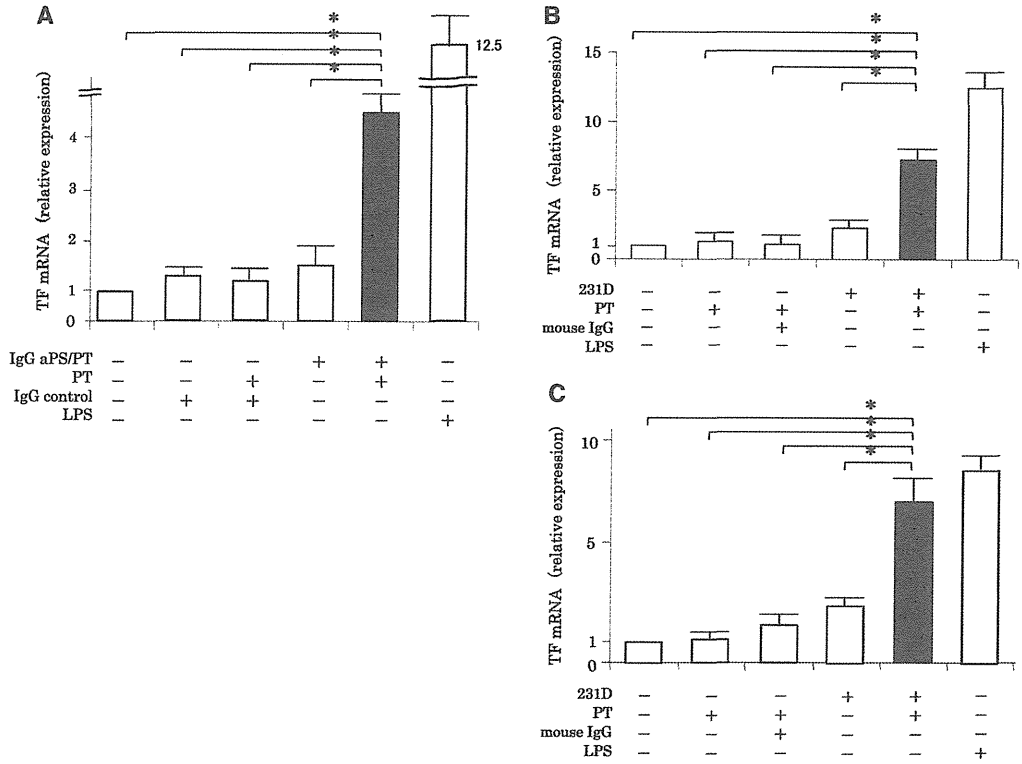
FIG. 1 Upregulation of TF mRNA expression by aPS/PT.



RAW264.7 cells or PBMCs obtained from normal healthy controls were exposed to the substances described below for 5 h. IgG extracted from APS patients positive for aPS/PT (IgG aPS/PT) or IgG extracted from healthy controls (IgG control) was added at 500 μg/ml. Prothrombin (PT), 231D and mouse control IgG (mouse IgG) were added at a concentration of 10 μg/ml and LPS was added at a concentration of 100 mg/ml. *$P < 0.005$. Vertical axes represent the relative expression levels of TF mRNA determined by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. (**A**) The relative TF mRNA expression levels in PBMCs treated with an IgG fraction from aPS/PT-positive patients or an IgG fraction from healthy controls. (**B**) Relative TF mRNA expression levels in PBMCs treated with 231D or mouse control IgG. (**C**) The relative TF mRNA expression levels in RAW264.7 cells treated with 231D or mouse control IgG were measured.

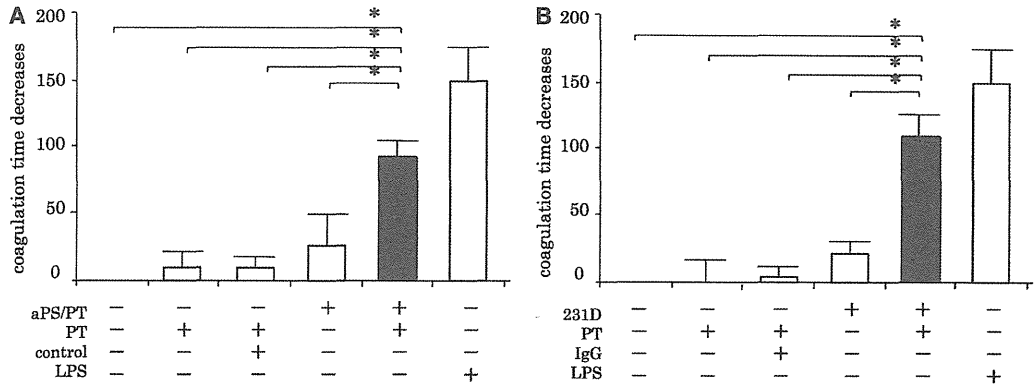
prothrombin (PT + 231D *vs* baseline, 231D alone and PT + control IgG: 2.5 ± 0.7 *vs* 1, 1.2 ± 0.3 and 1.31 ± 0.24; all, $P < 0.005$). The p38-specific inhibitor SB203580 significantly reduced TF mRNA overexpression in 231D-treated HUVECs [SB203580 (+) *vs* SB203580 (–): 1.4 ± 0.2 *vs* 2.5 ± 0.7; $P < 0.005$]; however, its inactive analogue SB202474 did not affect TF mRNA overexpression.

The expression levels of vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PCAM-1) and endothelin-1 mRNA were significantly upregulated 2- to 3-fold in HUVECs treated with 231D in the presence of prothrombin. However, in the absence of prothrombin, 231D did not affect the expression of these adhesion molecules (PT + 231D *vs* baseline, 231D alone and PT + control IgG; VCAM-1: 2.1 ± 0.6 *vs* 1, 1.4 ± 0.1 and 1.1 ± 0.2, PCAM-1: 2.8 ± 0.3 *vs* 1, 1.3 ± 0.0 and 1.8 ± 0.3, selectin: 2.0 ± 0.4 *vs* 1, 1.3 ± 0.2 and 1.1 ± 0.4; all, $P < 0.01$).

Discussion

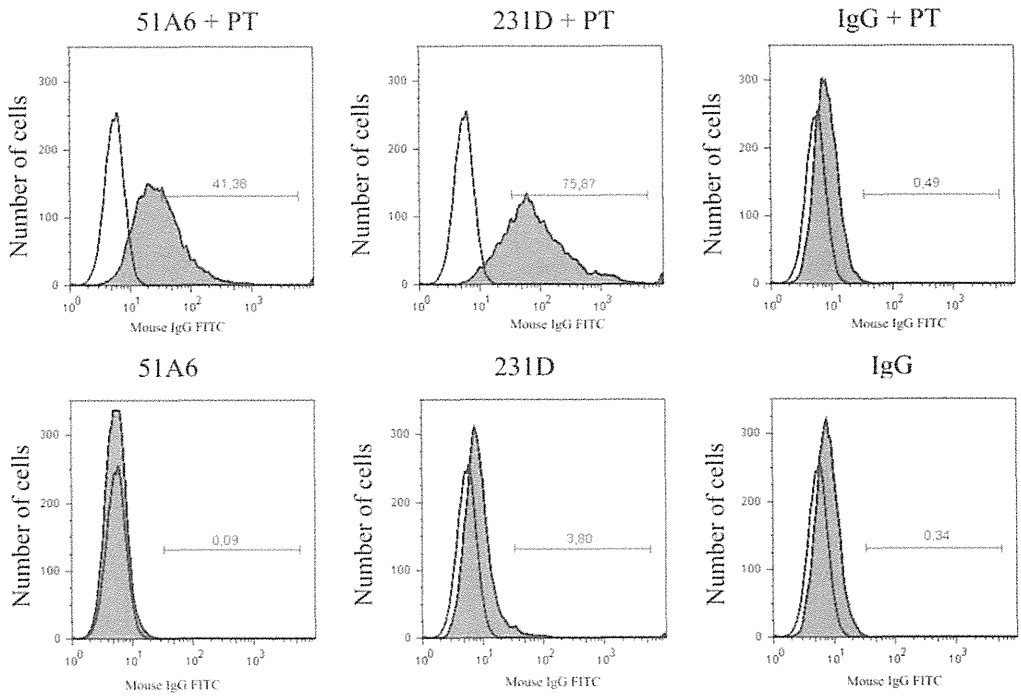
In this study we showed that IgG fractions and monoclonal aPS/PT induced TF in procoagulant cells. Monoclonal aPS/PT bound to monocytes using prothrombin as a cofactor for binding. Further, we demonstrated that treatment by aPS/PT induces the phosphorylation of p38 MAPK in these procoagulant cells. The coagulation process *in vivo* is complicated and various cells or molecules other than monocytes or endothelial cells are involved. Obviously there are certain limitations in discussing aPS/PT-induced thrombosis from the current results. However, our results showed that TF, the key protein in the coagulation pathway, is overproduced by its main sources in the circulation, monocytes and endothelial cells. Upregulation of blood-borne TF indicates increased procoagulant activity that is considered one of the most important characteristics of aPL-induced thrombosis.

FIG. 2 Procoagulant activity of cells treated with aPS/PT.



