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CONCISE COMMUNICATION

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Association between autoantibodies to the Ku protein and DPB1*

The Ku protein, a heterodimer consisting of 70-kd (p70) and 80-kd (p80) polypeptide subunits, binds free ends of double-stranded DNA (dsDNA). Once associated with DNA it creates a binding site for the catalytic subunit of the holoenzyme known as DNA-dependent protein kinase. This enzyme is essential for repairing dsDNA breaks that occur during radiation injury and V(D)J recombination (1).

Autoantibodies to the Ku protein were identified originally in 9 individuals among a randomly selected group of 330 Japanese patients (3%) with various connective tissue diseases studied with a classic immunodiffusion assay. Six of the patients who tested positive for autoantibodies came from a subgroup of 11 individuals (55%) with polymyositis–scleroderma (PM–scleroderma) overlap syndrome (2).

A somewhat different picture of anti-Ku autoantibodies emerged from studies of patients in the US. Reeves observed anti-Ku autoantibodies in the sera of 39% of patients with systemic lupus erythematosus (SLE), 55% of patients with mixed connective tissue disease, and 40% of patients with scleroderma, using an enzyme-linked immunosorbent assay (3). These antibodies also appear to be much more common among African American patients than white patients with SLE (4). Using immunoprecipitation assays, Francoeur et al observed anti-Ku antibodies in 10% of patients with SLE and in no samples obtained from patients with scleroderma (5). These observations suggest that anti-Ku antibodies have unique clinical associations in different racial groups, but further studies applying the same assay systems to different populations simultaneously will be required to confirm this speculation.

In the last several years, it has become clear that autoantibodies to nucleoproteins are antigen driven and require T helper cell support. Therefore, variations of autoantibody correlations in different patient groups seem likely to reflect racial differences in distribution of major histocompatability complex (MHC) phenotypes and the pattern of peptide antigens that are presented to T cells. We have now explored this idea through a genotypic analysis of all patients with anti-Ku autoantibodies at our institution in Japan.

A total of 750 Japanese patients were screened for autoantibodies in a radioimmunoprecipitation assay (6), and 21 were found to have anti-Ku autoantibodies. The presence of these antibodies was confirmed in an immunoblot assay using extracts of HeLa cells. The clinical diagnosis was established from a review of the medical record (Table 1). None of these patients had familial relationships. Clinically, 13 patients had PM or overlap syndromes with myositis (5 had PM–scleroderma, 4 had PM–scleroderma–SLE overlap, 2 had PM–SLE overlap, and 2 had PM), 5 had SLE, 2 had autoimmune hepatitis, and 1 had scleroderma according to established classification criteria (7–10). Forty-six healthy unrelated Japanese individuals served as control subjects. The HLA class II (DRB1, DQA1, DQB1, and DPB1) alleles were identified from restriction fragment length polymorphisms of polymerase chain reaction–amplified genomic DNA (11).

The HLA class II genotypes of all 21 patients are shown in Table 1. DRB1*0901 (62% of subjects versus 28% of controls; $P = 0.009$, odds ratio [OR] = 4.1), DQA1*0302 (62% versus 59%), and DQB1*0303 (62% versus 30%) were elevated in the study group, but none of these associations were statistically significant. However, DPB1*0501 was present in all patients with anti-Ku autoantibodies, compared with 59% of control subjects. This association was significant ($P = 0.0016$, OR 30) and remained significant ($P = 0.03$) when corrected for the number of alleles examined. Thirteen of the 21 patients (62%) with anti-Ku antibodies had myositis. Ten of these individuals (77%) had the class II haplotype of DRB1*0901-DQA1*0302-DQB1*0303, compared with 38% of anti-Ku–positive patients without myositis and 28% of controls ($P = 0.004$, OR 8.5). Four patients were homozygous for DRB1*0901, DQA1*0302, and DQB1*0303, but we found no indication of more severe disease in this group.

