Statistical analyses

Data were statistically evaluated using SPSS Statistics (IBM, Tokyo, Japan). Fisher exact probability tests were used for comparison of frequencies. Mann–Whitney U tests were used for comparison of ELISA units. *P* values of less than 0.05 were considered significant.

Results

Measurement of anti-PM/Scl antibodies by ELISA

For the screening of anti-PM/Scl antibodies in large numbers of serum samples, we developed an ELISA system that uses biotinylated recombinant PM/Scl-100 and PM/Scl-75. We screened a total of 600 serum samples obtained from patients with various systemic autoimmune diseases and an additional 36 serum samples from healthy volunteers for both antibodies. Based on the cutoff levels at 5 SDs above the mean value, nine (1.5%) and seven (1.2%) patients were positive for anti-PM/Scl-100 and anti-PM/Scl-75 antibodies, respectively (Figure 1). Five patients (A, C, D, E and F) had both antibodies, four (B, G, H and I) had only anti-PM/Scl-100 antibodies, and two (J and K) had only anti-PM/Scl-75 antibodies. When the cutoff was set at 3 SDs above the mean value, one sample from a patient (L mentioned in Figure 2) with overlap syndrome was just below the cutoff for both antibodies. Subsequently, serum samples from these 12 patients were used for immunoprecipitation to confirm whether they were truly positive for the anti-PM/Scl antibodies. An additional 36 samples from healthy volunteers showed levels below the cutoff for both antibodies.

Immunoprecipitation using recombinant PM/Scl protein and radiolabeled cellular protein

After the initial screening by ELISA, we investigated antibodies against PM/Scl in sera from 11 anti-PM/Scl-100 and/or anti-PM/Scl-75-positive patients and 1 equivocal patient for their ability to immunoprecipitate biotinylated recombinant PM/Scl-100 and PM/Scl-75 and radiolabeled cellular PM/Scl. All nine anti-PM/Scl-100-positive sera in ELISA immunoprecipitated biotinylated recombinant PM/Scl-100, whereas five of the seven anti-PM/Scl-75-positive sera in ELISA immunoprecipitated biotinylated recombinant PM/Scl-75 (Figure 2, TnT-IPP). Sera that were anti-PM/Scl-75-positive in ELISA but -negative in IPP (J and K) were negative for anti-PM/Scl-100 antibodies in ELISA and IPP. Serum of patient L with equivocal ranges in both ELISAs immunoprecipitated neither recombinant PM/Scl-100 nor PM/Scl-75 (data not shown).

To determine whether the positive sera in ELISA immunoprecipitate the PM/Scl complex, we applied conventional IPP using radiolabeled HeLa cell extract (Figure 2, HeLa-IPP). All nine sera (patients A to I) that had reacted with the recombinant PM/Scl-100 also immunoprecipitated a cellular 100-kDa protein. Eight of these sera also immunoprecipitated a 75-kDa protein,

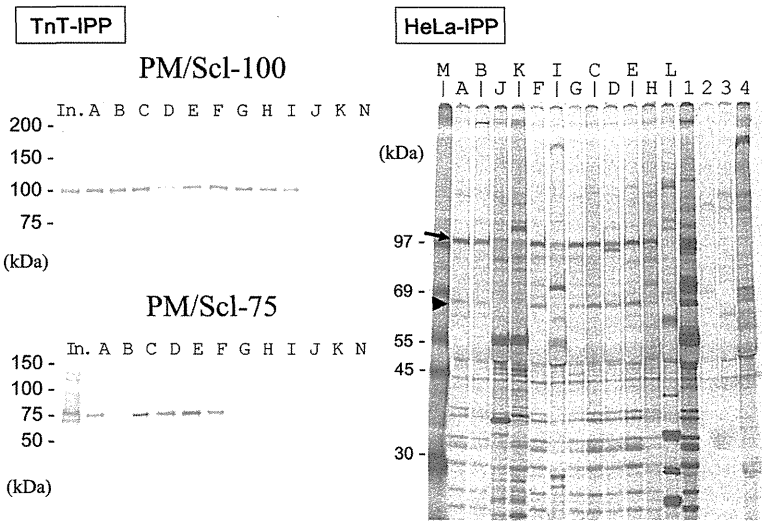

Figure 2 Detection of anti-PM/Scl antibodies in immuoprecipitation analysis. TnT-IPP: immunoprecipitation of biotinylated recombinant PM/Scl-100 and PM/Scl-75. Recombinant proteins were subjected to 4% to 20% SDS-PAGE and analyzed by immunoblotting with streptavidin-alkaline phosphatase and substrate. In., the input was half the dose for immunoprecipitation. Lanes A to K correspond to the anti-PM-Scl-100 and/or –75-positive patients shown in Figure 1. Lane N: healthy control serum. HeLa-IPP: immunoprecipitation analysis using radiolabeled HeLa cell extracts. Lanes A to L correspond to the patients shown in Figure 1 and Table 1. Lanes A to K correspond to anti-PM-Scl-100 and/or –75-positive patients shown in Figure 1. Lane M: [Methyl-^{14}C] methylated protein MW markers (PerkinElmer Japan, Yokohama, Japan). Lane L: anti-U1-RNP-positive serum with equivocal titers for both antibodies in ELISA. Lanes 1 to 4 show the reference sera; lane 1, anti-PM/Scl-positive serum; lane 2, anti-MDA5-positive serum; lane 3, anti-TIF1-γ-positive serum; lane 4, anti-Mi-2-positive serum. Arrow and arrowhead corrspond to the PM/Scl-100 and PM/Scl-75 antigens, respectively. IPP, immunoprecipitation; TnT, *in vitro* translation and transcription product.

but one another (patient I) did not. Two sera that were positive only for anti-PM/Scl-75 in ELISA (patients J and K) immunoprecipitated neither the 100-kDa nor the 75-kDa protein. The serum from an overlap syndrome patient (L) with an equivocal level of both anti-PM/Scl-100/75 antibodies in ELISA was negative in IPP. According to these results, eight sera (patients A to H) were judged to be positive for anti-PM/Scl antibodies, as was one other serum (patient I), which reacted to recombinant PM/Scl-100 and which immunoprecipitated a 100-kDa cellular protein.

Indirect immunofluorescence staining patterns of anti-PM/Scl-100 and/or anti-PM/Scl-75-positive sera

In IIF analysis, the eight sera (patients A to H) that immunoprecipitated the 100-kDa and 75-kDa proteins showed nucleolar patterns (Table 1). The serum (patient I) that only immunoprecipitated the 100-kDa protein showed a speckled pattern without nucleolar staining. Two sera (patients J and K) that immunoprecipitated neither the 100-kDa nor the 75-kDa protein, also showed no nucleolar patterns.

Clinical and laboratory data for anti-PM/Scl-positive patients

The nine patients with anti-PM/Scl were four with UCTD, three with DM (including one with CADM), one with limited cutaneous SSc and one with SS. The clinical features of these patients are summarized in Table 1. The prevalence of anti-PM/Scl in UCTD (25%) is significantly higher than that of DM (2.4%, $P = 0.0032$), SSc (0.5%, $P = 0.000066$), SS (1.2%, $P = 0.0018$), SLE (0%, $P = 0.00012$), OL (0%, $P = 0.045$) and healthy control (0%, $P = 0.0067$). Although the numbers of examined sera are very small, no patients with anti-PM/Scl antibodies are found among patients with PM or OL. Four patients with UCTD are clinically heterogeneous; two are suspected of having SLE, one of having SS and one of having rheumatoid arthritis (RA). All but one are young adult women. No common clinical features, including Raynaud's phenomenon and abnormal nailfold capillaries, are present among these four patients.