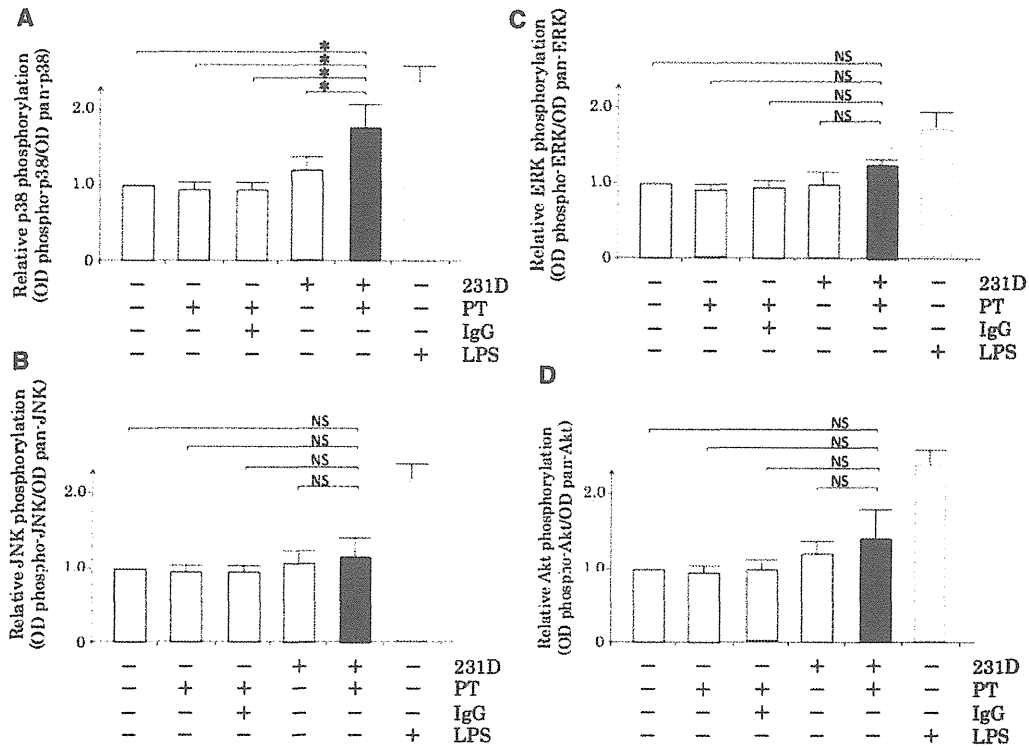
PBMCs obtained from normal healthy donors were exposed to the substances described below for 5 h. The cells were washed and then added to normal healthy plasma, and coagulation time was measured. The reduction in coagulation time was calculated by subtracting the coagulation time of each treated sample from that of unstimulated cells. The bars represent the mean ± S.E. of three independent experiments. *$P < 0.005$. (**A**) PBMCs treated with IgG fractions from patients positive for aPS/PT (aPS/PT) or IgG fraction from healthy controls (control) (500 μg/ml) in the presence or absence of prothrombin (PT). (**B**) PBMCs treated with 231D or mouse control IgG (IgG) (10 μg/ml).

FIG. 3 Binding of aPS/PT to the surface of RAW264.7 cells.



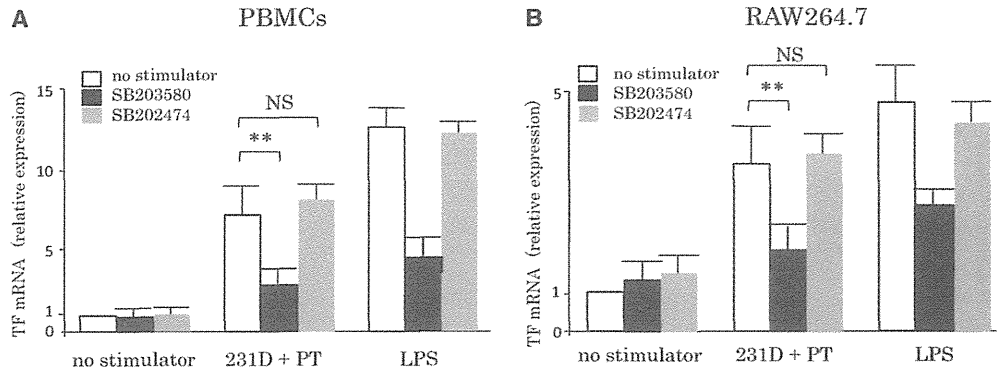
Murine monoclonal anti-prothrombin antibodies (51A6, 231D) and control murine IgG (IgG) were added to RAW264.7 cells at a concentration of 7.5 μg/ml with or without prothrombin (PT) (10 μg/ml), and then incubated for 4 h. After incubation, FITC-conjugated anti-mouse IgG antibody was added to the cell suspension and then analysed with a FACS analyzer. The vertical axes represent the number of cells and the horizontal axes represent the FITC fluorescence intensity.

FIG. 4 Quantitative analysis of serine-threonine kinase phosphorylation in aPS/PT-treated cells.



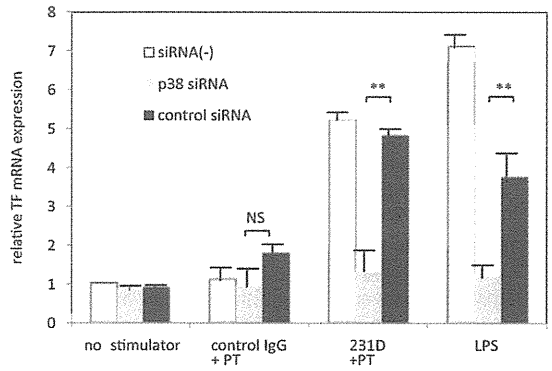
RAW264.7 cells were exposed to the substances described below for 15 min. Prothrombin (PT), 231D and mouse control IgG (IgG) were added at a concentration of 10 μg/ml and LPS was added at a concentration of 100 ng/ml. The relative OD ratio of each sample was measured and calculated as described in the Materials and methods section. $^{*}P < 0.005$, NS: not statistically significant.

FIG. 5 p38 MAPK inhibition assay in aPS/PT-treated cells.



PBMCs and the mouse monocyte cell line, RAW264.7, were exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. Cells were pretreated with the p38-specific inhibitor SB203580 (1 μM) or its inactive analogue SB202474 (1 μM) for 30 min followed by treatment with stimulators. Vertical axes represent the relative expression levels of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. $^{**}P < 0.001$, NS: not statistically significant. (**A**) The relative TF mRNA expression levels in PBMCs. (**B**) The relative TF mRNA expression levels in RAW264.7 cells.

FIG. 6 RNA interference of p38 MAPK in aPS/PT-treated cells.



PBMCs were pretreated for 72 h with siRNA of p38 MAPK or the control and subsequently exposed to stimulators for 5 h. Prothrombin (PT) and 231D were added at a concentration of 10 μg/ml and LPS was added at 100 ng/ml. The vertical axis represents the relative expression level of TF mRNA detected by real-time PCR. The bars represent the mean ± S.E. of three independent experiments. $^{**}P < 0.001$, NS: not statistically significant.

231D and 51A6 significantly bound to the membranes of monocytes in the presence of prothrombin. The binding of 231D to the cell surface was clearly stronger than that of 51A6. This observation was similar to our previous report that 231D had stronger binding to the PS/PT complex than 51A6 [13], suggesting that the monoclonal antibodies bind to prothrombin complexed with phosphatidylserine on the cell surface.

Our data suggest that TF production induced by aPS/PT in procoagulant cells is mainly induced via activation of the p38 MAPK pathway, which is similar to past reports showing that p38 MAPK was the main pathway of aCL/β_2GPI-induced cell activation. It is interesting that antibodies recognizing different proteins seem at least partially to utilize a common signalling pathway. Our findings are in agreement with the clinical observation that the manifestations of APS do not differ in patients with different antibody profiles.

Protein kinases are key regulators of cellular signalling, inflammation, cell differentiation and cell death. Thus they have been attractive targets for the treatment of neoplasms and inflammatory diseases [25–27].

p38 MAPK belongs to the MAPK signal protein family and is strongly activated by environmental stress or inflammatory cytokines such as TNF-α, IL-1β and IL-18 [28–30]. Consequently p38 MAPK activation is considered critical for physiological immune responses, and p38 MAPK dysfunction is related to the pathology of autoimmune diseases other than APS [31–33].

In the present study, phosphorylation of signal proteins, such as those in the MAPK protein family and serine/threonine kinases, was screened in aPS/PT-treated cells using a proteome array and major signals were quantitatively measured by ELISA tests. No proteins other than p38 MAPK were found to be phosphorylated. Further, specific p38 MAPK inhibitors or knockdown of p38 MAPK mRNA effectively inhibited procoagulant cell activation. Therefore p38 MAPK is suggested as a major signal protein for the activation of aPS/PT-induced procoagulant cells. Although a previous study showed that ERK activation was observed in cells treated with IgG fractions from APS patients [22], an ERK inhibitor did not abolish TF expression in procoagulant cells, suggesting that ERK does not play a major role in cell activation. We also did not detect the ERK phosphorylation in our aPS/PT-treated cells.

The two major aPLs, aCL/β_2GPI and aPS/PT, are suggested to activate procoagulant cells primarily through p38 MAPK phosphorylation, therefore inhibition of p38 MAPK appears to be a promising modality for the treatment of APS. Since p38 MAPK contributes to various cell activities, its non-specific inhibition might result in severe complications. In fact, clinical trials of p38 inhibitors for a variety of diseases have been carried out; however, most of the trials encountered several complications and were unsuccessful [34]. A more realistic and practical strategy would be to target a more specific molecule involved in the activation of aPL-induced procoagulant cells.

Some reports have demonstrated possible receptors for aCL/β_2GPI-induced cell activation on procoagulant cells. Annexin A2 is a receptor for tissue plasminogen activator and plasminogen that is found on the surface of ECs and monocytes, and on the brush-border membrane of placental syncytiotrophoblasts, all of which are recognized targets of pathogenic aPLs [35, 36]. Annexin A2 interacts with the β_2GPI-aCL/β_2GPI complex on EC and monocyte surfaces, mediating cell activation [37–39]. The involvement of annexin A2 in aPL-mediated pathogenic effects has been reported *in vitro* and *in vivo* [40, 41]. However, it is still not clear whether such a receptor is actually involved in cell activation because annexin A2 is not a transmembrane protein. Further, it has been proposed that activation of the signalling responses required another transmembrane adaptor protein(s) that associates with annexin A2 on the EC surface [42].