Studies of HLA associations with anti-Ku autoantibodies are limited. Yaneva and Arnett reported that the HLA class II antigen DQw1 was present in 17 of 19 anti-Ku positive patients (89%), compared with its frequency in local white (58%) and African American (61%) controls ($P = 0.01$, relative risk 5.8) (12). Although this allele occurs at increased frequency in patients with SLE, it is not associated with myositis and scleroderma. In the present study, the most striking finding is the universal occurrence of DPB1*0501 in 21 consecutive patients with anti-Ku autoantibodies. The DRB1*0901-DQA1*0302-DQB1*0303 haplotype also correlates with myositis in this patient cohort. Both DPB1*0501 and the DRB1*0901-DQA1*0302-DQB1*0303 haplotype are more common in the Japanese population than in the white population (13). It should be noted that DPB1*0501 is also a risk factor for Graves' disease in Japan (14). These findings suggest that there is a common immunogenetic background for Graves' disease and the anti-Ku autoimmune response. Therefore, these associations help to rationalize the earlier findings that anti-Ku autoantibodies are more clearly associated with myositis among the Japanese population.

Among the patients studied here, 9 had PM–scleroderma overlap syndrome with anti-Ku antibodies but none had the anti–PM-Scl, specificity. In the US population, ~10% of patients with this syndrome develop anti–PM-Scl. We have examined >100 patients with this overlap syndrome, but none have had anti–PM-Scl, nor have any of the >3,000 patients screened in our clinical diagnostic laboratory. Therefore we believe this autoantibody is rare among Japanese individuals. An explanation may be that anti–PM-Scl antibodies have been linked with DR3, a phenotype that is uncommon in the Japanese population (13). In any case, the MHC phenotype appears to exert a stronger influence over expression of specific autoantibodies than over the emergence of individual autoimmune syndromes. Further studies including analysis of MHC-restricted T cell responses could provide important clues for understanding mechanisms of onset of the PM–scleroderma overlap syndrome and the expression of anti-Ku antibodies.

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Table 1. HLA class II genes in Japanese patients with anti-Ku autoantibodies*

Patient no.	Diagnosis	DRB1*	DQA1*	DQB1*	DPB1*
1	PM/SSc	0405/1101	0303/0505	0401/0301	0501/0402
2	PM/SSc	0901/080302	0302/0103	0303/0601	0501/0202
3	PM/SSc	0901/080302	0302/0103	0303/0601	0501/0201
4	PM/SSc	0901/0405	0302/0303	0303/0401	0201/0501
5	PM/SSc	0901/0901	0302/0302	0303/0303	0501/0402
6	PM/SSc/SLE	0901/0901	0302/0302	0303/0303	0501/0402
7	PM/SSc/SLE	0901/1401	0302/0104	0303/0503	0501/0201
8	PM/SSc/SLE	0901/1502	0302/0103	0303/0601	0501/0901
9	PM/SSc/SLE	0901/0901	0302/0302	0303/0303	0501/0201
10	PM/SLE	0901/0901	0302/0302	0303/0303	0501/0201
11	PM/SLE	0405/0405	0303/0303	0401/0401	0501/0301
12	PM	0901/0802	0302/030101	0303/0302	0501/4101
13	PM	0405/1502	0303/0103	0401/0601	0501/0901
14	SLE	0901/1501	0302/0102	0303/0602	0501/0501
15	SLE	1501/0802	0401/0102	0302/0602	0201/0501
16	SLE	0405/080302	0303/0103	0401/0601	0501/0501
17	SLE	0901/080302	0302/0103	0303/0601	0501/0201
18	SLE	080302/1302	0103/0102	0601/0604	0501/0401
19	SSc	0405/0405	0303/0303	0401/0401	0501/0201
20	AIH	0802/0802	030101/030101	0302/0302	0201/0501
21	AIH	0901/0802	0302/030101	0303/0302	0501/0501

* PM = polymyositis; SSc = systemic sclerosis (scleroderma); SLE = systemic lupus erythematosus; AIH = autoimmune hepatitis.

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Autoantibodies to a 140-kd Polypeptide, CADM-140, in Japanese Patients With Clinically Amyopathic Dermatomyositis

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Objective. To identify novel autoantibodies specific for dermatomyositis (DM), especially those specific for clinically amyopathic DM (C-ADM).

