sex, M/F, No.	10/7	1/7	.04
Clinical features, %			
Muscle involvement[a]	71	100	.14
Arthritis	12	0	>.99
Interstitial lung disease	12	13	>.99
Skin eruptions, %			
Punctate hemorrhages on the perionychium	24	88	.007

[a] Muscle involvement included muscle weakness and subclinical myositis.

Regarding laboratory findings, maximum serum creatine kinase levels were significantly lower in patients with anti–CADM-140 than in those with anti–Mi-2 and anti–155/140 ($P<.001$ for all comparisons). KL-6 is a mucin-like high-molecular weight glycoprotein that is strongly expressed on type II alveolar pneumocytes and bronchiolar epithelial cells. Serum KL-6 levels are associated with the activity and severity of ILD.[29] Serum KL-6 levels were higher in patients with anti–CADM-140 than in those with anti–Mi-2 and anti–155/140 ($P<.001$ for all comparisons).

TREATMENT AND PROGNOSIS IN PATIENTS WITH DM WITH DM-SPECIFIC Abs

The treatment regimens and prognosis of individual patients with anti–Mi-2, anti–155/140, and anti–CADM-140 are summarized in eTable 1, eTable 2, and eTable 3, respectively (available at http://www.archdermatol.com). Although most patients with anti–Mi-2 responded well to the initial therapy, 6 of 9 patients had a recurrence of muscle or skin involvement during follow-up. In 15 of 17 patients (88%) with anti–155/140 who had malignancy, treatment for malignancy did not improve the symptoms of DM. Effective initial and additional treatments in patients with anti–CADM-140 were not elucidated in this study (Table 5).

We assessed the survival rates between disease subsets (**Figure 2**). Overall cumulative survival from the time of DM diagnosis in all 77 patients with DM was 65% at 5 years. Survival in patients with anti–155/140 and those with anti–CADM-140 was 68% and 56% at 5 years, respectively. Both cumulative survival rates were significantly decreased compared with those for patients with anti–Mi-2 (P=.01 for both). The cumulative rates were not identical between patients with anti–155/140 and those with anti–CADM-140 because RP-ILD in patients with anti–CADM-140 often developed rapidly in a short period after onset of the disease.

The prognosis of patients with anti–Mi-2 was favorable: no patients had malignancy and only 1 had mild ILD (Table 3). Although 8 patients (32%) with anti–155/140 died mainly from progression of malignancy during follow-up, the prognosis of anti–155/140–positive patients without malignancy was favorable. No significant trend was observed concerning the type of malignancy in anti–155/140–positive patients. Although patients with anti–CADM-140 whose prognosis was poor had significantly increased serum KL-6 levels (P=.04), no apparent negative prognostic factors were noted in those with anti–CADM-140 who died (Table 5).

Table 5. Clinical and Laboratory Features in 43 Anti–CADM-140 Autoantibody–Positive Patients With Dermatomyositis by Prognosis

Variable	Dead (n=19)	Alive (n=24)	P Value
Age at onset, median (range), y	58 (30-76)	50 (15-74)	.06
Sex, M/F, No.	4/15	5/19	>.99
Clinical features, %			
Fever	74	75	>.99
Muscle involvement[a]	26	67	.01
Arthritis	32	50	.34
Interstitial lung disease	100	88	.24
Pneumomediastinum	32	13	.26
Type of ILD, %			
Rapidly progressive	89	54	.02
Classical	11	33	.14
Skin eruptions, %			
Heliotrope rash	42	63	.35
Facial erythema other than heliotrope rash	32	67	.06
Punctate hemorrhages on the perionychium	26	46	.19
Truncal erythema	16	46	.09
Skin ulcers	16	42	.10
Laboratory findings, mean (SD)			
KL-6, U/mL	2656 (1989)	1703 (1803)	.04
Initial treatment, %			
Prednisolone only	26	58	.06
Prednisolone and methylprednisolone pulse	63	29	.13
Additional treatment, %			
Methylprednisolone pulse	74	50	.06
Cyclophosphamide	53	42	.55
Cyclosporine	79	54	.12

Abbreviation: ILD, interstitial lung disease.
[a]Muscle involvement included muscle weakness and subclinical myositis.

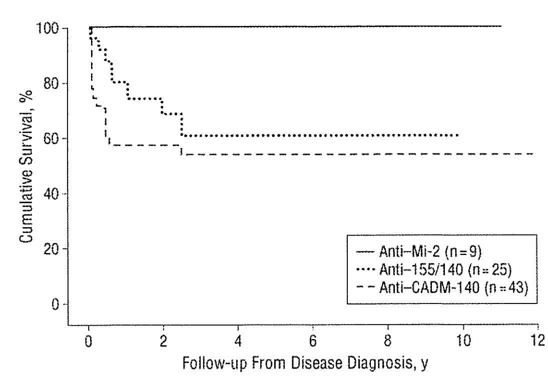

Figure 2. Cumulative survival rates from the time of diagnosis in 77 Japanese patients with dermatomyositis with serum anti–Mi-2, anti–155/140, and anti–CADM-140 autoantibodies. Cumulative survival rates were compared using log-rank tests.

CAUSE OF DEATH

Twenty-seven of 77 patients with DM died during follow-up. Of the 25 patients with anti–155/140, 7 died of malignancy and 1 died of bacterial pneumonia. Of the 43 anti–CADM-140–positive patients, 16 died of ILD and 1 died of *Pneumocystis jiroveci* pneumonia. One anti–CADM-140–positive patient died of disseminated intravascular coagulation. Thus, the major cause of death in patients with DM was associated with DM-related internal organ involvement. This is consistent with previous reports that malignancy and ILD are the major causes of death in patients with DM.

COMMENT

In this study, we compared clinical features and prognosis in adult Japanese patients with DM based on their DM-specific Abs. This study includes 3 major findings. First, to our knowledge, this is the first study to investigate the association of clinical features with 3 DM-specific Abs in adult Japanese patients with DM on a large scale. Second, the 3 DM-specific Abs are mutually exclusive and do not coexist. Third, each of these DM-specific Abs defines a clinically distinct phenotype and may work as a predictor of clinical complications and prognosis. Thus, classifying patients with DM based on their serum Ab profiles seems to be beneficial for focusing on their clinical features.

In an association of myositis-related Abs with the connective tissue diseases, PM/DM overlap was associated with anti-U1RNP and anti-Ku, and Abs detected in PM were predominantly anti-ARS and anti–signal recognition particle, as previously reported.[4,30,31] We also confirmed that anti–Mi-2, anti–155/140, and anti–CADM-140 were specific to DM in adult Japanese connective tissue diseases, and this finding was consistent with previous studies[5,16,20,30] assessing other ethnic groups.