The Toll-like receptor (TLR) family, in particular, TLR-2 and TLR-4 [43–45], may also play a role in the interaction of the β_2GPI-aCL/β_2GPI complex [42]. Adhered β_2GPI interacts with TLR-4 and aCL/β_2GPI cross-links β_2GPI and the TLR-4 complex, eventually triggering the signalling cascade activation. Moreover, TLR-4 is the putative adaptor protein for annexin A2 [38].

Further investigations have shown that megalin/gp330 [46] and apolipoprotein E receptor 2′ [47–49] are putative receptors for aCL/β_2GPI. Recently we identified the gelsolin/integrin α5β1 complex as a novel receptor of aCL/β_2GPI [50].

In contrast to the intensive investigation of aCL/β_2GPI thrombogenicity, no data are available on the mechanism of aPS/PT-dependent procoagulant cell activation. It is not yet known if aCL/β_2GPI and aPS/PT have a common

cell surface receptor and upstream signals of p38 MAPK. However, we believe that our data are the first to show the critical pathway of the procoagulant state related to antibodies against prothrombin.

There are several reports showing the correlation between aPS/PT and APS-related pregnancy morbidity [51–53] that are subject to further investigation to clarify the molecular mechanism of the manifestation. Identification and comparison of the receptors for aPS/PT and aCL/β_2GPI will help elucidate the pathogenicity of aPLs and the mechanisms of APS pathology.

Rheumatology key messages

- Phosphatidylserine-dependent aPT induced TF expressions on procoagulant cells *in vitro*.
- Similar to aCL, phosphatidylserine-dependent aPT induced cell activation via the p38 MAPK pathway.
- Cell activation via the p38 MAPK pathway may partially explain the pathogenesis of APS thrombosis.

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ORIGINAL ARTICLE

Prevalence and incidence of polymyositis and dermatomyositis in Japan

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Abstract

Objectives. To estimate the number of patients with polymyositis/dermatomyositis (PM/DM) in Japan and the prevalence rate and incidence rate of the disease.
Methods. The electronic database in the nationwide registration system on intractable diseases from 2003 to 2010 was utilized to identify the number of prevalent and incident cases of PM/DM. The electronic data entry rate was used to establish the total number of registered cases.
Results. The estimated total number of patients with PM/DM and the prevalence rate in Japan in 2010 were 17,000 and 13.2 per 100,000 population, respectively. The prevalence of PM/DM ranged from 10 to 13 per 100,000 population with a trend toward increasing over time. The incidence of PM/DM was estimated within the range 10–13 per 1,000,000 person-years, except for 2003.
Conclusions. We report the prevalence and incidence of PM/DM recently in Japan for the first time at the nationwide population level. Because the prevalence seems to be increasing recently, continued monitoring of these epidemiologic features is required.

Keywords

Epidemiology, Incidence, Nationwide survey, Polymyositis/dermatomyositis (PM/DM), Prevalence

History

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Introduction

Polymyositis (PM) and dermatomyositis (DM) are chronic idiopathic inflammatory disorders, affecting the skeletal muscles, the skin and other organs. They are rare, but their chronic intractable nature has a significant impact on the utilization of medical care resources, the patients' activities of daily living, and their quality of life. The epidemiologic features of PM/DM, such as prevalence and incidence, are not well documented. In order to understand the clinical and public health importance and to plan for disease control and prevention, it is essential to estimate incidence and prevalence rates, and to know the total number affected in the population.

As PM/DM are rare diseases, only limited epidemiologic studies have been undertaken, and mostly the incidence investigated in Western countries [1–9]. Few studies have been conducted in Asian populations [10, 11]. Prevalence data on PM/DM are even more scarce [4, 11–13]. PM/DM incidence and prevalence reported in the literature have been estimated only in relatively small populations, and are likely to have correspondingly large variance. For rare diseases, epidemiological observations in large populations are required for accuracy. The incidence of PM/DM in Japan has not been estimated to date in any nationwide survey. The prevalence of PM/DM in Japan was estimated from a nationwide survey in 1991 [13]. However, no other reports on prevalence in Japan have appeared since. It is therefore worthwhile to estimate the recent incidence and prevalence of PM/DM in Japan at the nationwide population level.

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The National Program on Rare and Intractable Diseases was launched by the government in Japan in 1972 to promote research on a number of rare and intractable diseases [14]. It increased support for patients by subsidizing their health care expenditure and provides a nationwide registration system for diseases including PM/DM, systemic lupus erythematous, systemic sclerosis (SSc) and some other autoimmune diseases. In the present study, we used this database to estimate the total numbers of patients with PM/DM in Japan, and the prevalence and incidence rates of the disease.

Materials and methods

Data sources

The database in a nationwide registration system established by the Japanese government for patients with intractable diseases including PM/DM was utilized for estimating prevalence and incidence. Patients with PM/DM desiring a subsidy for their medical care, must apply for aid. If the application is accepted, the recipient is recorded in the registry. The duration of the subsidy is 1 year, effective from October to September of the following year. Renewed application and re-registration in the system are required every year, if the patients wish to continue to receive financial support. The details of the registration system have been described elsewhere [14]. Diagnoses of patients with PM/DM were established according to the standard criteria ordained by the Ministry of Health, Labour and Welfare (MHLW) of Japan [15]. Approximately 40–80% of registered patients' data were converted into electronic form, which we utilized to collect the characteristics of each patient. This electronic database has been available for epidemiological research since 2003.With permission from the MHLW, we used the data from Japanese fiscal years 2003 to 2010.

The fiscal year means from April to March of the following year. The electronic converted data indicate the year of disease onset for each patient.

Statistical analysis

We calculated the electronic data entry rate as the number of patients whose data were converted into electronic form divided by the total number of patients enrolled in the registration system for each fiscal year from 2003 to 2010. The latter information is reported in MHLW's Report on Public Health Administration and Services [16]. As the number of registered patients with PM/DM is reported together with SSc in the report, we added the number of patients with PM/DM and SSc together in the electronic converted data to give the electronic data entry rate. Thus, the electronic data entry rate is the number of PM/DM and SSc patients whose data were converted into electronic form divided by the number of all registered patients with PM/DM and SSc. Because we could not extract the electronic data entry rate exclusively for PM/DM, we assume that the data entry rate of PM/DM is not different from that of PM/DM and SSc combined. We also assume that both the entry rate of new recipients and annual renewals are the same, and also that they are identical with the rate of all patients (new and renewals summed together) in each year.

The number of PM/DM cases in each year to estimate prevalence was the number of PM/DM patients whose data were converted into electronic form divided by the electronic data entry rate.

The number of PM/DM cases to calculate incidence was estimated as follows: for the number each year, we used the onset year of the electronic converted patients. The number of patients initially registered and converted into electronic form in fiscal year-j and onset at year-i, Nij, divided by the electronic data entry rate in fiscal year-j are summed for each year-i to yield the number of incidence cases in year-i as the following equation shows:

$$Ai = \sum_{j=i-1}^{2010} \frac{Nij}{Pj}$$

i = onset year (i = 2003, 2004,..., 2010)

j = initially registered fiscal year (j = 2003, 2004,..., 2010)

$i \leq j$

Ai: number of incidence cases in year-i

Nij: number of patients initially registered and converted into electronic form in fiscal year-j and onset at year-i

Pj: electronic data entry rate in fiscal year-j

The Japanese census population in 2005 and 2010 and the estimated population in the other years are used as the denominator of the prevalence rate and incidence rate. All statistical analyses were performed with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

All data provided by the MHLW are anonymous, and researchers cannot access personal information about any of the patients.

Results

Table 1 shows the electronic data entry rate of PM/DM and SSc, and estimated number of patients with PM/DM in fiscal years 2003–2010. The number of patients with PM/DM and SSc whose data were converted into electronic form ranged from 16,388 to 33,309 each year, with the highest number in 2009. Dividing by the total number of registered patients with PM/DM and SSc enrolled in the registration system which was obtained from the MHLW's Report on Public Health Administration and Services, the electronic data entry rates were obtained. They ranged from 39% to 80%, highest in 2009. The number of registered patients with PM/DM whose data were converted into electronic form ranged from 6,328 to 13,710 which was divided by the entry rate to yield the prevalent number of patients each year. The estimated number of PM/DM patients in 2010 was 17,000, ranging in each year from 2003 to 2010 from 13,000 to 17,000, thus tending to increase over the years. These numbers can be considered to represent the total number of registered patients in the whole of Japan (population 127 million). The estimated prevalence of PM/DM in Japan in 2010 was thus 13.2 per 100,000 population. The prevalence of PM/DM ranged from 10 to 13 per 100,000 population over the years 2003–2010, increasing over time. Thus, prevalence increased 1.3-fold during this time.

The incidence of PM/DM in each year from 2003 to 2010 was estimated from the number of cases initially registered in that year, and converted into electronic form. The figures in Table 2 show the number of patients initially registered and electronically converted, excluding the cases with disease prior to 2003. The number of cases in each year is separated according to their year of onset and divided by the electronic data entry rate of that registration year to yield the estimated number of incidence cases in each year from 2003 to 2010. This ranged from 900 to 1,700 per year (Table 3). The incidence rate of PM/DM in Japan was estimated as ranging from 10 to 13 per 1,000,000 person-years (except for

Table 1. Electronic data entry rate for PM/DM and SSc (on June 2011), and estimated prevalence of PM/DM in fiscal years 2003–2010.