Of the 126 DM patients, there are 8 anti-nucleolar antibody (ANoA)-positive patients, of whom 3 patients, all men, had anti-PM/Scl antibodies. Of the 123 anti-PM/Scl-negative DM patients, only 32 are men ($P = 0.020$). These three patients were complicated with ILD. The clinical manifestations of ILD for these three patients were improved by oral prednisolone and immunosuppressive agent therapy, and their ILD did not have a fatal outcome. Additionally, the complication of internal malignancy (mesopharynx and prostate) was also recognized in two patients three years before or after the disease onset. ILD and internal malignancy are

Table 1 Connective tissue disease manifestations of anti-PM/Scl-100-ELISA- and/or anti-PM/Scl-75-ELISA-positive patients

Patient	Age in years	Sex M/F	Diagnosis	IIF pattern[a], titer	ELISA PM/Scl-100/ PM/Scl-75	TnT-IPP PM/Scl-100/ PM/Scl-75	HeLa-IPP PM/Scl-100/ PM/Scl-75	other auto-antibodies	clinical features
A	52	F	SS	nucleolar, 1:2560	+ / +	+ / +	+ / +		dry eye, dry mouth
B	62	F	lSSc	nucleolar, 1:320	+ / -	+ / -	+ / +		Raynaud's ph, sclerodactyly
C	54	M	CADM	nucleolar, 1:640 diffuse, 1:80	+ / +	+ / +	+ / +		ILD, Gottron papules, mechanic's hands
D	69	M	DM	nucleolar, 1:640 diffuse, 1:80	+ / +	+ / +	+ / +		ILD, Gottron sign, mechanic's hands, V-neck sign, dysphagia, pharyngeal Ca
E	67	M	DM	nucleolar, 1:1280	+ / +	+ / +	+ / +		ILD, Gottron sign, Heliotrope rash muscle weakness, prostate Ca
F	73	F	UCTD	nucleolar, 1:640	+ / +	+ / +	+ / +		ILD, dry eye, dry mouth
G	33	F	UCTD	nucleolar, 1:640 diffuse, 1:80	+ / -	+ / -	+ / +		morning stiffness, polyarthralgia
H	31	F	UCTD	nucleolar, 1:160 speckled, 1:80	+ / -	+ / -	+ / +		polyarthralgia, photosensitivity
I	31	F	UCTD	speckled, 1:80	+ / -	+ / -	+ / -		oral ulcer, photosensitivity
J	24	F	UCTD	diffuse, 1:640 cytoplasmic, 1:160	- / +	- / -	- / -	SS-A	dry eye, dry mouth
K	23	F	SLE	diffuse, 1:320 cytoplasmic, 1:80	- / +	- / -	- / -	SS-A ribosomal P	polyarthralgia, malar rash, photosensitivity, leukopenia

[a] 'diffuse' and 'speckled' in the IIF pattern, respectively, refer to nuclear diffuse and nuclear speckled patterns. CADM, clinically amyopathic DM; Ca, carcinoma; DM, dermatomyositis; ILD, interstitial lung disease; lSSc, limited cutaneous SSc; ph, phenomenon; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; UCTD, undifferentiated connective tissue disease.

more frequent in anti-PM/Scl-positive DM patients than in anti-PM/Scl-negative DM patients, but not significantly ($P = 0.060$ and $P = 0.072$, respectively).

Besides the three DM patients with ILD, one patient who had UCTD was also complicated with ILD. Although the anti-PM/Scl-100 ELISA units of these four patients with ILD were not higher than those of five anti-PM/Scl-positive patients without ILD (mean 26.7 versus 62.0), anti-PM/Scl-75 titers of the four patients with ILD were significantly higher than those of five patients without ILD (mean 86.0 unit versus 0.96 unit, $P = 0.027$ by Mann–Whitney U test).

Discussion

The anti-PM/Scl antibody is a well-known ANoA and a serological marker of OL and other systemic autoimmune diseases such as SSc, PM and DM alone [15]. This antibody is common in the West. For example, it

Table 2 Frequencies of anti-PM/Scl antibodies in disease subsets

Frequencies of anti-PM/Scl antibodies in disease subsets						
Study	Marguerie	Mahler	Rozman	Hanke		Maes
Reference	[13]	[39]	[40]	[41]		[42]
Year	1992	2005	2008	2009		2010
Country	UK	Various	Europe	Germany		Belgium
Anti-PM/Scl detection	CIE	PM1-α ELISA	LIA	LIA		PM1-α ELISA
Patient selection and numbers of patients	1689 SLE	205 SSc	625 SSc	280 SSc[b]		70 SSc
	879 SSc[a]	114 SLE		88 RA		66 SLE
	256 PM or DM	40 PM		72 SLE		35 SS
		40 PM/SSc		49 SS		24 RA
						23 DM
						13 PM
						11 MCTD
Anti-PM/Scl-positive patients	27 PM (or DM)/SSc	22 PM/SSc (55%)	1 PM (7.7%)	Anti-PM/Scl-75	Anti-PM/Scl-100	3 SSc (4.3%)
	4 SSc	27 SSc (13%)	18 SSc (2.9%)	29 SSc (10%)	20 SSc (7.1%)	
	1 PM	3 PM (7.5%)	1 DM (1.7%)	3 RA (3.4%)	3 SLE (4.2%)	
				1 SLE (1.4%)	1 SS (2.0%)	
Study	Mierau	Koschik	Mehra	D'Aoust	Kazi	Muro
Reference	[31]	[57]	[58]	[17]	[18]	The present study
Year	2011	2012	2013	2014	2014	
Country	Germany	USA	Australia	Canada	Japan	Japan
Anti-PM/Scl detection	ID	ID	LIA	PM1-α ELISA	IIP	ELISA, IPP
Patient selection and numbers of patients	863 SSc	2425 SSc	528 SSc	763 SSc	Kanazawa cohort	223 SSc
					316 SSc	126 DM
					Keio cohort	123 SLE
					272 SSc	88 SS
						17 overlap
						16 UCTD
						7 PM
Anti-PM/Scl-positive patients	42 SSc (4.9%)	75 SSc (3.1%)	Anti-PM/Scl-75	55 SSc (7.2%)	0	4 UCTD (25%)
			66 SSc (12.5%)			3 DM (2.4%)
			Anti-PM/Scl-100			1 SS (1.1%)
			26 SSc (4.9%)			1 SSc (0.4%)

[a]Since the numbers of myositis overlap patients were not given, the frequencies of the antibodies in disease subsets were not calculated; [b]51 overlap and 16 undifferentiated connective tissue disease patients were included.

CIE, counter immunoelectrophoresis; DM, dermatomyositis; ID, immunodiffusion; IPP, immunoprecipitation; LIA, line immunoassay; MCTD, mixed connective tissue disease; PM, polymyositis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; UCTD, undifferentiated connective tissue disease.

was the third most-found, followed by anti-centromere and anti-topoisomerase I antibodies, in a large cohort of SSc patients in Germany [31]. However, large studies of Japanese patients with SSc showed this antibody to be absent [32,33], and a recent study noted that 0/588 Japanese patients with SSc had anti-PM/Scl antibodies [18]. Large-cohort studies using sera from more than 200 connective tissue disease patients in the literature are summarized in Table 2, although some studies with mostly overlapped patients are omitted. Anti-PM/Scl antibodies are strongly linked to HLA-DRB1*0301 [34], which is very rarely found in Japanese, with a prevalence of only 0.14%, according to an online database [35] (HLA Laboratory, Kyoto, Japan); however, the contribution of this finding remains unknown. Since we had found three patients to have strong ANoA in IIF analysis during our recent studies on myositis-specific or associated autoantibodies [36-38], we aimed to investigate anti-PM/Scl antibodies in our large cohort of systemic autoimmune disease.