Clinical characteristics of patients with anti–Mi-2 in this study were generally consistent with previous studies that anti–Mi-2 is associated with typical cutaneous lesions and mild to moderate muscle involvement and responds well to corticosteroid treatment.[5,11-13] However, recurrence of skin or muscle involvement might not be as rare as expected. Thus, although the overall prognosis is favorable, it is important to keep in mind that intractable myositis and rashes can occur, and careful observation is needed for monitoring flare-ups of the disease.

In this study, malignancy preceded DM in 6 patients, and the 2 conditions were simultaneously diagnosed in 10 patients. Malignancy was found after the diagnosis of DM in only 1 patient. This contrasts somewhat with previous studies[32,33] reporting that the diagnosis of DM is made before the development of malignancy in at least half of patients. Regarding patients with anti–155/140 Abs, Chinoy et al[34] reported that malignancy preceded the onset of DM in only 1 of 8 patients. Two patients had DM and malignancies at the same time. The remaining 5 patients developed malignancies shortly after the diagnosis of DM. Although we currently cannot explain why the discrepancy that the diagnosis of malignancy preceded the onset of DM in a large portion of patients in our study, the discrepancy may have resulted from the timing of screening for malignancy because the interval between detection of the 2 conditions was short in most cases. Also, the discrepancy about the association of the diagnoses of malignancy with the onset of DM may be affected by center-based bias in collecting samples. Alternatively, ethnicity might account for the present data. More studies are required to confirm the relationship between the onset of DM and the development of malignancy.

Previous studies[15,16,20] have described at least 2 different subsets in patients with anti–155/140: adult malignancy-associated DM and juvenile DM. Although anti–155/140 was associated with malignancy, 32% of patients (8 of 25) did not have malignancy in this study. Although most patients with malignancy were elderly and male, clinical features were generally similar between patients with malignancy and those without except for punctate hemorrhages on the perionychium. In addition, clinical features in anti–155/140–positive adult patients without malignancy were similar to those seen in juvenile patients in that they had more extensive skin involvement, such as Gottron papules, over a wider distribution.[16,20] It is unclear why transcriptional intermediary factor 1-γ is common as a major autoantigen in these 2 groups (adult malignancy and juvenile). Gunawardena et al[30] proposed the possibility that some perturbation of transcriptional intermediary factor 1-γ in proliferating cells combined with a more efficient anticancer response by a younger immune system may be important. Of interest is that patients with malignancy had a lower frequency of punctate hemorrhages on the perionychium compared with those without malignancy. Peripheral circulatory disturbances, including vasculopathy and microcirculation injury, are considered to be a hallmark of autoimmune connective tissue diseases. The lower frequency of punctate hemorrhages on the perionychium in patients with malignancy might be explained by the different mechanisms in developing skin and by muscle involvement between those with malignancy and those without, although both groups had the same Abs against transcriptional intermediary factor 1-γ. Further investigation is needed to reveal the pathogenesis of these 2 subsets.

Interstitial lung disease is a crucial complication for patients with DM because the prognosis of ILD in DM varies. The severity, clinical course, and prognosis of ILD in DM vary, and ethnicity seems to affect the clinical presentation. In the United States, the frequency of ILD in patients with clinically amyopathic DM is low, and the prognosis is favorable if they do not have a malignancy.[35-37] On the other hand, patients developing RP-ILD have been frequently reported not only in Japanese individuals but also in Chinese individuals and those of other Asian ethnicity. For example, Lee et al[38] reported 2 cases of idiopathic inflammatory myopathy with diffuse alveolar damage. Ye et al[39] also reported that 21 of 28 patients with clinically amyopathic DM had ILD, and, even in classical DM, 50% of patients had ILD. The Asian population might be sensitive to lung damage accompanied by genetically susceptible factors because severe acute respiratory syndrome caused by a coronavirus prevailed predominantly in eastern Asia.[40]

It is considered that the Abs present are associated with a type of ILD. Interstitial lung disease in anti-ARS–positive patients is characterized by the chronic course of the disease and elevation of the diaphragm.[41] Detection of anti–CADM-140 is extremely important because patients with anti–CADM-140 can frequently develop RP-ILD. Therefore, predictors of poor prognosis in this subgroup are needed. Skin ulcers, arthralgia or arthritis, lower arterial PO_2, and higher lactate dehydrogenase levels are considered to be risk factors for poor prognosis.[39] It is also reported that anti–CADM-140–positive patients with RP-ILD have rashes typically seen in DM.[24] In other studies,[42,43] spontaneous pneumomediastinum or pneumothorax is a severe complication and may indicate poor prognosis. However, the clinical phenotype was otherwise similar between patients with a poor prognosis and those with a favorable prognosis in this study.

To establish the treatment for RP-ILD is another urgent issue that needs a solution. Cyclophosphamide and cyclosporine are recommended in the early phase of the disease.[41,44] In contrast, Lee et al[38] reported that 4 patients with RP-ILD received 1 course of intravenous cyclophosphamide therapy and additional cytotoxic agents, such as azathioprine, cyclosporine, and methotrexate, but none responded. In this study, we could not elucidate definite predictors of poor prognosis and recommended treatment. Thus, it might be required to attempt the maximum possible combination of immunosuppressive treatments when patients with anti–CADM-140 present signs of developing RP-ILD.

In conclusion, classifying patients with DM according to their DM-specific Abs may guide the physician to focus on particular manifestations with high risk during follow-up of individual patients. However, the detection of DM-specific Abs is limited only to certain facilities because it requires a complicated technique. Establishment of a system to screen DM-specific Abs, such as an enzyme-linked immunosorbent assay, is needed. We acknowledge several limitations of this study. First, it included a relatively small number of patients with PM as a control because most enrolling institutions were dermatology departments. Second, we did not include juvenile patients with DM and other juvenile patients with connective tissue diseases. Third, anti–NXP-2 Abs were not included in this study because they are extremely rare in Japanese patients with DM. In addition, most of the facilities enrolled in this study were referral centers. Therefore, the possibility of center-based bias in collecting samples cannot be ruled out. More studies are needed for a better general understanding of patients with DM-specific Abs.