	PM/DM and SSc			Estimation of prevalence of PM/DM		
Fiscal year	No. of electronic entries of PM/DM and SSc patients[a]	Total No. of registered patients with PM/DM and SSc[b]	Electronic data entry rate[c]	No. of electronic entries of PM/DM patients[d]	Estimated number of patients with PM/DM[e]	Estimated prevalence (per 100,000 population)[f]
2003	20,162	31,829	0.633	8,332	13,163	10.3
2004	21,709	32,944	0.659	9,043	13,722	10.7
2005	22,057	34,592	0.638	9,327	14,619	11.4
2006	20,031	36,110	0.555	8,139	14,665	11.5
2007	16,388	37,975	0.432	6,328	14,648	11.5
2008	20,242	39,970	0.506	7,919	15,650	12.3
2009	33,309	41,648	0.800	13,710	17,138	13.4
2010	16,528	42,233	0.391	6,618	16,926	13.2

PM/DM, polymyositis/dermatomyositis; SSc, systemic sclerosis.

[a]The number of patients with PM/DM and SSc whose data were converted into electronic form.

[b]Total numbers of registered patients with PM/DM and SSc enrolled in the registration system, obtained from the Report on Public Health Administration and Services [16].

[c]a/b.

[d]The number of patients with PM/DM whose data were converted into electronic form.

[e]d/c.

[f]The Japanese census population in 2005 and 2010 and estimated population in the other years are used as the denominator of the prevalence rate.

Table 2. Number of initially registered patients with PM/DM whose data were converted into electronic form and whose year of onset year was 2003–2010.

Initially registered fiscal year	No. of patients
2003	338
2004	668
2005	921
2006	683
2007	715
2008	956
2009	1,240
2010	861
Total	6,382

PM/DM, polymyositis/dermatomyositis.

2003). The incidence rates in 2009 and 2010 are lower than in the other years.

Discussion

In this study, we estimated the number of patients with PM/DM in Japan, and also calculated prevalence and incidence of the disease based on the nationwide survey. We estimated the number of patients currently affected (e.g. prevalent cases) in Japan and its prevalence per 100,000 population. We also estimated the incidence of the disease in Japan at the nationwide population level for the first time. These results provide basic information for disease control and prevention and planning public health policy.

PM/DM are rare diseases and earlier reports on incidence and prevalence are limited. The reported incidence of PM/DM ranges from 2 to 10 per 1,000,000 person-years in different populations between the 1940s and the 1990s [1–11]. Furthermore, there is a trend toward increasing incidence over time also in these studies. Earlier prevalence data are also very limited. Estimates of prevalence from the USA [4] and Japan [11–13] range between 2.4 and 9.9 per 100,000 population. Different diagnostic and classification criteria were employed in these studies, partly explaining the diverse reported incidence and prevalence rates in these studies. Some reports determined incidence from retrospective hospital-based studies in which the true incidence of PM/DM may have been underestimated. Furthermore, as the number of patients included in most of these earlier studies was small, only 40–100, the estimates may be relatively unreliable. The increasing incidence over time may be due to an increased physician awareness of the disease, progress in diagnostic techniques or increased availability of tests and better medical records, but could also reflect a true increase in disease occurrence.

The estimated prevalence per 100,000 population of PM/DM in Japan was 10–13 in 2003–2010, tending to increase over the years. The incidence of PM/DM per 1,000,000 person-years was estimated as 10–13, except in 2003. The prevalence and incidence of PM/DM in our study are higher than in most previously reported estimates. These differences may be due to the lack of standardization of the diagnostic criteria employed, as well as different ethnicities of the patients. Easier access to medical care via the public insurance system and financial support program in Japan may be one more reason for the higher estimates in this country. Cases in this study were identified by the standard diagnostic criteria ordained by the MHLW of Japan [15]. The significance of our study depends on the clearly defined standard criteria and large scale population review. The estimated incidence is based on a large number of approximately 6,400 patients during 8 years (Table 2).

A comparable total number of PM/DM patients in Japan are also available from a nationwide survey which accumulated number of patients reported from random-sampled hospitals from all over Japan, although the methodology of that survey was different from our own. The total number of patients with PM/DM in Japan was estimated at 3,000 (95% confidence interval 2,800–3,300) for PM and the same number (95% confidence interval 2,800–3,200) for DM, in 1991 [13]. Our estimate for PM/DM in Japan was 17,000 in 2010. This implies an increase of 2.8-fold (17,000/6,000) between 1991 and 2010. Our study also determined an increase in PM/DM prevalence over time. In addition to the above-mentioned factors which manifest as apparent increases in incidence rate, such as the use of different diagnostic criteria and physician awareness of the disease, better prognosis as result of improved treatment might also account for this trend.

Our estimated incidence values in 2009 and 2010 are slightly lower than 2008. This could be an underestimate because cases which would be registered from 2011 onward were not included.

There are some limitations to this study. First, there may be a possible bias due to the use of data from the Japanese government's registration system. Regarding the accuracy of the data we used, some degree of over- and/or underdiagnosis may exist, although specialist committees organized in each prefectural government check the diagnoses according to the standard criteria ordained by the government. With respect to coverage of the patients, most are expected to be diagnosed and registered in this system, but clearly we cannot be sure of the number of omissions. Patients who do not need financial support, some pediatric patients and patients whose medical expenses are covered by local government in an alternative financial support system will not apply to the system we used and thus be missing from the database. Thus, our study may underestimate prevalence. Second, we could not distinguish between PM and DM prevalence and incidence separately. This is because the national registration system combines these two diseases for administrative reasons, even though they are subgrouped

Table 3. Estimated number of incidence cases and incidence rate of PM/DM by onset year.

	Initially registered fiscal year									
Onset year	2003	2004	2005	2006	2007	2008	2009	2010	Total[a]	Incidence per 1,000,000 person-years[b]
2003	466	209	69	40	23	32	23	3	865	6.8
2004	68	750	260	63	60	26	21	15	1,263	9.9
2005		55	1,016	204	63	61	25	49	1,473	11.5
2006			99	886	301	99	46	46	1,477	11.6
2007				38	1,146	356	78	61	1,679	13.1
2008					63	1,253	290	110	1,716	13.4
2009						63	1,026	442	1,531	12.0
2010							41	1,476	1,517	11.8

PM/DM, polymyositis/dermatomyositis.

[a]Estimated number of incidence cases.

[b]The Japanese census population in 2005 and 2010 and estimated population in the other years are used as the denominator of the incidence rate.

based on distinct clinical and pathologic features. If we use the information of the skin manifestation of all the patients, PM and DM could be separated. Although the skin information of some proportion of patients is not well described in the database, further consideration may solve the problem in further study.

In conclusion, we provide estimates of the recent prevalence of PM/DM, and the incidence of the disease in Japan for the first time at the nationwide population level. As prevalence has been showing an increasing trend recently, a continuing concern must be to maintain monitoring of these epidemiologic features.

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Conflict of interest

H. Kohsaka has served as a consultant to Chugai Pharmaceutical and has received research grants from Eisai Pharmaceutical and Takeda Pharamaceutical. All other authors have declared no conflicts of interest.

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ORIGINAL ARTICLE

Serum levels of tenascin-C in collagen diseases

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ABSTRACT

Tenascins are a family of large multimetric extracellular matrix (ECM) proteins. Among them, large molecular weight variant tenascin-C is known to be specifically expressed in pathological conditions. However, no link between tenascin-C and collagen diseases has been established. The aim of our study was to determine the serum tenascin-C levels in patients with various collagen diseases, and to evaluate the possibility that serum levels of tenascin-C can be a useful marker for collagen diseases, correlating with the pathogenesis. Serum tenascin-C levels of 33 patients with scleroderma (SSc), 10 patients with scleroderma spectrum disorder (SSD), 15 patients with localized scleroderma (LSc), 12 patients with dermatomyositis (DM), 10 patients with systemic lupus erythematosus (SLE) and 15 healthy controls were measured with specific enzyme-linked immunosorbent assays. Serum tenascin-C levels were significantly elevated in patients with SSc, SSD and LSc than in healthy controls. Significantly higher total skin thickness score or higher incidence of pitting scars/ulcers and diffuse pigmentation were observed in SSc patients with elevated tenascin-C levels than in those with normal levels. Our study suggests that serum tenascin-C levels are increased in fibrotic conditions, and that tenascin-C contributes to the pathogenesis of vascular damage as well as fibrosis in SSc patients. Clarifying the role of tenascin-C in the pathogenesis of collagen diseases may lead to a new therapeutic approach.

Key words: autoimmune disease, dermatomyositis, fibrosis, scleroderma, systemic lupus erythematosus.

INTRODUCTION

Collagen diseases include various systemic disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), mixed connective tissue disease (MCTD) or Sjögren's syndrome (SjS). Dysregulation or degeneration of extracellular matrix (ECM) including collagen is thought to be one of the common denominators and may occur in diverse tissues; for example, joint destruction of RA is caused by intra-articular ECM degradation.[1] SSc is characterized by collagen accumulation in the skin.[2] Furthermore, lung fibrosis is observed in patients with PM/DM.[3] However, most aspects of these changes are poorly understood, and its mechanism needs to be clarified.

Tenascin is a family of large multimetric ECM glycoproteins. Vertebrates express four tenascins in their connective tissues: tenascin-C, -R, -X and -W.[4] Among them, tenascin-C is composed of a TA domain at the N-terminus, epidermal growth factor (EGF)-like sequences, fibronectin type III-like (FNIII) repeats, and a fibrinogen-like domain.[5,6] The molecular weight is variable by the alternative splicing of the FNIII repeats. A small molecular weight variant of tenascin-C lacking FNIII repeats by the splicing is known to be present in normal tissues, whereas the large molecular weight variants containing 1–7 FNIII repeats are specifically found in several pathological conditions including osteoarthritis.[7,8] The skin also express both variants of tenascin-C. Hasegawa *et al.*[7] reported there were no difference in the distribution of the tenascin-C variants between in healthy skin, cutaneous wounds, psoriatic lesions and epidermal tumors. However, no link between tenascin-C and collagen diseases has been established, and function of tenascin-C in collagen diseases has not been clarified. Accordingly, in this study, we determined the serum levels of large molecular weight variant tenascin-C in patients with various collagen diseases, and try to evaluate the possibility that tenascin-C contributes to the pathogenesis of collagen diseases and that serum levels of tenascin-C can be a useful marker for collagen diseases.