Although LIA for anti-PM/Scl-75 and −100 antibodies and PM1-α ELISA have often been used recently [16,31,39-43], the latter is not available in Japan and the former is not cost-effective, costing around 13,000 yen/sample (Cosmic Corporation, Tokyo, Japan). For our in-house ELISA, the anti-PM/Scl-75 assay was found to be inferior to the anti-PM/Scl-100 assay both in sensitivity and specificity, according to the results of protein-IPP, which is widely accepted as a reference method for detecting several markers for SSc and PM/DM. Originally, most PM/Scl-positive sera have been shown to contain anti-PM/Scl-100 and about 50% to 60% of the sera have been shown to react with PM/Scl-75 [11,12,44,45]. Raijmakers and colleagues showed that PM/Scl-75 contains a previously unidentified N-terminal region that is important for the antigenicity of the protein [46]. This longer form, named PM/Scl-75c, was as reactive as PM/Scl-100 to sera from PM/SSc overlap patients in ELISA (28% and 25%, respectively) [46]. Subsequently, Hanke and colleagues showed the prevalence of anti-PM/Scl-75c to be higher than that of anti-PM/Scl-100 (10.4% versus 7.1%) in LIA using sera from 280 SSc patients [41]. There are several possible explanations for the lower prevalence of anti-PM/Scl-75 than anti-PM/Scl-100 in this study. The cDNA in this study, PM/Scl-75c-β, has a 17 amino acid insertion at the C-terminus which could introduce conformational changes in epitope [47]. In the study of Hanke and colleagues, recombinant PM/Scl-75 was expressed by a baculovirus [41]. The discrepancies might also be due to racial differences or clinical backgrounds. In a validation study by Jaskowski and colleagues, the anti-PM/Scl-100 LIA had better agreement for the detection of anti-PM/Scl with IPP as the reference method than with PM/Scl-75 LIA and PM1-α ELISA [48].

In this study, eight of nine anti-PM/Scl antibody-positive sera exhibited nucleolar staining in IIF analysis. Some studies have shown that anti-PM/Scl-positive sera do not always demonstrate a nucleolar staining pattern in IIF [16,17,47,49]. Interestingly, one ANoA-negative serum with anti-PM/Scl reacted with PM/Scl-100 but not with PM/Scl-75. Intramolecular epitope spreading from the initial response against PM/Scl-100 to a successive response by other exosomal components has been recognized, as have many other autoantibody responses [50]. Figure 3 shows a four-way Venn diagram depicting the overlap between anti-PM/Scl-100 by TnT-IPP, anti-PM/Scl-75 by TnT-IPP, anti-PM/Scl by cellular IPP and anti-nucleolar pattern by IIF. ANoA-positive anti-PM/Scl antibodies all immunoprecipitated both 100- and 75-kDa proteins in HeLa-IPP, whereas only one ANoA-negative anti-PM/Scl antibody (patient I) immunoprecipitated only a 100-kDa protein in HeLa-IPP. Since sera from Patient I immunoprecipitated several other polypeptides, the nuclear speckled staining of this patient in IIF may correspond to antibodies against these proteins. Moreover, anti-PM1α reactivity has been reported in apparently ANA-negative samples [16,17,49]. Although future studies are necessary to address whether monospecific anti-PM/Scl-100 antibodies show nucleolar staining in IIF, we can conclude that IIF is not a sensitive immunoassay for the detection or screening of anti-PM/Scl antibodies.

The findings of anti-PM/Scl antibodies in UCTD patients are of clinical importance. The classification criteria of UCTD are not well established [51]. Since our UCTD patients were not diagnosed with definite

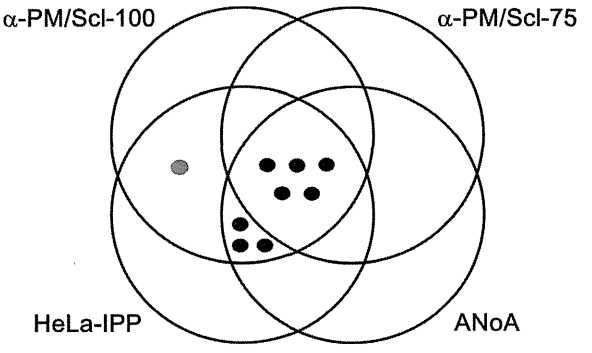

Figure 3 Venn diagram showing the overlap between anti-PM/Scl antibodies by different assays. Anti-PM/Scl-100 and anti-PM/Scl-75 circles show the antibody-positive patiens determined by immunoprecipitation with the corresponding recombinant protein. The HeLa-IPP circle shows anti-PM/Scl-positive patients determined by immunoprecipitation with HeLa cellular protein. Black dots show the patients with both anti-75 kDa and anti-100 kDa antibodies, and the gray dot shows the patient with only anti-75 kDa antibody in HeLa-IPP. The ANoA circle shows the patients demonstrating anti-nucleolar patterns as observed by indirect immunofluorecence.

connective tissue disease even if the new set of RA criteria [52] and the preliminary criteria for the very early diagnosis of SSc [53] were used, we applied the preliminary classification criteria of UCTD suggested by Mosca and colleagues [27]. Four UCTD patients with anti-PM/Scl were all so-called 'stable UCTD'. Their disease courses were stable over a period of more than three years without internal organ involvement, except for one, whose ILD was nonspecific interstitial pneumonia that did not exacerbate for more than ten years. Interestingly, Cordiali-Fei and colleagues [54] reported that anti-PM/Scl responses were mainly associated with Italian patients with UCTD, which was defined by the same criteria used in our study. They found 5 patients with anti-PM/Scl in 23 patients with UCTD (22%), a frequency that is almost the same as that of our study.

Of second importance are the three anti-PM/Scl-positive DM patients. Myalgia or muscle weakness varied, and the levels of creatine kinase ranged from normal levels to more than 2,000 IU/L. Patient C in this study is the second reported case of CADM, to the best of our knowledge, following the first case described by Lega and colleagues [55]. A previous study of 20 PM/DM patients with anti-PM/Scl demonstrated that anti-PM-Scl is not necessarily a marker for good prognosis in patients with PM/DM, because lung and esophageal involvement were found (in 75% and 20%, respectively), as was internal malignancy (in 15%) [56]. Also in our study, all three patients were complicated with ILD and required combined therapy of steroids and immunosuppressive agents. Although they were all alive during the observation periods, the prognosis of ILD in anti-PM/Scl-positive DM patients cannot be determined due to the very limited numbers examined and the limited observation periods (maximum 45 months). Two of these patients were also complicated with localized cancer without metastasis. In a previous study, two out of twelve antibody-positive patients with DM had mechanic's hands [56]. Very interestingly, also in our study, two patients exhibited mechanic's hands in addition to sole hyperkeratotic rhagadiform symptoms.

Conclusions

Our study of Japanese patients with various systemic autoimmune diseases confirms that anti-PM/Scl antibodies also exist in these patients. ELISA with PM/Scl-100 recombinant protein was useful in detecting anti-PM/Scl antibodies. Anti-PM/Scl was not always specific for DM or SSc; it was also present in various autoimmune conditions, including UCTD. All the anti-PM/Scl-positive DM cases were complicated with ILD and/or cancer, while no life-threatening internal organ involvement was found in other anti-PM/Scl-positive cases. Considering the higher prevalence of anti-PM/Scl in UCTD, this autoantibody may be more important in systemic autoimmune disease clinics than we expected. Further studies in larger cohorts are necessary to define the clinical significance of anti-PM/Scl antibodies in Japanese patients with each autoimmune condition. Future collaborative studies for evaluating our sera with LIA and PM-1α ELISA promise to be interesting.