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ORIGINAL PAPER

Cyclosporin A induces the unfolded protein response in keratinocytes

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Abstract Psoriasis vulgaris is a chronic inflammatory disorder of the skin, in which activation of keratinocytes and crosstalk between keratinocytes and T cells or dendritic cells are considered to be involved in the pathogenesis of psoriasis vulgaris. Cyclosporin (Cy) A, an immunomodulator, has been used for the treatment of psoriasis vulgaris, but the mechanism of its action on keratinocytes has not been well elucidated as its function on T cells is well known. Previous study indicated that the expression of the unfolded protein response (UPR) markers, GRP78/Bip and HRD1 were poorly expressed in psoriasis vulgaris. To investigate if the UPR in keratinocytes is involved in the pathogenesis of psoriasis vulgaris we assessed immunocytochemistry of normal human skin and psoriatic lesions, quantitative PCR of keratinocyte cell line (HaCaT) treated with TGFβ. Moreover, to elucidate how CyA effects on the UPR in keratinocytes, we set out quantitative PCR and western blotting, HaCaT and squamous cell carcinoma cell lines (HSC-1) treated with CyA and CyA analog, cyclosporin D. Furthermore, the siRNA-mediated knockdown effect of cyclophilin (Cyp) A, Cyp B and Cyp C on HaCaT cells were also examined. As a result, the UPR was downregulated in keratinocytes from psoriatic lesions, characterized by immunocytochemical staining of GRP78/Bip, CHOP/GADD153, HRD1 and C/EBPβ. TGFβ induced UPR markers in HaCaT cells. CyA treatment and siRNA-mediated knockdown of Cyp B induced the UPR in HaCaT cells or HSC-1 cells. Altogether, we demonstrate that in psoriasis vulgaris CyA or reduction in Cyp B by RNA interference might induce the UPR in keratinocytes.

Keywords Bip/GRP78 · CHOP/GADD153 · Cyclosporine A · Endoplasmic reticulum stress · Psoriasis · Unfolded protein response

Abbreviations

ATF6	Activating transcription factor 6
Bip	Immunoglobulin heavy chain-binding protein
C/EBPβ	CCAAT/enhancer-binding protein beta
CHOP	C/EBP homologous protein
ER	Endoplasmic reticulum
GADD153	Growth arrest and DNA damage inducible gene 153
GRP78	Glucose-regulated protein 78 kDa
HRD	Hydroxymethylglutaryl-CoA reductase degradation
PDI	Protein disulfide isomerase
PERK	PKR-like endoplasmic reticulum kinase
TGF-β	Transforming growth factor-β
UPR	Unfolded protein response
XBP-1	X box-binding protein 1
CyA	Cyclosporine A
Cyp	Cyclophilin

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Introduction

Psoriasis vulgaris is a common, chronic inflammatory skin disease. Histologically, it is characterized by keratinocyte hyperproliferation with the loss of differentiation and infiltration of immune cells, especially neutrophils and T lymphocytes, into the dermis and epidermis. There is accumulating evidence that these infiltrating immune cells and their release of inflammatory cytokines play a critical role in the pathogenesis of psoriasis vulgaris, by interacting with and activating keratinocytes [14]. A subset of T cell-producing interleukin-17 (Th17) can stimulate keratinocytes to express proinflammatory cytokines [21]. Interestingly, IL-17 mRNA is highly expressed in T cells derived from psoriatic lesions. Macrophage-derived IL-12 and IL-23 enhanced inflammatory skin disease [9, 21]. Therefore, current treatments for psoriasis vulgaris focus on the modulation of the inflammatory immune response from T cells, dendritic cells and keratinocytes. TGFβ signal is important for keratinocyte differentiation, and decrease in TGFβ signal is suggested in proliferating keratinocyte in psoriasis by showing reduced expression of TGFβ signal related molecules [4, 10, 24].

Cyclosporin A (CyA) is often used in the treatment of autoimmune diseases, rheumatoid arthritis, atopic dermatitis and psoriasis. CyA exerts a direct effect on molecular events required for the activation of T cells and may act in the recruitment of antigen-presenting cells into the epidermis [3]. CyA is also distributed in the keratinocytes in psoriatic tissues, and is supposed to reduce keratinocyte proliferation [22], although how it affects the cell growth remains to be clarified.

CyA is known to bind to intracellular cyclophilins [5]. Cyclophilin A (Cyp A) is the major intracellular receptor for CyA [6]. Cyp A acts primarily in the cytosol, whereas Cyp B is mainly in the ER lumen and Cyp C localizes both to the cytosol and ER lumen [8, 16]. Recent studies have demonstrated that many cyclophilins function as chaperones in the endoplasmic reticulum (ER) [1].

The ER plays an important role in regulating protein homeostasis. Perturbations in the efficiency of protein folding result in the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER). An overload of unfolded or misfolded proteins in the ER caused by various triggers, including ultraviolet and oxidative stress is called ER stress. The unfolded protein response is a cellular recovery mechanism activated by ER stress [26, 27]. In the ER, transactivation of the chaperone Bip/GRP78 is initiated by its dissociation from the ER transmembrane proteins IRE1, PERK, and ATF6. IRE1 catalyzes the splicing of XBP1 mRNA. Activated PERK kinase phosphorylates eIF2α resulting in reduced protein synthesis and the formation of stress granules containing stalled transcripts. Phosphorylation of eIF2α promotes the translation of ATF4. Activated ATF6 translocates from the ER to the Golgi, where it is cleaved by two proteases, SP1 and SP2. Mature remodeled XBP1, ATF4 and ATF6, which are now activated cytosolic DNA-binding domains, translocate to the nucleus and induce a gene expression program called the unfolded protein response (UPR). UPR genes include Bip/GRP78, HRD1, CHOP/GADD153, Cyp B and C/EBPβ.

We previously identified a potential role for UPR in the differentiation of normal epidermal keratinocytes [17, 18]. The aim of this current report is to show that the inadequate differentiation of keratinocytes in psoriatic lesions could be associated with a defective UPR, and that CyA is expected to be able to improve this UPR-dependent dysfunctional differentiation by forming a complex with Cyp B.

Materials and methods

Human skin samples

Normal human skin was obtained from that around a benign scalp tumor of a 16-year-old patient and from the left anterior arm of a 37-year-old healthy individual after obtaining written informed consent [13]. Psoriasis vulgaris tissues were isolated from 10 patients (mean age 56.4 years, SD 13.5) after obtaining written informed consent. This study was approved by the Ethics Committee of Nagoya University, Graduate School of Medicine, and was conducted according to the principles of the declaration of Helsinki.