METHODS

Clinical assessment and patient material

Serum samples were obtained from 33 SSc patients, 12 DM patients and 10 SLE patients. Patients with SSc or SLE fulfilled the criteria proposed by the American College of Rheumatology (ACR).[9,10] SSc patients were grouped according to the classification system proposed by LeRoy *et al.*[11] Fourteen patients had diffuse cutaneous SSc (dcSSc) and 19 patients had limited cutaneous SSc (lcSSc). Fifteen patients with local-

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ized scleroderma (LSc), and 10 patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought to develop SSc in the future based on the criteria as described below, were also included in this study.[12–14] DM patients were diagnosed based on the criteria proposed by Bohan and Peter.[15,16] Patients who had received any treatments including immunosuppressive agents or corticosteroid were excluded. Clinical and laboratory data reported in this study were obtained at the time of serum sampling. Fifteen control serum samples were also collected from healthy age- and sex-matched volunteers. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. All serum samples were stored at −80°C prior to use.

Antinuclear antibodies

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence using HEp-2 cells as the substrate and double immunodiffusion, as described previously.[14]

Determination of serum tenascin-C levels

Concentration of large molecular weight (~250–400 kDa) tenascin-C was measured with a solid phase sandwich enzyme-linked immunoassay kit (IBL, Gunma, Japan) using two different antibodies to detect high molecular weight variance specifically.[17] Briefly, mouse monoclonal antibody against human FNIII-C domain was precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated mouse antibody to human EGF-like domain. Tetra methyl benzidine (TMB) is used as a coloring agent (chromogen). The absorbance at 450 nm was measured, and the concentration of tenascin-C in each sample was determined by interpolation from a standard curve.

Diagnostic method of SSD using the point system

For the diagnosis of SSD patients, total points were calculated as the sum of the following five factors:[12,18,19]

1. Extent of skin sclerosis: 1 point for swollen fingers, 3 points for sclerodactyly alone, 5 points for skin sclerosis limited to the extremities and face, and 10 points for truncal sclerosis.
2. Pulmonary changes: 2 points for pulmonary fibrosis accompanied by normal vital capacity (≥80%), and 4 points for pulmonary fibrosis accompanied by decreased vital capacity (<80%).
3. ANA: 5 points for positive anti-topoisomerase-I, 3 points for positive anticentromere or anti-U1 RNP antibody, 2 points for antinuclear antibody and 1 point for other positive ANA.
4. Pattern of Raynaud's phenomenon: 1 point for biphasic and hemilateral, or monophasic (pale or purple only) and bilateral, 2 points for biphasic (two of the above colors) and bilateral, and 3 points for triphasic (pale purple-red).
5. Nail fold bleeding (NFB): 1 point for NFB in one or two fingers, and 2 points for NFB in three or more fingers.

A score of 5–8 points are consistent with SSD, and those with point 9 or more points are consistent with SSc.

Statistical analysis

Statistical analysis was carried out with Mann–Whitney *U*-test to compare medians in each patient group, and Fisher's exact probability test to compare percentages. $P < 0.05$ was considered significant.

RESULTS

Serum concentrations of tenascin-C

The serum levels of large molecular weight tenascin-C variant in 33 SSc patients, 12 DM patients, 10 SLE patients and in 15 healthy control subjects are shown in Figure 1. Fifteen LSc patients and 10 SSD patients, who did not fulfill the criteria of SSc but were thought to develop SSc in the future, were also included in this study.[12–14]

Mean serum levels were significantly higher in SSc patients than in the healthy control subjects (40.5 ± 33.7 vs 13.6 ± 8.2 ng/mL, $P < 0.0001$). When SSc patients were classified into lcSSc and dcSSc as described in Methods, both dcSSc patients and lcSSc patients had significantly higher tenascin-C levels than healthy controls (46.9 ± 5.6 vs 13.6 ± 8.2 ng/mL, $P < 0.0001$; 36.4 ± 43.0 vs 13.6 ± 8.2 ng/mL, $P = 0.033$, respectively). Also, tenascin-C levels in LSc patients as well as SSD patients were significantly elevated than those in healthy controls (39.1 ± 36.8 vs 13.6 ± 8.2 ng/mL, $P = 0.002$; 43.4 ± 47.6 vs 13.6 ± 8.2 ng/mL, $P = 0.007$, respectively). Meanwhile, there was no significant difference in

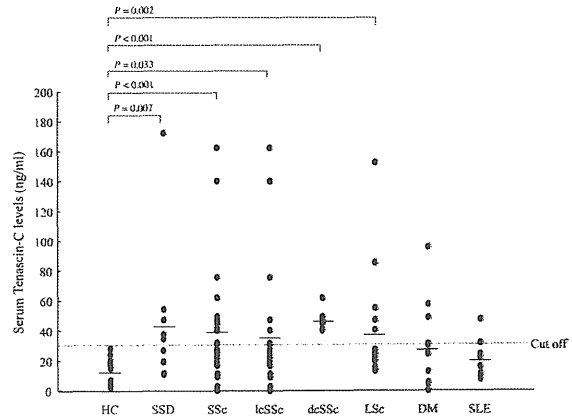

Figure 1. Serum concentrations of large molecular weight tenascin-C variant in patients with scleroderma spectrum disorder (SSD, $n = 10$), systemic sclerosis (SSc, $n = 33$), localized scleroderma (LSc, $n = 15$), dermatomyositis (DM, $n = 12$) or systemic lupus erythematosus (SLE, $n = 10$), and healthy control subjects (HC, $n = 15$). Serum concentrations of tenascin-C determined by enzyme-linked immunoassay are shown on the ordinate; the horizontal dotted line indicates the cut-off levels. Bars show means. *P*-values determined using Mann–Whitney *U*-test. dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc.

the values between healthy controls and patients with DM or SLE (13.6 vs 27.9 ng/mL; 13.6 vs 21.0 ng/mL, respectively).

When the cut-off value was set at 30.06 ng/mL (mean + 2 standard deviations of the controls), increased serum concentrations of tenascin-C were found in 22 of the 33 SSc patients (66.7%), five of the 15 LSc patients (33.3%), five of the 10 SSD patients (50.0%), four of the 12 DM patients (33.3%), and two of the 10 SLE patients (20.0%). To note, all of 14 dcSSc patients (100%) showed increased serum tenascin-C levels.

Correlation of serum tenascin-C levels with clinical manifestations and laboratory data in patients with collagen diseases

Table 1 shows the association of serum tenascin-C levels with the clinical and laboratory features in SSc patients. In patients with elevated tenascin-C levels, the ratio of dcSSc : lcSSc was significantly higher compared with that in patients with normal levels (diffuse : limited, 14:6 vs 0:13, $P < 0.0001$), consistent with the result that serum tenascin-C levels in all dcSSc patients were above the cut-off value (Fig. 1). Also, modified Rodnan total skin thickness score (MRSS) were significantly higher in patients with elevated tenascin-C levels than in those with normal tenascin-C levels (16.9 vs 2.8, $P = 0.001$). In addition, pulmonary fibrosis, pitting scars/ulcers and diffuse pigmentation were also found at the significantly higher prevalence in patients with elevated serum levels than those without (52.9% vs 7.7%, $P = 0.0174$; 66.7% vs 16.7%, $P = 0.0106$; 53.8% vs 0%, $P = 0.0436$, respectively). Serum levels of tenascin-C were previously reported to be elevated in patients with pulmonary hypertension or cardiac failure.[20,21] In our study, elevated right ventricular systolic pressure by echocardiography was seen more frequently in SSc patients with increased serum tenascin-C than in those without (36.8% vs 8.3%), but not statistically significant. Heart involvement was increased in patients with increased serum tenascin-C than those without (31.6% vs 25.0%), albeit insignificantly. Consistently, serum tenascin-C levels tended to be elevated in SSc patients with pulmonary hypertension or heart involvement than those without (53.2 vs 31.4 ng/mL; 52.4 vs 38.6 ng/mL, respectively), but there was no statistical significance.

On the other hand, there were no correlations between points calculated as described above and serum tenascin-C levels in SSD patients (Table 2). In addition, we did not find significant correlation between serum tenascin-C levels and clinical characteristics in DM patients (Table 3) and SLE patients (Table 4). However, although not statistically significant, DM and SLE patients with elevated serum tenascin-C levels tended to show higher prevalence of pulmonary fibrosis than those with normal levels (66.7% vs 20.0%; 50.0% vs 0%, respectively).

Table 1. Correlation of serum tenascin-C levels with clinical and serological features in patients with systemic sclerosis

	Patients with elevated tenascin-C levels ($n = 20$)	Patients with normal tenascin-C levels ($n = 13$)
Age at onset (years)	58.4	61.5
Duration of disease (years)	4.3	6.6
Type (diffuse/limited)	14:6**	0:13
MRSS	16.9**	2.8
Clinical features		
Pitting scars/ulcers	66.7*	16.7
Nail fold bleeding	44.4	0.0
Raynaud's phenomenon	94.1	92.3
Telangiectasia	17.6	18.1
Contracture of phalanges	92.3	100.0
Calcinosis	0.0	0.0
Diffuse pigmentation	53.8*	0.0
Short SF	92.3	71.4
Sicca symptoms	35.7	85.7
Organ involvement		
Pulmonary fibrosis	52.9*	7.7
Mean% VC	94.6	101.4
Mean% DLCO	83.6	82.9
Elevated RVSP (>30 mmHg)	36.8	8.3
Esophagus	29.4	16.7
Heart	31.6	25.0
Kidney	0.0	0.0
Joint	44.4	66.7
Thrombosis	0.0	0.0
ANA specificity		
Anti-topoisomerase I	41.2	63.6
Anticentromere	47.1	18.2
Anti-U1 RNP	17.6	18.2

*$P < 0.05$; **$P < 0.01$ versus patients with normal tenascin-C levels using Fisher's exact probability test or Mann–Whitney *U*-test. Unless indicated, values are percentages. ANA, antinuclear antibodies; DLco, diffusion capacity for carbon mono-oxidase; MRSS, modified Rodnan total skin thickness score; RVSP, right ventricular systolic pressure; SF, sublingual frenulum; VC, vital capacity.