Abbreviations

ACR: American College of Rheumatology; ANoA: anti-nucleolar antibody; ARS: aminoacyl tRNA synthetase; CADM: clinically amyopathic dermatomyositis; CIE: counter immunoelectrophoresis; DM: dermatomyositis; ELISA: enzyme-linked immunosorbent assay; EULAR: European League Against Rheumatism; ID: immunodiffusion; IIF: indirect immunofluorescence; ILD: interstitial lung disease; IPP: immunoprecipitation; LIA: line immunoassay; OL: overlap syndrome; PM: polymyositis; RA: rheumatoid arthritis; RLU: relative luminescence unit; Scl: scleroderma; SD: standard deviation; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; SSc: systemic sclerosis; TnT: *in vitro* translation and transcription product; UCTD: undifferentiated connective tissue disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM performed the serological analyses and the analysis of the data. YH and TM performed immunoprecipitation with the radiolabeled extract and interpreted the data. KS and YO provided sera for the analyses. MA made intellectual contributions and helped to prepare the manuscript. All authors were involved in the conception, design, and interpretation of data and in drafting the article and revising it critically for important intellectual content. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Epidemiologic analysis of the clinical features of Japanese patients with polymyositis and dermatomyositis

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Abstract

Objectives: This study aimed to investigate the clinical characteristics of polymyositis/dermatomyositis (PM/DM) in Japan by analyzing data from the nationwide registration system.
Methods: The data of the registration system in 2009 were analyzed to investigate patient numbers, sex, clinical symptoms, therapies, complications, and prognosis of PM/DM.
Results: The total number of PM/DM cases was approximately 17,000, and the female/male sex ratio was 2.7:1. Almost all patients improved as a result of therapy, but many suffered from sequelae such as muscle weakness.
Conclusions: The results characterize significant aspects of Japanese PM/DM patients. However, a further prospective survey is required to clarify the true epidemiology and natural history of PM/DM.

Keywords:

Clinical features, Epidemiology, Nationwide registration system, Polymyositis and dermatomyositis (PM/DM)

History

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Introduction

Polymyositis and dermatomyositis (PM/DM) are systemic inflammatory diseases involving the skeletal muscles, skin, and other organs [1–3]. Although the causes of PM/DM remain unclear, some autoimmune processes are implicated [1,3,4]. The prognosis of PM/DM was very poor before the introduction of corticosteroids [5]. PM/DM is classified as intractable diseases by the Ministry of Health, Labour and Welfare (MHLW) of Japan [6,7]. Nevertheless, there have been no nationwide epidemiologic surveys since 1991 [8], which reported 3000 cases of PM and DM each. However, the numbers of cases in recent years are expected to differ greatly.

The National Program on Rare and Intractable Diseases was initiated in Japan in 1972, and a nationwide registration system was implemented for patients with intractable diseases, including PM/DM. The PM/DM Subcommittee in the Research Committee on Autoimmune Diseases convened in 2011, and a nationwide epidemiologic survey of PM/DM was brought up as an important issue. First, the data of the registration system from fiscal years 2003–2010 were analyzed for epidemiologic investigation [7]. In the present study, this paper is based on the analyzed these data in order to elucidate the clinical features of Japanese PM/DM patients.

Materials and methods

This study utilized the data from a database in a nationwide registration system established by the Japanese government for patients with intractable diseases, including PM/DM. In Japan, registered patients with PM/DM receive subsidized medical care. As the subsidy period is one year, patients must renew their application and registration to the system every year. The details of the registration system have been described previously [7].

The diagnosis of PM/DM is based on the PM/DM criteria recommended by the MHLW of Japan [9]. Information regarding clinical findings essential for diagnosing PM/DM must be indicated in the registration form. These items include (1) cutaneous changes such as heliotrope erythema and Gottron's papule and signs; (2) proximal muscle weakness in the upper or lower extremities; (3) muscle pain and tenderness; (4) elevation of serum myogenic enzymes such as creatine kinase; (5) myopathic changes in electromyography; (6) arthritic inflammation or pain without bone erosion; (7) symptoms of systemic inflammation such as fever, elevation of C-reactive protein, and erythrocyte sedimentation rate; (8) serum Jo-1 antibody; (9) myopathic changes in muscle biopsy; and (10) interstitial pneumonitis. According to the Japanese PM/DM criteria, patients presenting with more than 4 items among items 2–9 can be diagnosed with PM; furthermore, a patient meeting the criteria for PM plus item 1 can be diagnosed with DM [9].

The registration form requires information about treatments and their effects as well as complications. Treatments included corticosteroids, non-steroid anti-inflammatory drugs (NSAIDs), corticosteroid pulse, immunosuppressants, and plasma exchange. Complications included infections, gastrointestinal ulcers, diabetes mellitus, hypertension, compression fractures, bone necrosis, myocardial infarction, cerebral infarction, malignant neoplasms, and disseminated intravascular coagulation syndrome. To compare the proportions of complications in between the newly registered patients and the renewed patients, we estimated their prevalence by using chi-square test with the significance level of 0.05.

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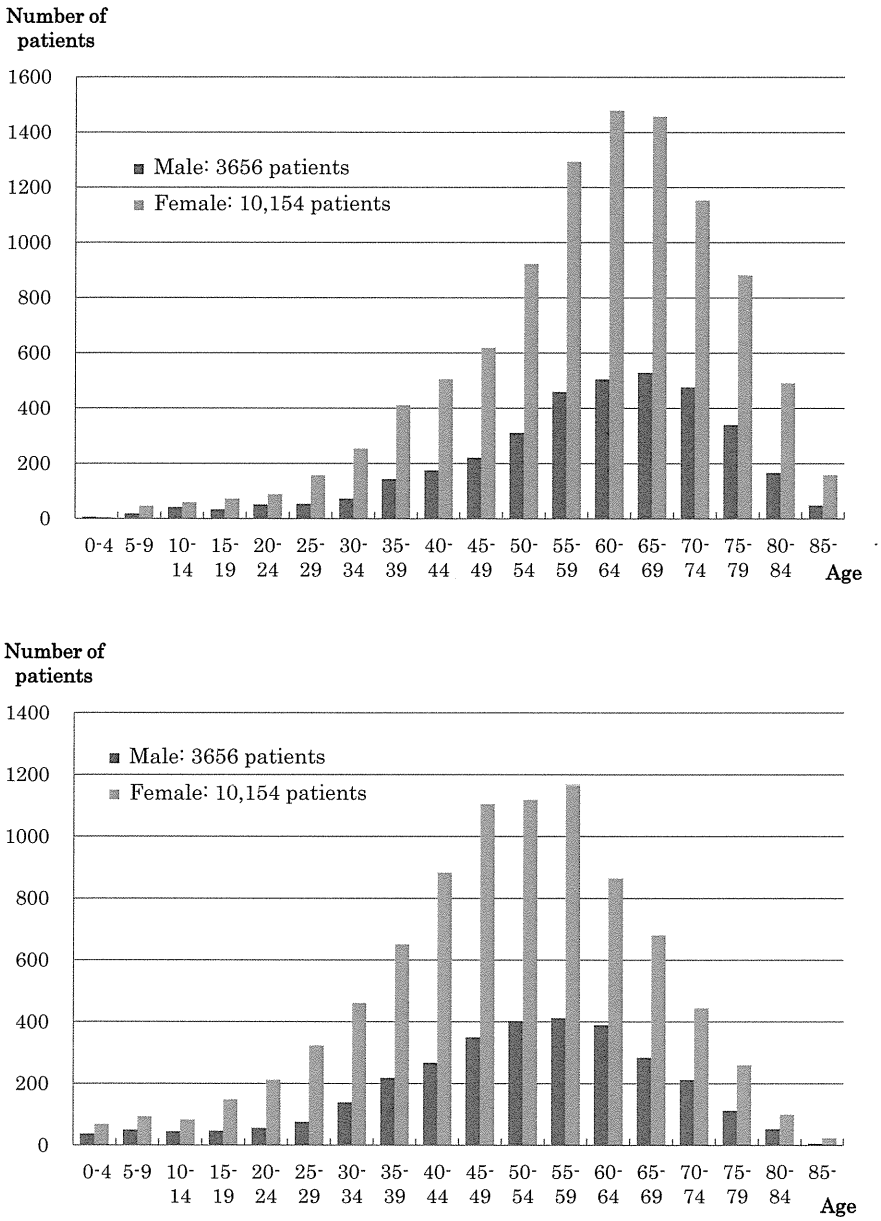

Figure 1. (A) There were a total of 13,710 registered PM/DM patients in 2009 (3656 men and 10,054 women). Female patients were predominant in all age groups, and the male/female ratio was 1:2.7. (B) Two-thirds of PM/DM patients developed symptoms from 40 to 60 years of age. About 30% and 3% of patients experienced onset at >60 and <15 years of age, respectively.