Antibodies and reagents

The following polyclonal antibodies were purchased from commercial sources: anti-GRP78/Bip, anti-CHOP/GADD153 and anti-C/EBPβ (Santa Cruz Biotecnology, Santa Cruz, CA), anti-HRD1 (Abgent, San Diego, CA), and anti-K1/K10 (Chemicon International, Temecula, CA). TGFβ was purchased from Wako (Osaka, Japan). CyA and CyD powder, kindly provided by Novartis International AG, Basel, Switzerland were dissolved in dimethyl sulfoxide at a concentration of 12 mg/ml. Dimethyl sulfoxide was diluted in parallel to serve as a control. Tunicamycin was purchased from Sigma–Aldrich. (St. Louis, MS). The sequences of the Taqman probes for real-time PCR are shown in Table 1.

Immunocytochemistry

Immunocytochemistry of normal skin and psoriasis sections were performed as previously described with slight

Table 1 DNA sequences of the probes for the quantitative real-time PCR

	Forward	Reverse	Taqman
GRP78	CGGCCGCACGTGGA	CAACCACCTTGAACGGCA	CCCGTCTGTGCAGCAGCAGGACATCAA
CHOP	TGCAGATTCACCATTCGGTC	AGGAATCGAGCGCCTGAC	CAGAGCTCGGCGAGTCGCCTCTACTT
XBP1-s	ATGCCCTGGTTGCTGAAGAG	GAGATGTTCTGGAGGGGTGAC	CCTGCACCTGCTGCGGACTCAG
Mxd1	CAATGAAATGGAGAAGAATAGAC	GTAGTGTGTCGACTTGATTC	TCATCTTCGCTTGTGCCTGGA
HRD1	GCTTTGTTGCACTCTTCACTCTTC	GAGATGTTGGGGCTGCGTTC	AGTCCACACGGTCCTCAGCCAGCC
GAPDH	TGGGCTACACTGAGCACCAG	CAGCGTCAAAGGTGGAGGAG	TCTCCTCTGACTTCAACAGCGACACCC
CypA	CCTGGCATCTTGTCCATGG	CAGTCTTGGCAGTGCAGATGA	TGGACCCAACACAAATGGTTCCCAGT
CypB	CAGCAAATTCCATCGTGTAATCA	TTTCCTCCTGTGCCATCTCC	TGATCCAGGGCGGAGACTTCACCA
CypC	AGCTCCAAGCAACTGATGGG	TTTTCACGTCTATCTTGCCACTG	ACCGTCCACTCACCAACTGCTCGA

modification [23]. In brief, sections of 6-μm thickness were cut from paraffin blocks of each sample. After immersion in 0.4% pepsin for 30 min, endogenous peroxidase was blocked by incubating for 20 min in 0.3% H_2O_2/methanol solution and then washed once in PBS with 0.01% Triton X-100. The sections were incubated with PBS with 4% bovine serum albumin (BSA) for 30 min and then conjugated with the first antibodies (10 ng/ml) in PBS containing 1% BSA over night at 4°C. After washing repeatedly with PBS, the secondary antibodies were applied for 1 h at room temperature. The secondary antibodies used were biotinylated anti-rabbit immunoglobulin or anti-mouse immunoglobulin antibodies. After washing repeatedly in PBS buffer, the sections were immersed in VECTASTAIN Elite ABC reagent for 30 min. The staining was stopped by rinsing with distilled water and the antibody complex was made visible by adding 1% H_2O_2. In a case a section shows the weak staining much more than in two layers defined as a positive staining pattern.

Cell culture

The keratinocyte cell line, HaCaT, was kindly provided by Dr. N. Fusenig [2]. HSC-1 cell line (human cutaneous squamous cell carcinoma cells) was purchased from Japanese Collection of Research Bioresources (Osaka, Japan). HaCaT and HSC-1 cells were maintained in Dulbecco's modified Eagle Medium (Sigma–Aldrich Co) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2.

Western blotting

Western blotting was performed as previously described [19]. Samples were resolved in 2× Laemmle sample buffer. The immunoblots were detected by LumiVision PRO HSII (Aisin Seiki, Aichi, Japan), and the densitometry analysis of the immunoreactive protein bands was performed using Lumi Vision Analyzer 2.1 software (Aisin Seiki).

siRNA transfection

The target sequence for siRNA was selected from a cDNA library corresponding to the coding region of human Cyp A (GenBank accession no. NM021130), Cyp B (GenBank accession no. NM000942) and Cyp C (GenBank accession no. NM000943) genes. The siRNA duplexes used were synthesized by Nippon EGT (Tokyo, Japan): Cyp A (5′-GAU GAG AAC UUC AUC CUA AAT-3′): Cyp B (5′-GGA UUU GGC UAC AAA AAC ATT-3′); Cyp C (5′-UCA AGG AUU UCA UGA UUC ATT-3′) and control CD4 (5′-ACA AGG AAG UGU CUG UAA ATT-3′).

HaCaT cells in culture medium containing fetal bovine serum were plated in 12-well plates at a density of the confluency mentioned in the protocol of HiPerfect Reagent (Qiagen, Valencia, CA). Cells were transfected with the siRNA constructs using the transfection reagents indicated, according to the manufacturer's protocol. For all experiments, transfection efficiency was maintained at 50–90%, and no detectable cellular toxicity was observed.

Real-time qPCR study

Total RNA was extracted from the cells using the RNeasy® Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA using the random primer procedure and the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan) following the manufacturer's protocol. Real-time quantitative PCR was performed in a Sequence Detector System (Mx3000P Real-Time PCR System and software; Stratagene, La Jolla, CA). Amplification was performed in a final volume of 25 μl containing 20 ng of cDNA from the reverse transcribed reaction primers (Table 1), TaqManProbes and 12.5 μl TaqMan® universal PCR Master Mix

(Roche, Branchburg, NJ). The gene expression values were normalized using the housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase to correct even minor variations in mRNA extraction and reverse transcription.

Statistical analysis

Statistical analysis was performed using Student's t test; P values of <0.05 was considered statistically significant.