DISCUSSION

Both small and large molecular weight variants of tenascin-C are usually secreted as hexamers from the cells. They may be degraded by matrix metalloproteinases to be an entire or partial peptide of monomer or polymer in the serum, although the processing of extracellular tenascin-C is still unknown.[22] In this study, we have presented two major findings. First, serum levels of the large molecular weight tenascin-C variant in SSc was elevated compared with those in healthy controls. The hallmarks of SSc are fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack and vascular damage, leading to abnormal accumulation of the ECM and tissue fibrosis.[17,23] Considering that the percentage of dcSSc, MRSS and the incidence of pulmonary fibrosis or pitting scar/ulcers is higher in patients with elevated tenascin-C levels than in those without, tenascin-C may contribute to the pathogenesis of vascular damage as well as fibrosis in this disease.

Second, serum tenascin-C levels in SSD and LSc as well as SSc were elevated compared with healthy controls, while the values in DM and SLE patients were not increased. LSc is a

Table 2. Correlation of serum tenascin-C levels with clinical signs or symptoms in patients with scleroderma spectrum disorder

Patient no.	Tenascin-C levels (ng/mL)	Sex/age	Skin sclerosis	Pulmonary changes	ANA	Raynaud's phenomenon	NFB	Point
1	47.16	F/80	–	–	ACA	Triphasic	1 or 2 fingers	7
2	19.24	F/58	Swollen	–	ACA	Triphasic	1 or 2 fingers	8
3	54.12	F/44	–	–	Topo-I	–	–	5
4	34.6	F/62	–	+, VC ≥ 80%	ACA	Triphasic	–	8
5	19.52	F/47	–	–	Topo-I	–	–	5
6	10.8	F/68	–	–	Anti-U1-RNP	Triphasic	–	6
7	37.56	F/45	–	–	Topo-I	–	–	5
8	11.6	F/57	Swollen	–	Others	Triphasic	1 or 2 fingers	6
9	26.88	F/48	Swollen	–	Others	Triphasic	–	5
10	172.28	F/75	–	+, VC ≥ 80%	ACA	Triphasic	–	8

ACA, anticentromere antibody; ANA; antinuclear antibodies; NFB, nail fold bleeding; Topo-I, anti-topoisomerase I antibody; VC, vital capacity.

Table 3. Correlation of serum tenascin-C levels with clinical and serological features in patients with dermatomyositis

	Patients with elevated tenascin-C levels (n = 3)	Patients with normal tenascin-C levels (n = 9)
Age at onset (years)	43.3	64.4
Duration of disease (years)	5.7	4.0
Skin eruptions		
Gottron's papules	100.0	62.5
Heliotrope coloration	100.0	66.7
Laboratory features		
IgG (mg/dL)	1610.3	1460.8
CK (IU/L)	7258.0	2890.6
Myoglobin (ng/mL)	2207.1	863.4
Aldolase (U/L)	204.0	19.4
ANA	50.0	75.0
Organ involvement		
Pulmonary fibrosis	66.7	20.0
Cancer	0.0	0.0
Dysphagia	33.3	40.0

Unless indicated, values are percentages. ANA; antinuclear antibodies; CK, cytokeratin; IgA, immunoglobulin A.

localized form of SSc lacking visceral involvement, while SSD is thought to be the incomplete/earliest form of SSc as described above. LeRoy *et al.*[24] also reported similar condition as 'limited SSc'. Thus, our results showed that serum tenascin-C concentration is increased specifically in fibrotic conditions. Progressive fibrosis in SSc is often irreversible, and there are urgent needs to develop new strategies to diagnose patients as early as possible and follow up carefully. For that purpose, the concept of SSD should be characterized more clearly. Clarifying the role of tenascin-C in SSD may lead to further understanding of its pathogenesis and the mechanism that triggers the development of SSc.

Dermatomyositis and SLE patients with elevated serum tenascin-C levels showed higher prevalence of pulmonary fibrosis, but we did not find statistical significance. This may be because of the small number of patients, and serum tenascin-C levels may be increased even in the fibrotic conditions of DM or SLE. Further investigation with a larger sample is needed in the future.

Table 4. Correlation of serum tenascin-C levels with clinical and serological features in patients with systemic lupus erythematosus

	Patients with elevated tenascin-C levels (n = 2)	Patients with normal tenascin-C levels (n = 8)
Age at onset (mean years)	45.0	41.0
Duration of disease (mean months)	21.0	22.7
Clinical features		
Fever	50.0	16.7
Skin changes	100.0	100.0
Alopecia	50.0	50.0
Photosensitivity	50.0	50.0
Organ involvement		
Pericarditis	50.0	0.0
Neuropsychiatric involvement	50.0	50.0
Arthritis	50.0	60.0
Pulmonary fibrosis	50.0	0.0
Renal failure	50.0	16.0
Thrombosis	0.0	0.0
Laboratory features		
Antiphospholipid antibodies	0.0	33.3
Leukopenia	50.0	50.0
Lymphopenia	14.0	0.0
Decreased C3	50.0	37.5
Decreased C4	50.0	85.7
Decreased CH50	100.0	100.0
dsDNA (IU/mL)	0.0	50.0
Proteinuria	0.0	33.3

Unless indicated, values are percentages.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Down-regulation of miR-223 contributes to the formation of Gottron's papules in dermatomyositis via the induction of PKCε

Background: Dermatomyositis (DM) is characterized by skin manifestations accompanying and preceding muscle weakness. Gottron's papules, one of the skin manifestations, are of great diagnostic value because they are specific to DM. However, the pathogenesis of Gottron's papules remains unclear. *Objectives:* We investigated the expression pattern of miRNAs in Gottron's papules of DM patients and evaluated the possibility that miRNAs play a role in its pathogenesis. *Materials and methods:* miRNAs were extracted from skin tissues and sera of patients with DM, clinically amyopathic DM (CADM) and healthy controls. To identify pathogenic miRNAs, we performed miRNA PCR array analysis. The results were confirmed by *in situ* hybridization, immunohistochemistry, immunoblotting and transient transfection of siRNAs or miRNA inhibitors. *Results:* PCR array analysis using tissue miRNAs demonstrated the miR-223 level was markedly decreased in Gottron's papules of DM and CADM *in vivo*, but not in psoriasis skin. The protein expression of PKCε, a predicted target of miR-223, was increased in DM/CADM skin. The transfection of a specific inhibitor of miR-223 in keratinocytes led to up-regulation of the PKCε protein, and resulted in increased cell proliferation. On the other hand, cell numbers were significantly decreased when cells were transfected with siRNA for PKCε. The serum miR-223 concentration was decreased in DM/PM patients, particularly in CADM patients, compared with healthy controls. *Conclusions:* A decreased miR-223 expression and the subsequently increased PKCε levels may therefore play a key role in the pathogenesis of Gottron's papules.

Key words: microRNA, autoimmune disease, collagen disease, keratinocyte, serum

MicroRNAs (miRNAs) are small endogenous RNA molecules, about 22-25 nucleotides in length, which can regulate gene expression post-transcriptionally [1]. More than 1,000 miRNAs have been so far identified in humans [1, 2]. Thus, miRNAs are thought to be the most abundant class of regulators, and have been implicated in various cellular activities, including the immune response, cell development, cell differentiation, cell growth control and apoptosis.

The miRNAs are thought to be involved in the pathogenesis of various autoimmune diseases. For example, miR-146, miR-155 and miR-124 are reported to be associated with rheumatoid arthritis, while miR-574 and miR-768-3p are associated with Sjögren's syndrome. Similarly, miR-196a, miR-17-5p and miR-146 may contribute to the etiology of SLE, whereas miR-29a and miR-206 may play a role in systemic sclerosis [3-5]. However, little is known about the role of miRNAs in dermatomyositis (DM). Eisenberg *et al.* found several miRNAs that were dysregulated in the muscle tissues of DM patients [6]. However, the role of miRNAs in the cutaneous lesions of DM still remains poorly understood.

DM is diagnosed based on characteristic skin manifestations accompanying and preceding muscle weakness [7-10]. DM patients with clinically and histopathologically typical cutaneous lesions, but without myositis, are diagnosed as clinically amyopathic DM (CADM). The skin manifestations of DM and CADM include Gottron's papules, heliotrope rash, V-neck sign or shawl sign. The histopathological features of these eruptions include basal layer liquefaction degeneration, mild lymphocyte infiltration or dermal mucin deposition. Among the skin manifestations, Gottron's papules have diagnostic value because they are frequently specific to DM. Acanthosis, papillomatosis and hyperkeratosis as well as the basal layer liquefaction, inflammation and mucin deposition are distinctive features seen in Gottron's papules [11]. However, the pathogenesis of Gottron's papules, especially the cause

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of acanthosis or hyperkeratosis, remains unclear. In this study, we examined the expression pattern of miRNAs in the Gottron's papules of DM patients, and evaluated the possibility that miRNAs play a role in the pathogenesis of the disease.

Materials and methods

Patient material

Skin specimens were obtained from the Gottron's papules of 6 DM patients and 5 CADM patients. All DM patients fulfilled the criteria proposed by Bohan and Peter [7, 8]. The patients with CADM were diagnosed according to the previously described criteria [17]. Control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin grafting. The control and patient samples were collected and fixed in formaldehyde immediately after resection.