Every year, around 40–80% of registered patients' data are digitized. With permission from the MHLW of Japan, we analyzed the electronic data from fiscal years (i.e. April–March) 2003–2010 [7]. As 80% of the data were digitized in 2009, the present study analyzed the 2009 data to evaluate the clinical features of PM/DM patients in Japan.

Results

We previously reported the prevalence and incidence of PM/DM in Japan [6,7]. Here, we present the detailed clinical features in the same Japanese PM/DM cohort [7]. There were approximately 17,000 patients with PM/DM in Japan in 2009, and approximately new 1500 PM/DM patients were registered in this year. The patients with PM and DM in the newly registered patients can be defined by the presence or absence of cutaneous findings. If we defined in the newly registered patients in 2009 that PM is all of three symptoms in the item 1 (i.e. cutaneous findings) negative patient, and that DM is one or more symptoms in the item 1 positive patient, the numbers of PM and DM were 647 and 773, respectively. Furthermore, the number of the patient that could not differentiated as DM or PM because some of the three cutaneous findings are not mentioned was 39 (data not shown). The patients in all age groups were predominantly female, and the overall male/female ratio about 1:2.7 (Figure 1A). Two-thirds of PM/DM patients developed their symptoms in middle age (i.e. 40–60 years). Meanwhile, 30% and 3% of patients experienced disease onset at >60 and <15 years of age, respectively (Figure 1B).

The clinical features of the PM/DM patients newly registered in 2009 are shown in Table 1. Frequent clinical observations in the newly registered patients included proximal muscle weakness in the upper or lower extremities, muscle pain or tenderness, and elevation of serum myogenic enzymes. Half of the patients presented with some inflammation and interstitial pneumonitis.

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Table 1. Clinical features of the newly registered and renewed PM/DM patients in 2009.

	Clinical features	Newly registered ($n = 1459$)								Renewed ($n = 12,251$)							
		(+)		(−)		Unknown		Defect		(+)		(−)		Unknown		Defect	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1	Dermatitis																
	a. Heliotrope eruption	441	30.2	971	66.6	34	2.3	13	0.9	1390	11.3	9903	80.8	71	0.6	887	7.2
	b. Gottron's papules	610	41.8	803	55	31	2.1	15	1	1897	15.5	9370	76.5	85	0.7	899	7.3
	c. Erythema on extensor surface of extremities	606	41.5	807	55.3	38	2.6	18	1.2	2127	17.4	9158	74.8	59	0.5	907	7.4
2	Proximal muscle weakness in upper or lower extremities	1280	87.7	163	11.2	10	0.7	6	0.4	6762	55.2	4565	37.3	41	0.3	883	7.2
3	Muscle pain or tenderness	1076	73.7	365	25	8	0.5	10	0.7	4282	35	6985	57	54	0.4	930	7.6
4	Elevation of serum myogenic enzymes	1248	85.5	175	12	4	0.3	32	2.2	3282	26.8	7644	62.4	234	1.9	1091	8.9
5	Myopathic changes in electromyography	647	44.3	160	11	631	43.2	21	1.4	1447	11.8	1572	12.8	8,051	65.7	1181	9.6
6	Arthritic inflammation or pain without bone erosion	619	42.4	728	49.9	83	5.7	29	2	3194	26.1	7205	58.8	788	6.4	1064	8.7
7	Symptoms of systemic inflammation																
	a. Fever	589	40.4	766	52.5	76	5.2	28	1.9	663	5.4	10,349	84.5	243	2	996	8.1
	b. Elevation of C-reactive protein	852	58.4	581	39.8	–	–	26	1.8	2329	19	8871	72.4	–	–	1051	8.6
	c. Increased erythrocyte sedimentation rate	782	53.6	430	29.5	–	–	247	16.9	2053	16.8	7689	62.8	–	–	2507	20.5
8	Serum Jo-1 antibody	210	14.4	1118	76.6	110	7.5	21	1.4	953	7.8	6515	53.2	3687	30.1	1096	9
9	Myopathic changes in muscle biopsy	544	37.3	132	9	743	50.9	40	2.7	2349	19.2	1093	8.9	7622	62.2	1187	9.7
10	Interstitial pneumonia	736	50.4	708	48.5	–	–	15	1	4538	37	6751	55.1	–	–	962	7.9

On the other hand, less than half of patients underwent electromyopraphy or muscle biopsy. The clinical features of renewed PM/DM patients in 2009 are shown in Table 1. Over half of the patients (55.2%) had residual proximal muscle weakness in the upper or lower extremities, 35% suffered from muscle pain and tenderness, and 37% suffered from interstitial pneumonitis.

The effectiveness of medical treatments in newly registered PM/DM patients in 2009 is shown in Table 2. Almost all patients (92.3%) received corticosteroid therapy, which ameliorated symptoms in 67.6% of them. Corticosteroid pulse therapy was administered to 24.7% of patients, which ameliorated symptoms in 65.6% of them. Moreover, 24.9% of patients received some immunosuppressants, which improved symptoms in 54.8% of them. The effectiveness of medical treatments during the course PM/DM in the renewed patients is summarized in Table 2. In corticosteroid therapy, 90% of renewed patients, which improved symptoms in 94.9% of them. Furthermore, 40.3% of patients required immunosuppressants, which ameliorated symptoms in 86.8% of them.

The complications in the newly registered and renewed patients in 2009 are summarized in Table 3. In the early phase, 16.7%, 13%, and 10.1% of newly registered PM/DM patients were complicated with hypertension, diabetes mellitus, and malignant neoplasms, respectively. On the other hand, the renewed patients had greater proportions of complications than the newly registered patients, including hypertension (27.7% vs. 16.7%), diabetes mellitus (21.8% vs. 13%), infections (12.9% vs. 6%), gastrointestinal ulcers (6.2% vs. 2.9%), compression fractures (12% vs. 2.3%), bone necrosis (5% vs. 0.6%), and cerebral infarction (2.7% vs. 1.6%). On the other hand, few renewed patients had malignant neoplasms (6.1% vs. 10.1%). From the results of statistical analysis, the prevalence of myocardial infarction and DIC did not show significant difference between newly registered and renewed PM/DM patients.

Discussion

This report is a follow-up of our previous studies on a Japanese PM/DM cohort [6,7], disclosing the clinical characteristics of PM/DM patients in Japan. The present results are the most up-to-date basic epidemiologic data of PM/DM patients in Japan. The total number of PM/DM patients in Japan in 2009 was estimated to be 17,000. This number represents a 2.8-fold increase compared with approximately 6000 patients in the 1991 PM/DM survey [8]. Moreover, in the newly registered patients in 2009, the number of DM was a little larger than the number of PM. Female patients were predominant: the male/female ratio was 1:2.7. Two-thirds of PM/DM patients developed symptoms in middle age (40–60 years), while no more than 30% experienced elderly onset (>60 years).