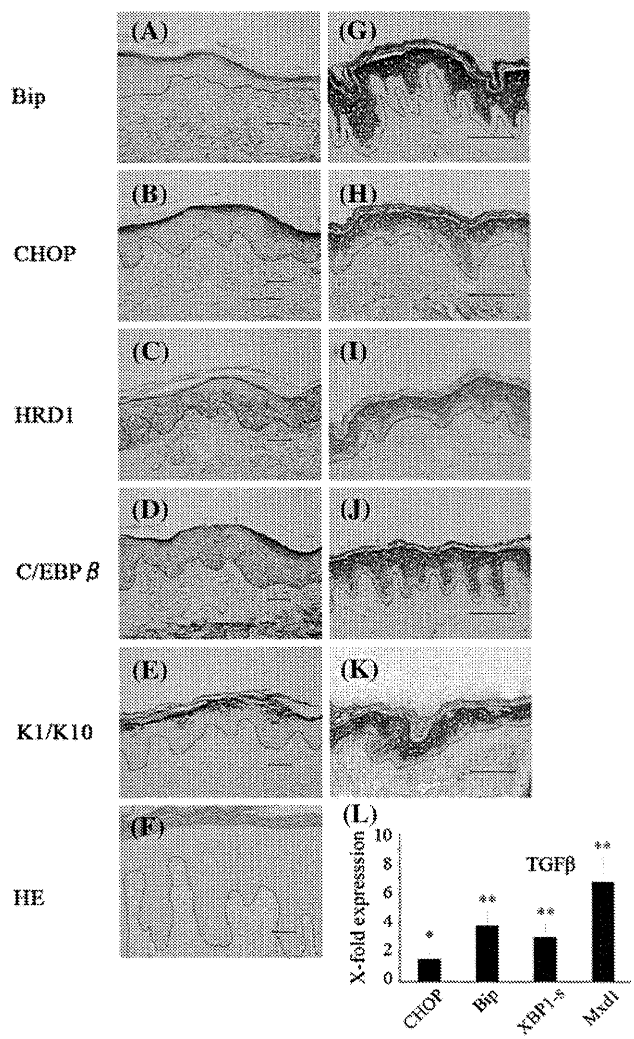

Fig. 1 Immunohistochemical staining of various UPR markers in psoriatic lesions and in normal skin. Immunocytochemical staining for GRP78/Bip (**a**), CHOP/GADD153 (**b**), HRD1 (**c**), C/EBPβ (**d**) and K1/K10 (**e**) on psoriasis sections and on normal human skin sections (**g–k**). Hematoxylin and eosin staining on a psoriasis section (**f**). *Scale bars* 100 μm. Note that the scale sizes are different for the psoriasis sections and normal skin sections. Derma-epidermal junction is dotted in all figures. Quantitative PCR revealed significantly induced UPR markers in HaCaT cells after treatment of TGFβ (5 ng/ml) for 24 h. Mxd1 is TGFβ-inducing gene

Results

Immunostaining of various UPR chaperone proteins in normal and psoriatic epidermis

Immunohistochemical staining for various UPR markers are shown in Fig. 1. In normal skin, GRP78/Bip showed strong cytoplasmic staining in all keratinocytes with increasing density toward the upper layers, but weak staining in a single layer of the basal cells (Fig. 1g). Staining pattern of GRP78/Bip in the psoriatic lesions has an enlarged area of low staining (Fig. 1a) as compared to the normal skin (Fig. 1g), which means that in the psoriatic lesions, GRP78/Bip expression was downregulated in the granular layers relative to the normal skin. When comparing the normal skin, psoriatic lesions exhibited weak staining of all UPR markers. We confirmed the staining pattern of the other UPR markers, CHOP/GADD153 (Fig. 1b), HRD1 (Fig. 1c) and C/EBPβ (Fig. 1d). The expression of those UPR markers was suppressed in the basal layer in the normal skin (Fig. 1g–j), however, the suppressed expression extended beyond the basal layer to prickle layers in psoriatic lesions (Fig. 1a–d). Staining pattern of each UPR marker in psoriatic lesions has much wider area of low staining than in normal skin. We determined the staining pattern as positive which showed the weak staining much more than in two layers. Table 2 summarizes immunocytochemical staining of ten psoriasis plates with the similar staining pattern to that of illustrated in Fig. 1a.

Previous studies indicated that the expression of UPR proteins in the suprabasal layers was reinforced in proportion to keratinization [18]. Therefore, K1 and K10, which were known to express only in well-differentiated suprabasal cells, were stained to examine the relationship between UPR and keratinocyte differentiation. As shown in Fig. 1e and k, the staining pattern for K1 and K10 in psoriatic lesions and normal skin were similar to that of UPR markers in the lesions and the normal skin, respectively.

Table 2 Numbers of the representative staining pattern out of 10 psoriasis tissues

Immunocytochemical staining	Positive
Bip	10/10
CHOP	10/10
HRD1	8/10
C/EBPβ	9/10

Immunocytochemical staining of 10 psoriasis plates are classified into the staining pattern as positive which showed the weak staining much more than in two layers

Furthermore, we investigated the effect of TGFβ stimulation on the UPR in HaCaT cells. We cultured HaCaT cells with TGFβ (5 ng/ml) for 24 h and measured the mRNA expressions of the UPR markers, CHOP/GADD153, GRP78/Bip and XBP1-s as compared to untreated cells (Fig. 1l). Those markers were significantly up-regulated after TGFβ stimulation. This result also suggests that the UPR is suppressed in psoriasis under which TGFβ signal is impaired. TGFβ administration could treat suppressed UPR condition in psoriatic lesion.