Methods. Autoantibodies were analyzed by immunoprecipitation in 298 serum samples from patients with various connective tissue diseases (CTDs) or idiopathic pulmonary fibrosis (IPF). Antigen specificity of the sera was further examined by immunoblotting and indirect immunofluorescence (IF). The disease specificity and clinical features associated with the antibody of interest were determined.

Results. Eight sera recognized a polypeptide of ~140 kd (CADM-140 autoantigen) by immunoprecipitation and immunoblotting. Immunoreactivity was detected in the cytoplasm, and indirect IF revealed a granular or reticular pattern. Anti–CADM-140 antibodies were detected in 8 of 42 patients with DM, but not in patients with other CTDs or IPF. Interestingly, all 8 patients with anti–CADM-140 antibodies had C-ADM. Among 42 patients with DM, those with anti–CADM-140 autoantibodies had significantly more rapidly progressive interstitial lung disease (ILD) when compared with patients without anti–CADM-140 autoantibodies (50% versus 6%; $P = 0.008$).

Conclusion. These results indicate that the presence of anti–CADM-140 autoantibodies may be a novel marker for C-ADM. Further attention should be directed to the detection of rapidly progressive ILD in those patients with anti–CADM-140 autoantibodies.

Polymyositis (PM)/dermatomyositis (DM) is a chronic inflammatory disorder that culminates in injury to the skin and muscle and, sometimes, is associated with interstitial lung disease (ILD) and/or neoplasia (1,2). A number of autoantibodies are associated with myositis, including those specific for aminoacyl–transfer RNA synthetase (anti-ARS) (3), signal recognition particle (anti-SRP) (4), and Mi-2 (5). These autoantibodies have proven to be clinically useful in the diagnosis and classification of these diseases and are predictive of responses to treatment.

It has been known for some time that certain patients may have the typical skin manifestations of DM but no evidence of myositis, a condition known as amyopathic DM. Recently, Sontheimer proposed the existence of a unique subgroup of patients with DM who have the clinical cutaneous features of DM but no evidence of clinical myositis symptoms for at least 2 years after the onset of skin manifestations (referred to as clinically amyopathic DM [C-ADM]) (6). In other words, C-ADM includes patients with amyopathic DM and patients with hypomyopathic DM (patients with subclinical signs of myositis and DM skin manifestations). Some patients with C-ADM, especially those in Japan (7), have been noted to develop rapidly progressive ILD. This condition in many of these patients is resistant to treatment, and fatal outcomes have been observed.

Because of the severity of ILD accompanying C-ADM, a marker autoantibody would be useful for early diagnosis and treatment monitoring. Potential marker autoantibodies have been described by Targoff et al, who, in a preliminary study, described specificity for a 95-kd Se protein, as well as an unidentified 155-kd protein (8). However, a full survey of the autoantibodies

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associated with C-ADM has not been performed. In the present study, we examined the sera from 15 Japanese patients with C-ADM to identify additional autoantibodies associated with this disease.

PATIENTS AND METHODS

Patients and sera. Serum samples were obtained from 255 randomly selected Japanese adult patients with connective tissue diseases (CTDs) who were being followed up in clinics at Keio University in Tokyo and collaborating medical centers. These sera were obtained, prior to therapy, from a cohort of 61 patients with PM, 42 with DM (including 15 with C-ADM), 50 with rheumatoid arthritis, 46 with systemic lupus erythematosus, 27 with mixed CTD/overlap syndrome, 22 with systemic sclerosis, and 7 with Sjögren's syndrome. Sera from 43 patients with idiopathic pulmonary fibrosis (IPF) and 16 normal human sera were used as control sera. The diagnosis of C-ADM was based on diagnostic criteria proposed by Sontheimer (6), i.e., DM patients with no clinical muscle symptoms for more than 2 years after the onset of skin manifestations.

The patients were diagnosed as having ILD according to the results of chest radiography, chest computed tomography (CT), and pulmonary function testing, which included the percent predicted values for forced vital capacity and diffusing capacity for carbon monoxide. A subset of patients with rapidly progressive ILD was defined as those presenting with progressive dyspnea and progressive hypoxemia, and a worsening of interstitial change on the chest radiograph within 1 month from the onset of respiratory symptoms.