Table 1 shows the proportions of each clinical symptom, which are clinical findings defined according to the Japanese PM/DM criteria. Proximal muscle weakness in the upper or lower extremities, muscle pain or tenderness, and elevation of serum myogenic enzymes were frequently observed in newly registered PM/DM patients; thus these symptoms can be thought of as the initial presenting symptoms of PM/DM. Although the degree of progression of interstitial pneumonitis is uncertain in some cases, 50.4% of newly registered patients were accompanied by interstitial pneumonitis.

Meanwhile, 44.3% and 37.3% of newly registered patients presented with myopathic changes on electromyography and muscle biopsy. Bohan and Peter's diagnostic criteria for PM/DM require myopathic changes on both electromyography and muscle biopsy [10]. Thus, more than half of the newly registered patients in this study would not be diagnosed with PM/DM according to

Table 2. Therapeutic effectiveness in newly registered and renewed PM/DM patients in 2009.

	Newly registered ($n = 1459$)								Renewed ($n = 12,251$)							
	(+)		(−)		Unknown		Defect		(+)		(−)		Unknown		Defect	
Treatments	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Corticosteroid	1347	92.3	99	6.8	–	–	13	0.9	11,029	90	276	2.3	–	–	946	7.7
Effectiveness	910	67.6	17	1.3	344	25.5	76	5.6	10,464	94.9	66	0.6	129	1.2	370	3.4
NSAIDs	212	14.5	1190	81.6	–	–	57	3.9	2830	23.1	7909	64.6	–	–	1512	12.3
Effectiveness	134	63.2	33	15.6	27	12.7	18	8.5	2490	88	65	2.3	145	5.1	130	4.6
Immunosuppressants	363	24.9	1054	72.2	–	–	42	2.9	4942	40.3	5988	48.9	–	–	1321	10.8
Effectiveness	199	54.8	6	1.7	120	33.1	38	10.5	4291	86.8	124	2.5	239	4.8	288	5.8
Corticosteroid pulse	360	24.7	1055	72.3	–	–	44	3	2806	22.9	8010	65.4	–	–	1435	11.7
Effectiveness	236	65.6	5	1.4	82	22.8	37	10.3	2495	88.9	39	1.4	54	1.9	218	7.8
Plasma exchange	5	0.3	1393	95.5	–	–	61	4.2	104	0.8	10,583	86.4	–	–	1564	12.8
Effectiveness	3	60	0	0	2	40	0	0	69	66.4	6	5.8	10	9.6	19	18.3
Other therapies	146	10	1146	78.5	–	–	167	11.5	2002	16.3	8242	67.3	–	–	2007	16.4
Effectiveness	81	55.5	6	4.1	35	24	24	16.4	1602	80	45	2.3	184	9.2	171	8.5

Table 3. Complications in newly registered and renewed PM/DM patients in 2009.

	$n = 1459$								$n = 12,251$								
	(+)		(−)		Unknown		Defect		(+)		(−)		Unknown		Defect		
Complications	*N*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*p* Values*
Infections	87	6	1333	91.4	24	1.6	15	1	1575	12.9	9708	79.2	88	0.7	880	7.2	$p<0.0001$
Gastrointestinal ulcers	43	2.9	1322	90.6	74	5.1	20	1.4	758	6.2	10,360	84.6	251	2	882	7.2	$p<0.0001$
Diabetes mellitus	190	13	1231	84.4	21	1.4	17	1.2	2671	21.8	8663	70.7	48	0.4	869	7.1	$p<0.0001$
Hypertension	244	16.7	1189	81.5	11	0.8	15	1	3391	27.7	7948	64.9	40	0.3	872	7.1	$p<0.0001$
Compression fractures	34	2.3	1383	94.8	26	1.8	16	1.1	1466	12	9750	79.6	164	1.3	871	7.1	$p<0.0001$
Bone necrosis	9	0.6	1401	96	32	2.2	17	1.2	612	5	10,436	85.2	309	2.5	894	7.3	$p<0.0001$
Myocardial infarction	20	1.4	1405	96.3	13	0.9	21	1.4	184	1.5	11,127	90.8	49	0.4	891	7.3	$p=0.6026$
Cerebral infarction	24	1.6	1398	95.8	16	1.1	21	1.4	329	2.7	10,963	89.5	61	0.5	898	7.3	$p=0.0103$
Malignant neoplasms	148	10.1	1119	76.7	161	11	31	2.1	744	6.1	10,412	85	212	1.7	883	7.2	$p<0.0001$
DIC	8	0.5	1406	96.4	21	1.4	24	1.6	30	0.2	11,283	92.1	36	0.3	902	7.4	$p=0.0901$
Others	167	11.4	975	66.8	28	1.9	289	19.8	2437	19.9	7812	63.8	165	1.3	1837	15	$p<0.0001$

*p Values were based on chi-square test, the significance level of 0.0.5.

the criteria. On the other hand, the Japanese PM/DM diagnostic criteria do not always require electromyological and pathological muscle evaluation. Indeed, the specificity of these Japanese criteria was 95.2% [9] and these two examination methods are invasive, but physiologically and pathologically evaluating muscle condition is important. This is a problem that should be resolved in order to ensure the diagnosis of PM/DM in Japan.

Regarding treatments, almost all patients received corticosteroids. Corticosteroids only ameliorated symptoms in 67.6% of patients in the early phase, whereas 94.9% of patients responded to corticosteroids during their overall clinical course. As other therapies such as immunosuppressants can be administered, the results indicate almost all Japanese PM/DM patients can achieve symptomatic amelioration. This therapeutic result is much better than previously reported results [5,11]. This encouraging result may be because the study analyzed PM/DM survivors, because only current patients were enrolled in the database; therefore, the data of deceased patients or patients not responding to therapy were not included, showing better therapeutic results. The differences of their doctor's evaluating method for judging the disease severity and effectiveness of the therapies might also be another reason of showing better therapeutic results in this study. On the other hand, half of the patients suffered from sequelae such as muscle weakness and interstitial pneumonitis despite responding well to therapy. Therefore, more effective therapeutic guidelines and standard evaluating methods for the disease severity must be established in order to maintain better patient condition.

Regarding complications, 10.1% of newly registered patients were accompanied by malignant neoplasms. Meanwhile, the renewed patients were often accompanied by infections, gastrointestinal ulcers, diabetes mellitus, hypertension, compression fractures, and bone necrosis during their clinical course. These complications are probably a result of corticosteroid therapy, because long-term corticosteroid use frequently induces these complications. On the other hand, the proportions of neoplasms and arteriosclerotic diseases such as myocardial and cerebral infarction were relatively low. Again, this is likely because only survivors were analyzed; moreover, patients with severe conditions might tend to withdraw from this registration system.

This study reports the basic epidemiologic characteristics of Japanese PM/DM patients, including numbers, major symptoms, diagnostic methods, therapies and their effectiveness, and complications. These findings are important for guiding medical and governmental strategies to control and prevent these diseases. However, this study has some limitations. One of them is that not all the patients with PM/DM in Japan registered this system. A main purpose of this registration for the patients is to receive subsidized medical care, so PM/DM patients who have minor symptoms and need not any treatments might not register this system. Moreover, affluent patients and the patients receiving other financial medical supports such as the children might not register the system, either. There seems many children receiving the supports in Japan might be a reason why this analysis showed no peak of the onset in children with DM. Furthermore, other limitations mentioned above are that this registration system only included the survivors and that the evaluating methods for the disease severity were not definite. Despite these limitations, a major strength of the present study is the very large sample size.

Regardless, the results should be interpreted cautiously. A prospective nationwide survey to investigate the prognosis, medical effectiveness, and complications of PM/DM in Japan is required to confirm the present results.

Conflict of interest

This study was supported by a Grant from the Ministry of Health, Labour and Welfare of Japan, for Research Committee on Autoimmune Diseases. None.