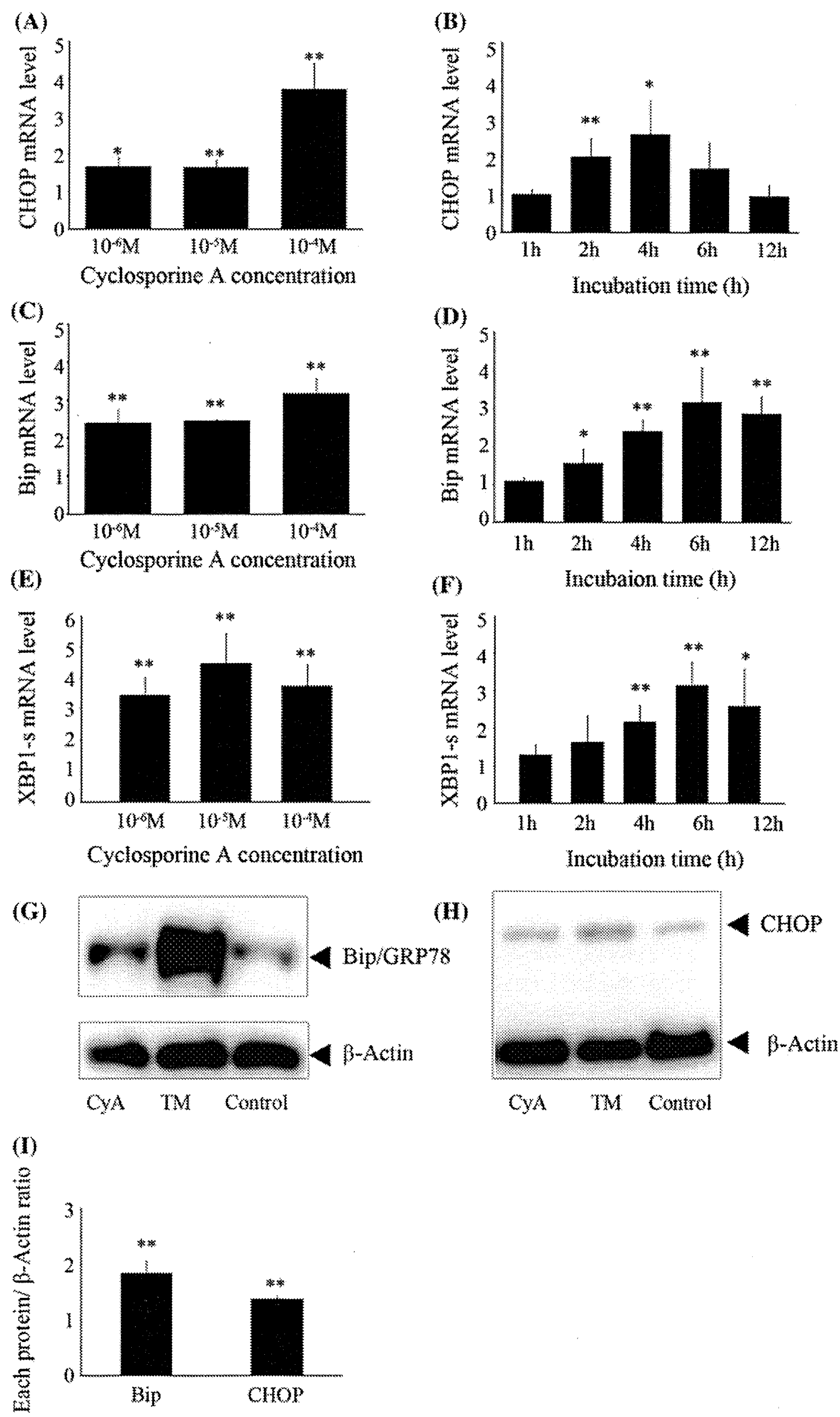

Fig. 2 CyA activated the UPR in HaCaT cells. HaCaT cells were treated with the indicated concentrations of CyA for 4 h and the mRNA expression of CHOP/GADD153 (**a**), GRP78/Bip (**c**) and XBP1-s (**e**) was measured by qPCR. The time course of UPR induction by 10^{-5} M CyA in the HaCaT cells were examined for 12 h by qPCR, CHOP/GADD153 (**b**), GRP78/Bip (**d**) and XBP1-s (**f**). Western blot analysis was performed using immunoreactive CHOP/GADD153, GRP78/Bip and β-actin antibodies (**g**, **h**). Quantitative densitometry of the UPR proteins in HaCaT cells incubated with CyA as compared to the control (**i**). Data shown are the mean ± SD of four independent experiments (*$0.01 < p < 0.05$, **$p < 0.01$)

Cyclosporin A induces the UPR in HaCaT cells

HaCaT cells were treated with CyA for 4 h and the level of CHOP/GADD153 and GRP78/Bip mRNA expression were assessed by quantitative PCR. mRNA expression of CHOP/GADD153 and GRP78/Bip were significantly upregulated in a dose-dependent manner (Fig. 2a, c). We found 0.5×10^{-6} M CyA also induced CHOP/GADD153 and GRP78/Bip mRNA (data not shown). To assess the kinetics of UPR induction, HaCaT cells were treated with 10^{-5} M CyA for up to 12 h. CHOP/GADD153 and GRP78/Bip mRNA started to increase after 2 h after the treatment and peaked at 4 and 6 h, respectively (Fig. 2b, d). We further recognized that 1 μg/ml tunicamycin could markedly enhance the mRNA expression of CHOP/GADD153 and GRP78/Bip in HaCaT cells after 4-h induction (data not shown). Examination of the protein levels of CHOP/GADD153, GRP78/Bip and tunicamycin recapitulated the mRNA expression (Fig. 2g, h). As shown in Fig. 2g and h, CyA induced the protein levels of CHOP/GADD153 and GRP78/Bip, which were less than those by tunicamycin induction. We measured the quantification of chemiluminescence signals of Fig. 2g and h by laser densitometry expressed as arbitrary units (Fig. 2i). The protein expression of GRP78/Bip and CHOP/GADD153 was significantly increased in HaCaT cells incubated with CyA as compared to the control. The bar graph of densitometry was expressed as each protein/β-actin protein amount ratio and then x-fold expression relative to the control.

We further confirmed CyA-mediated induction of other UPR markers, such as XBP1-s as shown in Fig. 2e and f and HRD1 (data not shown). The level of XBP1-s mRNA induced by CyA was significantly elevated and peaked at 6 h. HRD1 showed the similar increasing pattern to XBP1-s. The amount of HRD1 mRNA induced by CyA was significantly elevated in a dose-dependent manner and showed a 1.5-fold increase at 2–12 h as compared to the control (data not shown). We took further examination about CyA effect on the UPR in HSC-1 cells and obtained similar mRNA and protein changes to those in HaCaT cells (data not shown).

Cyclosporin D induces GRP78/Bip in HaCaT cells

We further investigated the ability of CyD, an analog of CyA, to induce the UPR in HaCaT cells. CyD is able to bind cyclophilins, but lacks the immunosuppressive properties which CyA has. As shown in Fig. 3, CyD induced the expression of CHOP/GADD153 and GRP78/Bip in a dose-dependent manner. The protein expression of GRP78/Bip in HaCaT cells incubated with CyD for 48 h also increased (Fig. 3c), but that of CHOP/GADD153 did not (Fig. 3d). We measured the quantification of chemiluminescence signals of Fig. 3c and d by laser densitometry expressed as arbitrary units (Fig. 3e). The bar graph of densitometry was expressed as each protein/