To collect sera, fresh bloods samples were obtained from 22 DM patients, 6 CADM patients and 4 polymyositis (PM) patients. The samples were incubated at room temperature for 30 minutes, then centrifuged at 1500g for 15 minutes. The clinical and laboratory data reported in this study were obtained at the time of serum sampling. Control serum samples were also collected from healthy age- and sex-matched volunteers. All serum samples were stored at –80°C prior to use. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study, according to the Declaration of Helsinki. Patients who had received treatments were excluded.

miRNA extraction from tissue and the PCR analysis of miRNA expression

Small RNAs were extracted from skin sections using miRNeasy FFPE kit (Qiagen, Valencia, CA, USA). Then, RNAs (100ng) were reverse-transcribed into first strand cDNAs with RT^2 miRNA First Strand Kit (Qiagen). For PCR Array, the cDNA was mixed with RT^2 Real-Time SYBR GREEN/ROX PCR Master Mix (Qiagen) and the mixture was added into 96-well RT^2 miRNA PCR Array (Qiagen) that included primer pairs for 88 human miRNAs. PCR was performed on Takara Thermal Cycler Dice (TP800®) following the manufacturer's protocol. The threshold cycle (Ct) for each miRNA was extracted using Thermal Cycler Dice Real Time System ver 2.10B. The raw Ct was normalized using the values of SNORD47, small RNA housekeeping gene stably expressed in the arrays.

For quantitative real-time PCR, Mir-X™ miRNA First-Strand Synthesis Kit (Clontech) was used for cDNA synthesis. The primers for miR-223 or U6 (Takara) and templates were mixed with SYBR Advantage qPCR Premix (Clontech). DNA was amplified for 40 cycles of denaturation for 5 seconds at 95 °C and annealing for 30 seconds at 60 °C. The transcript levels of miR-223 were normalized to those of U6.

miRNA extraction from serum and the PCR analysis of miRNA expression

The miRNA isolation from serum samples was performed with miRNeasy RNA isolation kit (Qiagen) following the manufacturer's instructions, with minor modifications [18]. In brief, 100 (L of serum were supplemented with 5 μL of 5 fmol/(L synthetic non-human miRNA (*C. elegans*, miR-54, Takara) as a control to provide an internal reference for normalization of technical variations between samples. After Qiazol solution (1 mL) was added and mixed well by vortexing, samples were incubated at room temperature for 5 minutes. Aqueous and organic phase separation was achieved by the addition of chloroform and centrifugation. The aqueous phase was applied to RNeasy spin column and RNeasy MinElute spin column. The miRNA was eluted from the column with nuclease-free water.

Mir-X miRNA First-Strand Synthesis Kit (Takara) was used to synthesize first-strand cDNA from the serum-derived miRNAs (about 100 ng). Quantitative real-time PCR with Takara Thermal Cycler Dice (TP800®) used primers and templates mixed with the SYBR Premix. The DNA was amplified for 50 cycles of denaturation for 5 seconds at 95 °C and annealing for 20 seconds at 60 °C. The transcript levels of miR-223 were normalized to those of cel-miR-54 in the same samples.

In situ hybridization

In situ hybridization was performed with 5'-locked digoxigenin-labeled nucleic acid (LNA) probes complementary to human mature miR-223 or a scrambled negative control (Exiqon, Vedbaek, Denmark) [19]. In brief, human skin sections were deparaffinized and deproteinized with protease K for 5 minutes. Slides were then washed in 0.2% glycine in PBS and fixed with 4% paraformaldehyde. Hybridization was performed at 48 °C overnight, followed by blocking with 2% fetal bovine serum and 2% bovine serum albumin in PBS and 0.1%Tween 20 (PBST) for 1 hour. The probe-target complex was detected immunologically by incubating the samples with a digoxigenin antibody conjugated to alkaline phosphatase, which acts on the chromogen nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany) for 48 hours. Slides were counterstained with nuclear fast red, and examined under a light microscope (OLYMPUS BX50, Tokyo, Japan).

Immunohistochemical staining

Wax-embedded sections (4 μm thick) were dewaxed in xylene and rehydrated in graded alcohols [20]. Antigens were retrieved by incubation with citrate buffer (pH6) for 5 minutes in a microwave oven. After endogenous peroxidase activity was inhibited, sections were blocked with 5% milk for 30 minutes and then reacted with the antibodies against PKCε (1:40, Santa Cruz Biotech, California, USA) overnight at 4 °C. After excess antibodies were washed out with PBS, samples were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies (Nichirei, Tokyo, Japan) for 1 hour. The reaction was visualized using the diaminobenzidine substrate system

(Dojin, Kumamoto, Japan). The slides were counterstained with Mayer's hematoxylin, and examined under a light microscope (OLYMPUS BX50, Tokyo, Japan).

Cell culture

Normal human epidermal keratinocytes (NHEKs) from 3 different donors were purchased from Lonza (Walkersville, MD). NHEKs were cultured in KBM-Gold Basal Medium with KGM-Gold SingleQuot Kit (Lonza) at 37 °C in a 5% CO_2 atmosphere.

Transient transfection

siRNAs were purchased from Dharmacon (Lafayette, CO). Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used as a transfection reagent. One day before transfection, NHEKs were cultured in KGM-Gold so that they were 30-50% confluent at the time of transfection. siRNAs were mixed with the transfection reagent and added to each well containing cells. Then, the NHEKs were incubated for 48-72 hours at 37 °C in 5% CO_2.
miRNA inhibitors were purchased from QIAGEN. For reverse transfection, The miRNA inhibitors were mixed with Lipofectamine RNAiMAX and then added when cells were plated, followed by incubation for 48-96 hours at 37 °C in 5% CO_2 [23, 24].

Cell lysis and immunoblotting

NHEKs were washed with PBS twice and lysed in lysis buffer (Denaturing Cell Extraction Buffer; Biosource International, Camarillo, CA). Aliquots of the cell lysates (normalized for protein concentrations) were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto PVDF filters. The PVDF filters were blocked in blocking One P buffer (Nacalai Tesque, Kyoto, Japan) for 1 hour and then incubated with primary antibody against PKCε (Sigma-Aldrich Japan, Tokyo, Japan) or β actin (Santa Cruz Biotechnology) overnight. The membranes were washed with Tris-buffered saline and 0.1%Tween 20 (TBS-T), probed with HRP-conjugated secondary antibody for 1 hour, and then washed with TBS-T again. The detection was performed using the ECL system (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's recommendations.

Cell counting

For proliferation assays, cells were plated in 24-well plates and detached from the wells by trypsin treatment. The cell number was counted using Coulter® Particle Counter (Beckman Coulter, Fullerton, CA) [25].

Statistical analysis

The data are expressed as the means ± standard deviation (SD) of at least 3 independent experiments. The statistical analyses were performed using the Mann-Whitney U-test for comparisons of medians. Values of $p<0.05$ were considered to be significant.

Results

The miRNA expression profile in Gottron's papules of DM and CADM

As an initial experiment to determine which miRNAs were involved in the pathogenesis of the cutaneous lesions in patients with DM or CADM, we performed miRNA PCR array analysis, which included 88 miRNAs involved in human cell differentiation and development *(table 1)*. The miRNA expression profiles in the Gottron's papules in the DM and CADM patients were compared with those of normal skin. There were several miRNAs that were overexpressed or suppressed specifically in DM and CADM skin *(table 1)*. Among them, we focused on miR-223, one of the miRNAs which was detected in normal skin but not in DM skin, and which was down-regulated in CADM skin. To confirm the result obtained by the miRNA PCR array, we performed quantitative real-time PCR analysis using specific primers for miR-223. As expected, miR-223 was found to be significantly decreased in DM and CADM compared with normal skin ($p = 0.006$ and 0.003, respectively, *figure 1*), but not in psoriasis skin, which is also characterized by the proliferation of epidermal cells. These results suggest that down-regulation of miR-223 is specific to DM and CADM. In addition, *in situ* hybridization showed that miR-223 expression was not found in the hyperproliferated epidermis of the Gottron's papules in DM *(figure 2figures 2A-B)* and CADM patients *(figure 2C)*, while the signal for miR-223 was evident in the basal layers of normal epidermis *(figure 2D)*.

Table 1. The expression profiles of miRNAs in the skin of DM and CADM as measured with the PCR array

miRNAs down-regulated in DM/CADM skin	Normal skin	DM	CADM
let-7a	-0.44	ND	ND
let-7e	2.81	ND	19.07
let-7g	1.52	ND	ND
miR-21	-2.09	ND	20.41
miR-22	2.27	ND	ND
miR-26a	-2.26	ND	ND
miR-92a	-0.88	ND	35.26
miR-146a	2.58	ND	23.59
miR-146b-5p	2.88	ND	ND
miR-223	1.88	ND	17.11
miR-378	2.19	ND	ND
miRNAs up-regulated in DM/CADM skin			
miR-218	ND	4.59	2.35
miR-503	ND	8.03	10.86

A mixture of equal amounts of miRNAs from 3 normal skin, 3 DM or 3 CADM samples was prepared, and the miRNA expression profile of each disease in vivo was evaluated using PCR array. The raw threshold cycle (Ct) was normalized using the values of small RNA housekeeping gene SNORD47. The ΔΔCt (the raw Ct of each miRNA – Ct of small RNA housekeeping gene) is shown. ND; not detected.