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The role of PSMB9 up-regulated by interferon signature in the pathophysiology of cutaneous lesions of dermatomyositis and lupus erythematosus

2) The running head: PSMB9 up-regulation in DM and LE skin

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9) What's already known about this topic?

- Dermatomyositis and systemic lupus erythematosus have common skin features, including dermal mucin deposition and interferon signature.

What does this study add?

- PSMB9 and versican V1, a core protein for glycosaminoglycan, were up-regulated while type I collagen was down-regulated in both DM and SLE skin.
- Interferon signature reduces collagen in dermal fibroblasts of DM and SLE skin, while PSMB9 overexpressed by interferon induces TGF-β2/β3 in epidermal keratinocytes, which results in the versican overexpression in dermal fibroblasts.
- The TGF-β2-ΔDiHS-diS1 pathway is the specific molecular changes to DM skin.

Summary

Background

Dermatomyositis (DM) and systemic lupus erythematosus (SLE) have common skin features, including dermal mucin deposition and interferon signature, although their roles are unknown.

Objective

To identify common or specific molecular changes in DM and SLE skin.

Methods

Proteomic analysis was performed utilizing DM and normal subject (NS) skin. Glycosaminoglycans were analyzed by high-performance liquid chromatography.

Results

The expressions of 61 proteins were upregulated or downregulated in DM skin compared to NS skin in the proteomic analysis. Among those proteins, PSMB9, an immunoproteasome subunit, was up-regulated in the epidermis of DM and SLE, but not in other skin diseases. Furthermore, versican V1, a core protein for glycosaminoglycans, was upregulated while type I collagen was downregulated in the dermis of DM and SLE. Interferon stimulated PSMB9 expression in cultured keratinocytes and reduced collagen expression in dermal fibroblasts, but did not affected on versican expression. The PSMB9 knockdown in keratinocytes led to the significant suppression of TGF-β2 and TGF-β3, inducers of versican synthesis. TGF-β3 expression was upregulated in both DM and SLE, while TGF-β2 expression was increased only in the DM epidermis. ΔDiHS-diS1, a component of heparan sulfate, was significantly increased only in DM. TGF-β2 expression significantly increased the ΔDiHS-diS1 expression in dermal fibroblasts in vitro.

Conclusions

The interferon signature in DM and SLE skin reduces collagen in dermal fibroblasts, whereas overexpression of PSMB9 induced by interferon stimulates versican inducers in epidermal keratinocytes. In addition, the TGF-β2-ΔDiHS-diS1 pathway may be the specific molecular change of the skin with DM.

Introduction

Dermatomyositis (DM) and systemic lupus erythematosus (SLE) are autoimmune disorders characterised by acute or chronic inflammation. They have several common features in the skin, including erythematous eruptions, photosensitivity and the Köbner phenomenon.[1-3] Recent studies have indicated that the gene expression profiles in DM and SLE patients are remarkably similar. For example, interferon (IFN)-related genes were reported to be overexpressed in the skin, muscle, and peripheral blood of DM and SLE patients.[4,5] Although IFN may be a key signal in the pathogeneses of both DM and SLE skin, the specific targets of the signaling have yet to be elucidated.

The histopathological findings from skin with DM and SLE include liquefaction degeneration of basal layer, mild infiltration of lymphocytes, and dermal mucin deposition,[6,7] but these findings are not specific to these diseases. Glycosaminoglycans (GAGs), histologically known as dermal mucin, usually exist by binding with core proteins, and the resulting complex is called proteoglycan. Excess mucin deposition in the skin with DM and SLE is speculated to be caused by increased GAGs production by dermal fibroblasts after some immunological stimulation or by decreased hyaluronidase-mediated resorption.[8] However, the detailed mechanism is still unknown.

Accordingly, DM and SLE are sometimes difficult to clinically and histopathologically distinguish in terms of cutaneous eruptions. Several previous reports attempted to identify specific changes in each disease, including microvasculopathy or immunocomplex deposition, but such candidates have not been clinically utilised. Compared with the many research studies that used muscle tissues, the kidney, or peripheral blood,[5,9,10] the number of researches that examined the skin of DM or SLE patients is limited. The aim of present study was to identify common molecular changes of the skin with DM and SLE, or specific molecular changes of the skin with DM, especially focusing on IFN signaling and mucin deposition.

Materials and Methods

Patients

Skin specimens were obtained from the involved skin of 25 DM, 20 SLE, 3 psoriasis (PSO), 3 atopic dermatitis (AD), 10 lichen planus (LP) and 10 erythema exsudativum multiforme (EEM) patients. Control skin samples were obtained from the routinely discarded skin of 15 normal subjects (NS) undergoing skin graft.

The DM patients were diagnosed based on the criteria proposed by Bohan and Peter.[11-13] Patients with SLE fulfilled the criteria proposed by the American College of Rheumatology (ACR).[14] We randomly and retrospectively collected samples from patients who visited our department from April 2008 to March 2014. Among various cutaneous lesions of DM or SLE, erythematous eruptions mainly caused by interface dermatitis were included in this study, and samples with panniculitis only without erythema were excluded. This study was approved by the Ethics Review Committee in Kumamoto University (No. 1452). Written informed consent was obtained according to the Declaration of Helsinki.

Liquid chromatography-tandem mass spectrometry-based quantitative proteomic analysis

Analysis of liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed in accordance with the protocol provided by iTRAQ® analysis service (Filgen, Nagoya, Japan). Proteins were extracted from each skin sample utilising T-PER tissue protein extraction reagent (Pierce, Rockford, IL, U.S.A.) with protease inhibitor cocktail (Sigma-Aldrich St. Louis, MO, U.S.A.). The extracted proteins were condensed, reductive alkylated, and digested with trypsin. The peptides were desalted by Sep-Pak® light C18 cartridge (Waters, Milford, MA, U.S.A.) and were labelled with iTRAQ reagent-mutiplex assay kit (AB Sciex). The labelled peptides from each sample were mixed and separated into 8 fractions using caution exchange buffer pack (AB Sciex). The mass spectrometric analysis was performed in the AB Sciex triple TOF 5600 system and DiNa system. The values were normalized via global normalization based on the measured median ratios.[15]

RNA isolation and quantitative real-time polymerase chain reaction

RNA extraction and quantitative real-time polymerase chain reaction (PCR) were performed as described previously.[16]

Immunoblotting

Immunoblotting was performed by using antibodies for proteasome subunit beta type 9 (PSMB9)/LMP2 (clone1c4, LSBio, Seattle, WA, U.S.A.), VCAN GAT (Origene, Rockville, MD, U.S.A.), collagen I Goat-UNLB (Southern Biotech, Birmingham, AL, U.S.A.) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).[17]

Measurement of collagen content

Collagen contents in paraffin-embedded skin sections were measured by quantitative micro-assay kit (Chondrex, Redmond, WA, U.S.A.) following the manufacturer's instructions.[18]

Immunohistochemical analysis

Immunohistochemistry was performed with primary antibodies for PSMB9, versican V1 or TGF-β2 (LSBio, Seattle, WA, U.S.A.).[19]

Cell cultures

Normal human epidermal keratinocytes, normal human dermal fibroblasts, and human immortalised keratinocytes cell line, HaCaT, were cultured as described previously.[19-21]

Transient transfection

siRNAs against PSMB9/LMP2 and control siRNA were purchased from Santa Cruz Biotechnology and Thermo, respectively. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, U.S.A.) was used as the transfection reagent.[22]

Quantification of GAGs

The quantification of GAGs was performed in accordance with the protocol provided by Glycoscience Laboratory (Tokyo, Japan). For the quantification of hyaluronic acid (HA), after degreasing, dehydration, and drying, each skin sample was digested by collagenase (Seikagaku Co, Tokyo, Japan) and actinase E (Kaken Seiyaku, Tokyo, Japan). Finally, the digests were boiled to extract HA. Then, HA polymer (Calbiochem, Cambridge, MA, U.S.A.) was added into each well of a 96-well amine-conjugated microtitre plate (Sumitomo Bakelite, Tokyo, Japan), followed by incubation with sodium cyanoborohydrate solution and diluted block ace solution (DS Pharma Biomedical, Osaka, Japan). Diluted samples were added into the wells followed by addition of biotinylated HA-binding protein solution (Hokudo, Sapporo,

Japan). After washing, samples were incubated with horseradish peroxidase (HRP)-conjugated streptavidin solution (Calbiochem) for 1 hour. TMB peroxidase substrate was added into each well, and incubated for 20 minutes. Absorbance at 450 nm was measured by using MTP-300 Microplate Reader (Corona Electric, Tokyo, Japan).