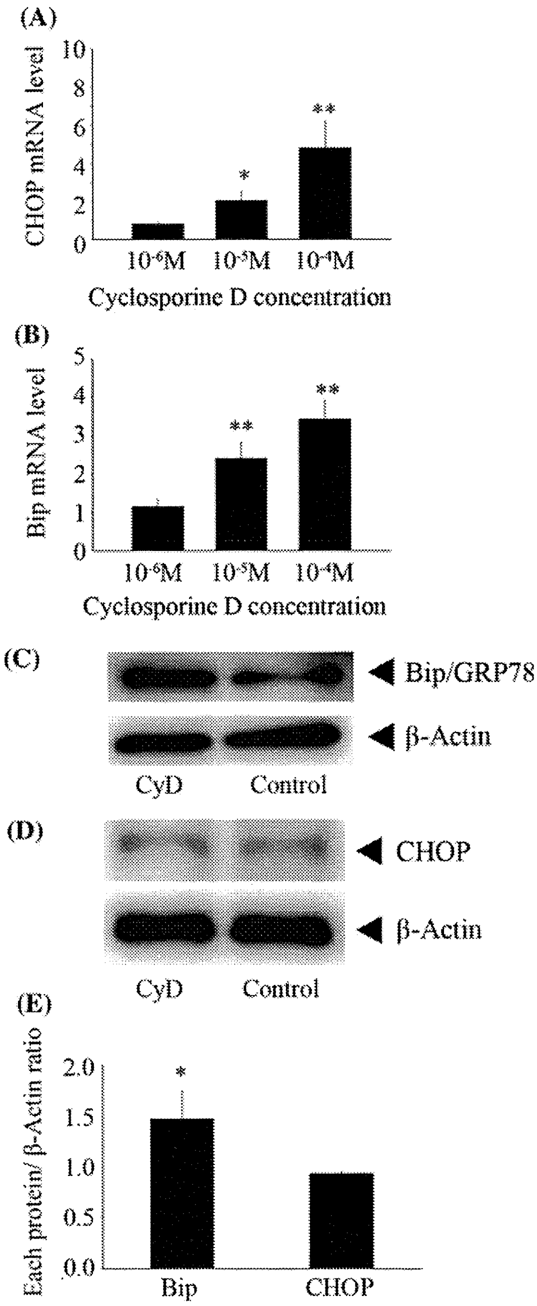

Fig. 3 Cyclosporin D, CyA analog, induced GRP78/Bip in HaCaT cells. HaCaT cells were treated with various concentration of cyclosporin D for 4 h. The expression of CHOP/GADD153 mRNA (**a**) and GRP78/Bip mRNA (**b**) were measured by qPCR and as compared to the untreated control cells ($n = 4$). Western blot analysis was performed using immunoreactive GRP78/Bip (**c**), CHOP/GADD153 (**d**) and β-actin antibodies. Quantitative densitometry of the UPR protein in HaCaT cells incubated with CyD as compared to the control (**e**). Data shown are the mean ± SD of four independent experiments (*$0.01 < p < 0.05$, **$p < 0.01$)

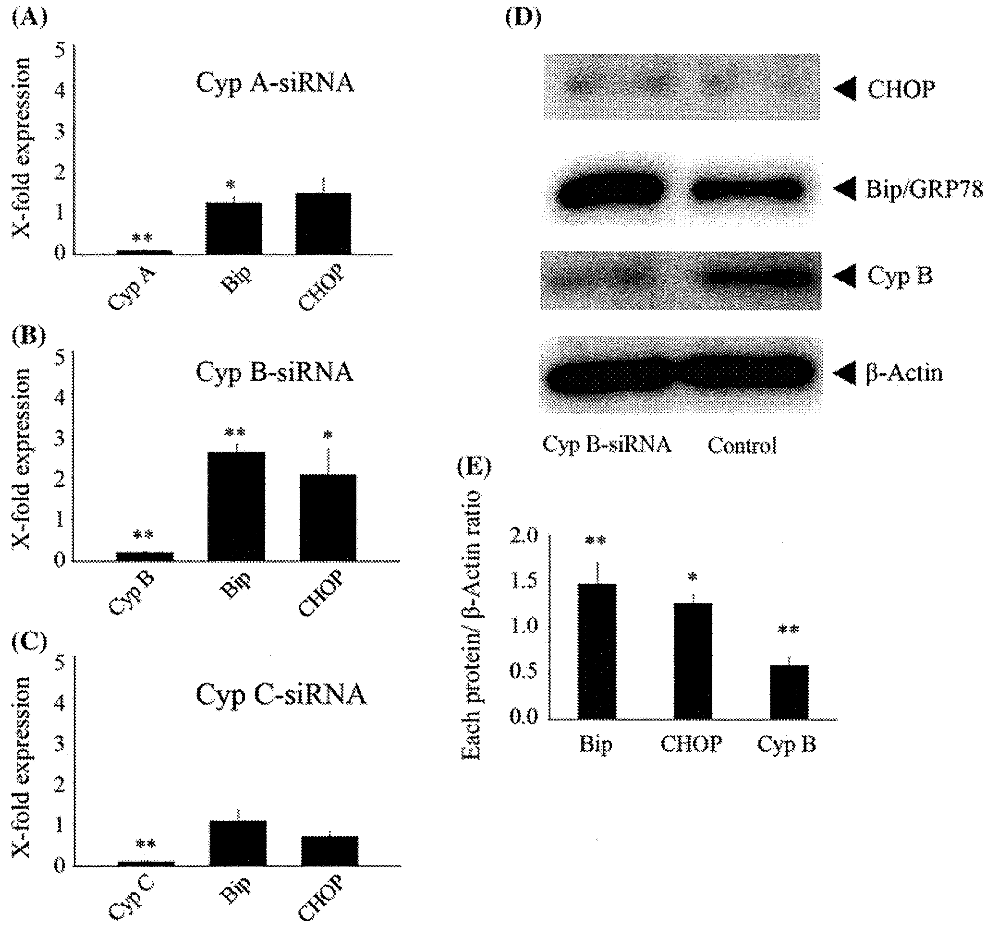

Fig. 4 Reduction in cyclophilin B induced the UPR. HaCaT cells were transfected with siRNA messenger RNA of Cyp A (**a**), Cyp B (**b**), Cyp C (**c**) or CD4. At 48-h of post-transfection, mRNA levels of Cyp A, Cyp B, Cyp C, GRP78/Bip and CHOP/GADD153 were measured by qPCR. The relative amounts as compared to CD4 transfected cells are given. Western blot analysis was performed using immunoreactive CHOP/GADD153, GRP78/Bip, Cyp B and β-actin antibodies (**d**). Quantitative densitometry of the UPR protein in HaCaT cells transfected with Cyp B siRNA as compared to CD4 siRNA-transfected cells (**e**). The data represent the means of four independent experiments. *Error bars* represent the SD (*$0.01 < p < 0.05$, **$p < 0.01$)

β-actin protein amount ratio and then x-fold expression relative to the control. The protein expression of GRP78/Bip was significantly increased in HaCaT cells incubated with CyD as compared to the control. As for the protein level of CHOP/GADD153, it did not show any significant change. CyD also induced CHOP/GADD153 and GRP78/Bip at mRNA and protein level in HSC-1 cells (data not shown).