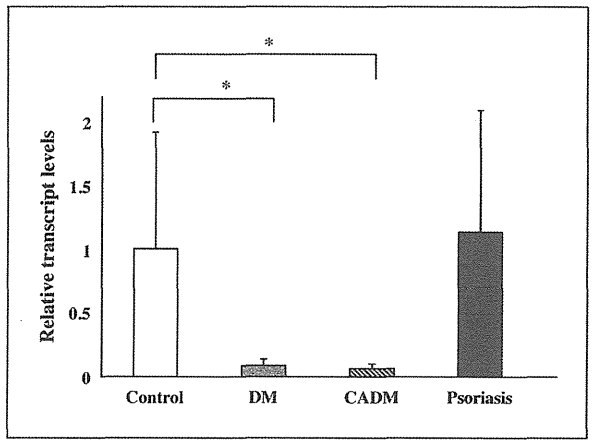

Figure 1. The expression levels of miR-223 in the skin of DM and CADM. The mean relative transcript levels of miR-223 in the tissues from 6 normal skin (NS), 6 DM, 5 CADM and 6 psoriasis patients were determined by quantitative real-time PCR. The bars show the means. The error bars represent SD of +1. The mean value in the NS samples was set at 1. *p<0.05 compared with the values in samples from NS.

Low miR-223 expression leads to cell proliferation via the induction of PKCε in DM and CADM skin

We expected that miR-223 might play a role in the pathogenesis of the cutaneous lesions of DM and CADM. According to TargetScan, the miRNA target gene prediction database (version 5.1, http://www.targetscan.org/), we found PKCε was one of the putative target genes of miR-223. Because PKCε was previously implicated as an effective promoter of keratinocyte growth *in vivo* and *in vitro* [26, 27], we hypothesized that decreased miR-223 expression would cause the induction of PKCε, resulting in the increased keratinocyte proliferation seen in the epidermis of Gottron's papules.

The immunohistochemical analyses revealed that the protein expression of PKCε in the hyperproliferated epidermis of Gottron's papules in DM patients was increased compared to that in normal skin *in vivo (figure 3)*. Next, to confirm that PKCε was a target of miR-223, NHEKs were transfected with miR-223 inhibitor, and the expression of PKCε was evaluated by immunoblotting. The inhibition of miR-223 *in vitro* resulted in a significant increase in the protein expression of PKCε ($p = 0.049$, *figure 4A*). Our results indicated that decreased miR-223 results in the overexpression of the PKCε protein in DM and CADM skin.

We next investigated whether miR-223 is involved in the keratinocyte proliferation via PKCε. A specific inhibitor of miR-223 significantly induced cell proliferation significantly ($p<0.001$, *figure 4B*). On the other hand, when PKCε was knocked down by a specific siRNA ($p = 0.016$, *figure 4C*), we observed a significant decrease in the cell number ($p = 0.021$, *figure 4D*). Taken together, these findings suggest that the abnormal keratinocyte proliferation in the epidermis of Gottron's papules may be caused by decreased miR-223 expression and subsequently increased levels of PKCε.

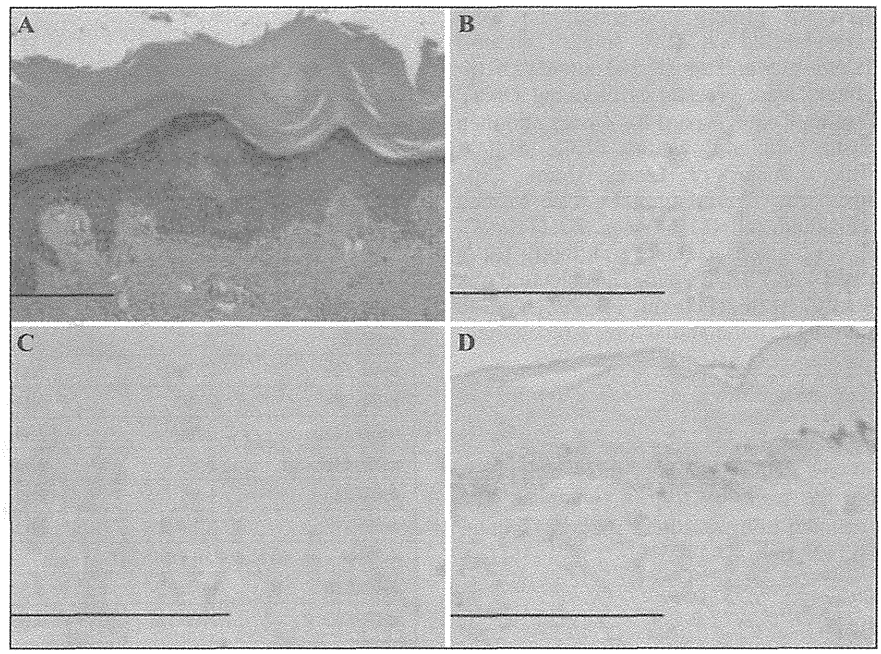

Figure 2. *In vivo* expression of miR-223 in the skin of DM and CADM. A) Representative histopathological findings of Gottron's papule. Skin biopsy sample was obtained from Gottron's papule of a DM patient and was stained with hematoxylin and eosin. Scale bar = 100 μm. **B-D)** *In situ* detection of miR-223 in paraffin-embedded, formalin-fixed tissues of DM skin **(B)**, CADM skin **(C)** and normal skin **(D)**. The Nuclei were counterstained with nuclear fast red. miR-223 is stained dark brown. Representative results from three independent experiments are shown. Scale bar = 100 μm.

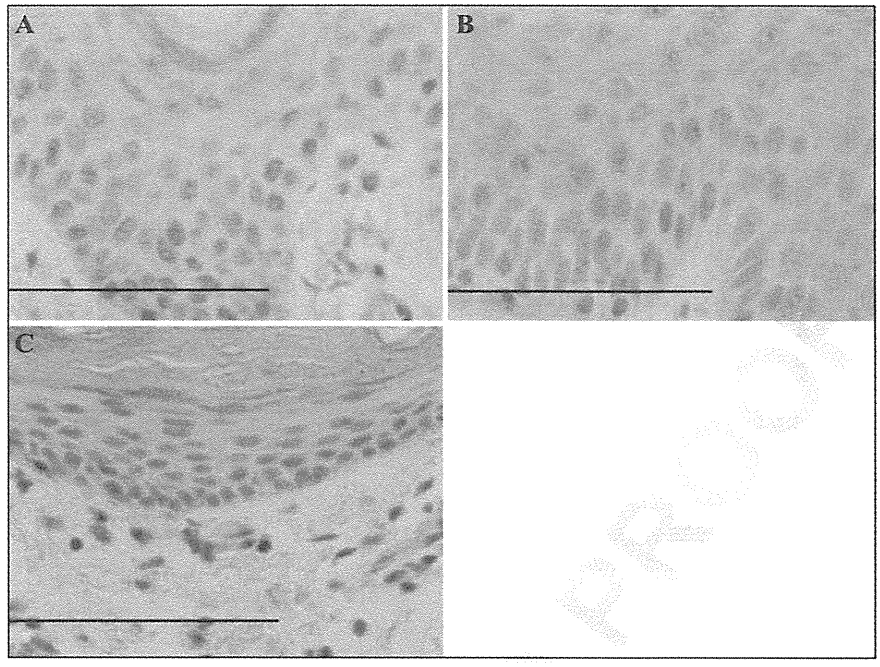

Figure 3. The expression pattern of PKCε in DM skin. Immunostaining for PKCε was performed using the tissue sections of DM skin (**A**), CADM skin (**B**), and normal skin (**C**). Scale bar = 100 μm.

Serum levels of miR-223 in DM patients

We also determined the serum concentration of miR-223 by quantitative real-time PCR and evaluated the possibility that the serum miR-223 levels can be a disease marker for DM. Serum samples were obtained from 22 patients with DM, 6 patients with CADM, 4 patients with PM and 19 healthy control subjects. The serum miR-223 levels were significantly decreased in patients with PM/DM/CADM compared with healthy controls by Mann-Whitney U-test ($p = 0.015$, *figure 5*). When patients were divided into groups based on the disease subtype, the serum miR-223 levels of CADM patients were significantly decreased compared with healthy controls ($p = 0.015$). On the other hand, although the miR-223 levels also tended to be decreased in patients with DM and those with PM, there were no statistically significant differences between controls and DM patients or between controls and PM patients *(figure 5)*. We next examined the correlation between the serum miR-223 levels and the clinical features of patients with PM/DM/CADM. As shown in *Table 2*, the disease duration (between symptom onset and the first visit to the hospital) was significantly shorter in patients with decreased miR-223 levels than in those with normal levels (2.8 *vs* 8.6 months, $p = 0.032$), indicating that those with decreased miR-223 levels may have more severe symptoms. Additionally, although there was no statistically significant difference ($p = 0.29$), the frequency of Gottron's papules tended to be increased in patients with reduced serum levels of miR-223 compared to those with normal levels, which is consistent with the array results indicating that miR-223 expression is reduced in Gottron's papules *in vivo*.

Discussion

This is the first study that has examined the miRNA expression specifically changed in DM skin. We herein demonstrated a role for the miR-223-PKCε pathway in keratinocyte proliferation and its contribution to the pathogenesis of Gottron's papules by uncovering three major findings.

We identified several miRNAs that were specifically overexpressed or suppressed in DM and CADM skin compared with normal skin by PCR array. Abnormal expression of miR-223 has been observed in a number of human diseases, such as rheumatoid arthritis, viral infections, sepsis, multiorgan failure, and cancer [28]. This is the first study that has indicated a role for miR-223 in DM. As a limitation of this study, because the array analysis was performed as a single experiment, the statistical significance could not be evaluated. Therefore, we confirmed the array result using real-time PCR and *in situ* hybridization, which also indicated the down-regulation of miR-223 expression in DM/CADM.

Second, in this study, we also found a new miRNA-mRNA target interaction in DM skin: the down-regulation of miR-223 leads to the overexpression of PKCε in keratinocytes. In addition, our results indicated that PKCε regulates keratinocyte growth, consistent with a previous report [26, 27]. PKCε is a molecule comprising 737 amino acids, and is found in diverse tissues, including neuronal, heart, immune, retinal, endothelial and epidermal cells. Our results revealed that PKCε is mostly localized in the proliferative basal layers in the epidermis, and suggested that the acanthosis and hyperkeratosis in Gottron's papules may be