Next, for the quantification of chondroitin sulfate (CS), the samples were treated with hyaluronidase (Amano enzyme, Nagoya, Japan). High-performance liquid chromatography (HPLC) analysis was used to confirm that HA was completely removed by analysis. CS in each sample was then digested into disaccharides with chondroichinase AC2 (Seikagagu Co.). The digests were collected via centrifuge and analysed by HPLC analysis.

Lastly, CS in samples was completely removed by chondroichinase ABC (Seikagagu Co.), which was confirmed by HPLC. Heparan sulfate (HS) in each sample was digested into disaccharides with hepatirinase (Seikagagu Co.) for the quantification of HS by HPLC.

Statistical analysis

Data presented as bar graphs are the means+standard deviation of at least three experiments. Statistical analysis was conducted by using the Mann-Whitney *U* test for the comparison of medians.

Results

Protein expression profiles in DM skin

First, quantitative proteomic analysis was performed utilising LC-MS/MS in order to determine whether molecules upregulated or downregulated in the skin with DM. Protein samples were obtained from 2 DM skin samples (DM1 and DM2) and 2 control skin samples of normal subjects (NS1 and NS2). The clinical features of the DM patients are shown in Supplemental Table. The NS samples were matched with the DM samples for age, gender, and biopsy site. The expression profiles of 3,268 proteins were compared between the DM and NS skin, and expression ratios >1.5 or $\leqq$0.67 were regarded as upregulated or downregulated, respectively. We identified 61 molecules whose expressions in both DM samples were upregulated or downregulated in comparison with both of the NS samples (Table 1). Twenty-seven of the 61 proteins were upregulated in DM skin, while 34 proteins were downregulated.

We selected 19 genes, which may be associated with the pathogenesis of DM (e.g., involved in immunity, antigen presentation, or IFN pathway), and their mRNA levels were

analysed by real-time PCR with an additional number of samples (randomly chosen 8 NS and 8 DM skin, Fig. 1a) to validate the LC-MS/MS analysis. Among the 19 genes, consistent with the LC-MS/MS results, versican variant 1 (versican V1), SPRR1B, THBS1, PSMB9, EIF1AX, and CTSL1 were significantly upregulated in DM skin compared to NS skin, whereas PIP, SPTAN1, COL1A2, COL1A1, and PI16 were significantly downregulated in DM skin. The biopsy sites (e.g., face, arm, hand), age, or gender did not affect the expression levels of these molecules.

Antibodies for aminoacyl-tRNA synthetase (ARS) have recently attracted attention as disease-specific/associated antibodies of myositis. However, the expressions of ARS-related genes including histidyl-tRNA synthetase (HARS) or tryptophanyl-tRNA synthetase (WARS) showed no significant differences between DM and NS based on real-time PCR (Fig. 1b) and LC-MS/MS results.

Expression of PSMB9, versican V1 and type I collagen in DM and SLE skin

Among these upregulated or downregulated molecules in DM skin, we first focused on PSMB9, an immunoproteasome subunit, because Nakajo-Nishimura syndrome, which is caused by PSMB8 mutations, demonstrates symptoms similar to those of DM and SLE (e.g., facial erythema, pernio-like eruptions, and myositis).[23] Real-time PCR using additional numbers of skin samples of DM (n=25), SLE (n=20) and NS (n=15) showed that PSMB9 mRNA levels were significantly upregulated in both DM and SLE skin compared to NS skin (Fig. 2a). Although we analysed the correlation between PSMB9 levels and biopsy sites (e.g., face, arm, hand), the types of cutaneous manifestations (e.g., heliotrope, Gottron's papule, malar rash, discoid lupus erythematosus), age, and gender, these factors did not significantly affect the PSMB9 levels. PSMB9 expression was not altered in PSO and AD, which were sometimes differential diagnoses of DM/SLE. Furthermore, to confirm the possibility that the PSMB9 upregulation was elicited by the pathophysiological mechanism of DM/SLE, samples of the other interface dermatitis were included, but PSMB9 mRNA levels in LP or EEM skin were not increased compared to NS skin. Thus, PSMB9 overexpression was rather specific to DM and SLE skin. PSMB8 mRNA levels tended to be elevated in DM and SLE skin, but no statistically significant differences were observed (Fig. 2b).

Next, we determined the levels of versican V1, a well-known core protein which binds with GAGs and forms proteoglycans. Dermal proteoglycan deposition is a common histopathological characteristic of DM and SLE skin. The mRNA levels of versican V1 were significantly upregulated in both DM and SLE skin compared to NS, AD and LP (Fig. 2c). In

contrast, we did not find any significant differences in the expression of versican V3, a variant that lacks GAGs attachment sites, or aggrecan, another known core protein, between DM and NS by real-time PCR (Fig. 1b).

Type I collagen is the most abundant extracellular matrix in the dermis of human skin and consists of COL1A1 and COL1A2. The mRNA levels of COL1A1 and COL1A2 were significantly downregulated in both DM and SLE skin compared to those in NS, AD and LP (Fig. 2d, e). Consistently, protein levels of type I collagen in vivo were decreased in both DM and SLE skin compared to NS skin and scleroderma skin, a positive control characterised by collagen overexpression (Fig. 2f). PSMB9 protein was increased in DM and SLE skin, but not scleroderma skin. We also performed quantitative analysis of collagen content in paraffin-embedded sections of randomly chosen 8 DM and 6 NS skin. A significant decrease in collagen level was observed in DM skin (Fig. 2g).

The localisation of increased PSMB9 and versican V1 in DM and SLE skin

To clarify the localisation of increased PSMB9 and versican V1 levels in DM and SLE skin, immunochemical staining of these molecules was performed utilizing skin specimens from DM (n=16), SLE (n=8) and NS (n=8). We found that PSMB9 expression was increased in the epidermis of both DM and SLE skin compared to NS skin (Fig. 3a). No apparent differences in PSMB9 staining of infiltrated cells were found between the dermis of DM, SLE or NS. We also tested other inflammatory diseases, including PSO, AD, LP, and EEM as well as vasculitis, lymphoma, and sarcoidosis, but PSMB9 expression was not increased in the epidermis of these diseases (data not shown). On the other hand, versican V1 expression was found between the collagen bundles of the dermis in DM and SLE skin, but not in NS skin (Fig. 3b).

Effects of IFN on the expression of PSMB9, collagen and versican V1

Our results suggested that PSMB9 was increased in the epidermis of DM and SLE, while type I collagen was decreased and versican V1 was increased in their dermis. Next, we determined whether these changes in DM and SLE skin were caused by IFN signaling. Immunoblotting showed that IFN-α and IFN-γ induced PSMB9 expression in cultured epidermal keratinocytes (Fig. 3c). Furthermore, IFN-α and IFN-γ reduced type I collagen expression in cultured dermal fibroblasts, while they did not affect versican V1 expression.