siRNA-mediated knockdown of cyclophilin B induces the UPR

Cyp A, Cyp B and Cyp C are major cellular targets of CyA. Therefore, we applied RNAi methodology to investigate the role of cyclophilins on the UPR in HaCaT cells. By introducing small interfering RNA (siRNA), the expression of endogenous Cyp A, Cyp B or Cyp C were suppressed in HaCaT cells (Fig. 4a–c). The mRNA expression of GRP78/Bip and CHOP/GADD153 in Cyp B siRNA-transfected cells were significantly increased to 2.1–2.6 folds as compared to the levels seen in CD4 siRNA-transfected HaCaT cells (Fig. 4b). In contrast, knockdown of Cyp A and Cyp C increased little or no expression of their mRNA (Fig. 4a, c). Western blot analysis showed that GRP78/Bip and CHOP/GADD153 protein products in HaCaT cells were actually increased after 48 h of Cyp B siRNA treatment (Fig. 4d). We measured the quantification of chemiluminescence signals of Fig. 4d by laser densitometry expressed as arbitrary units (Fig. 4e). The protein expression of GRP78/Bip and CHOP/GADD153 was significantly increased in Cyp B siRNA-transfected cells as compared to the control. In addition, the suppression level of Cyp B protein was also significant. The bar graph of densitometry was expressed as each protein/β-actin protein amount ratio and then x-fold expression relative to the control. This pattern of Western blot analysis was also consistent with the result obtained from HSC-1 cells (data not shown). These results indicate that among these cyclophilins, Cyp B, but not CypA and CypC, is involved in the CyA-induced UPR.

Combination of CyA and knockdown of Cyp B highly induces the UPR in HaCaT cells

To assess the contribution of both CyA and Cyp B on the UPR in vitro, HaCaT cells were transfected with Cyp B

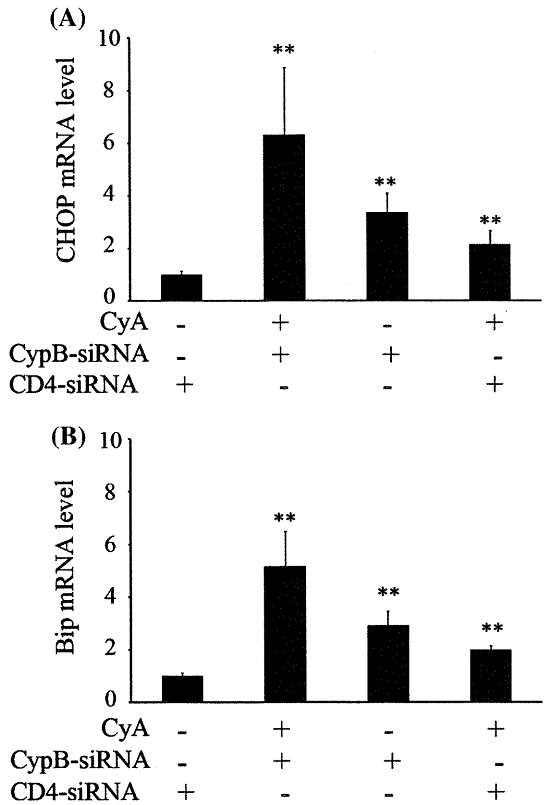

Fig. 5 Combination of CyA and knockdown of Cyp B highly induce the UPR in HaCaT cells. HaCaT cells were incubated for 48 h with Cyp B siRNA or CD4 siRNA, followed by 4 h 10^{-5} M CyA treatment. The expression of CHOP/GADD153 (**a**) and GRP78/Bip (**b**) were measured by qPCR and as compared to CyA untreated and CD4 siRNA treated as a control ($n = 4$) (*$0.01 < p < 0.05$, **$p < 0.01$)

siRNA for 48 h, followed by CyA treatment for 4 h, and mRNA expression of the UPR markers were evaluated. Treatment of CyA and Cyp B siRNA showed synergistic effects on the expression of CHOP/GADD153 (Fig. 5a) and GRP78/Bip (Fig. 5b) by 6.3-fold and 5.1-fold, respectively.

Discussion

We reported that the UPR is activated in differentiated epidermal keratinocytes recently [18]. Although CyA is believed to reduce proliferation of keratinocytes in psoriasis vulgaris, the mechanism of its action remains poorly defined. In this report, we showed that the UPR was downregulated in psoriatic lesions in keratinocytes in psoriasis vulgaris and that CyA induced the UPR in keratinocytes or reduction in Cyp B by RNA interference. Also, we found TGFβ induced the UPR markers in HaCaT cells.

The UPR in keratinocytes from psoriatic lesions is similar to that in squamous cell carcinoma lesions [18]. Activated Stat3 inhibits the UPR in cardiomyocytes, macrophage [11] and ER stress reduces Stat3 phosphorylation in HEK 293 and SHSY5Y [7]. Therefore, we expected that the UPR activation in keratinocytes from psoriatic lesions, after ER stress, reduced Stat3 phosphorylation similar to other cells. We confirmed the phosphorylation of Stat3 in the lesional keratinocytes of psoriasis vulgaris as described [15, 20].

CyA treatment or reduction in Cyp B by RNA interference of HaCaT cells led to the increase in the UPR. This result was consistent with that examined the HSC-1 cell line (Data not shown). In addition, treatment with a combination of CyA administration and Cyp B siRNA modulated the expression of a number of genes involved in keratinocyte differentiation. These genes included Kruppel-like factor 4, keratin K1 and K10 (Data not shown). The CyA analog, CyD, lacks immunosuppressive activity, but showed UPR inducing effects (as shown in Fig. 3). The point we would like to stress is that the functional mechanism of CyA and CyD for activating the UPR is not via the immunosuppressive pathway or T cell-mediated system. Therefore, it is reasonable to show in this study that Cyp A did not work well as a possible receptor for CyA and CyD for UPR inducing pathway because Cyp A is known to interact with CyA for the immunosuppressive pathway. And our observation is consistent with the report that knockdown of Cyp B induced the UPR in rat heart myoblasts [8].

Tunicamycin is known to be more efficient at inducing the UPR than CyA. Since the strong UPR activation by tunicamycin would trigger apoptosis, a mild UPR activation by CyA is considered to be important to contribute to cell homeostasis. Recently, it has been demonstrated that Cyp B makes a complex with GRP78/Bip and PDI in the ER [12]. Moreover, recent studies have shown that CD147 is an essential component of the cell-surface signaling receptor to Cyp B [25]. Whether the ER complex or the cell surface receptor is involved in the Cyp B mediated UPR induction is intriguing and needs to be elucidated.

This study indicates the UPR is attenuated in keratinocytes in lesional psoriasis epidermis. Furthermore, we demonstrated that UPR-inducing reagents which act through Cyp B may be good candidate drugs for the treatment of psoriasis vulgaris.

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