Immunoprecipitation. The immunoprecipitation assay was performed using extracts of the leukemia cell line, K562, as previously described (9). A total of 10 μl of patient serum was mixed with 2 mg of polypeptide A–Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) in 500 μl of immunoprecipitation buffer (10 m*M* Tris HCl, pH 8.0, 500 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, and then washed 3 times with immunoprecipitation buffer.

For polypeptide studies, antibody-coated Sepharose beads were mixed with 100 μl of ^{35}S-methionine–labeled K562 cell extracts derived from 2×10^5 cells, and rotated at 4°C for 2 hours. After 6 washes, the Sepharose beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer and the polypeptides were fractionated by 6% SDS–polyacrylamide electrophoresis gels. Radiolabeled polypeptide components were analyzed by autoradiography.

For analysis of RNA, the antigen-bound Sepharose beads were incubated with 100 μl of K562 cell extracts (6×10^6 cell equivalents per sample) for 2 hours at 4°C. To extract bound RNA, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast transfer RNA (10 mg/ml; Sigma, St. Louis, MO), and 300 μl of phenol:chloroform:isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added. After ethanol precipitation, the RNA was resolved using a 7*M* urea–10% polyacrylamide gel, which was subsequently silver-stained (Bio-Rad, Hercules, CA).

Immunoblotting. Immunoblotting analysis was performed using K562 cell extracts in a modification of the procedure described by Towbin et al (10).

Immunodepletion. A 10-μl aliquot of the prototype serum of autoantibodies to the 140-kd polypeptide was mixed with 2 mg of Sepharose beads and incubated for 2 hours at 4°C, followed by 3 washes with immunoprecipitation buffer. Another serum that recognized the 140-kd polypeptide was added in a dose-dependent manner (0 μl, 10 μl, 25 μl, and 50 μl) and then incubated. After 3 washes, immunoprecipitation for polypeptide analysis was performed as described above.

Indirect immunofluorescence (IF). Indirect IF was performed using HEp-2 cells and fluorescein-labeled anti-human immunoglobulin (Inova Diagnostics, San Diego, CA).

Clinical studies. The patients whose sera immunoprecipitated a 140-kd polypeptide were examined for their clinical symptoms, clinical course, muscle enzyme levels (creatine kinase [CK] and aldolase), results on chest radiographic and CT scans, and findings of skin pathology. An assessment of muscle weakness was performed using a manual muscle test (11). Some patients were also examined by electromyogram and muscle magnetic resonance imaging (MRI), and by pathologic analysis of the muscle.

Statistical analysis. The 2 groups of DM patients with or without autoantibodies to the 140-kd polypeptide were compared. The results of comparisons between groups were analyzed using the chi-square test, with Yates' correction where appropriate.

RESULTS

Detection of anti–140-kd polypeptide antibodies in patients with C-ADM. We screened 298 patient sera and 16 normal human sera by immunoprecipitation. Sera from 8 (19%) of 42 patients with DM immunoprecipitated a polypeptide of ~140 kd from ^{35}S-methionine–labeled K562 cell extracts (Figure 1A, lanes 1–8). All 8 patients were diagnosed as having C-ADM, a subtype of DM. In the analysis of RNA specificity, these sera did not immunoprecipitate any nucleic acid band, except for 1 patient's serum, which precipitated hYRNA of SSA/Ro components.

The C-ADM sera that immunoprecipitated the 140-kd polypeptide were also used to immunoblot K562 cell extracts. These sera from C-ADM patients displayed a similar reaction on immunoblot, with a polypeptide band of similar molecular weight (results not shown).

For identification of novel autoantibodies recognizing the 140-kd molecule, the polypeptide immunoprecipitated by the prototype serum was compared with antigens of similar molecular weight recognized by other known autoantibodies (Figure 1B). The protein recognized by the prototype serum migrated slightly faster than the 140-kd protein recognized by anti-MJ antibody (Figure 1B, lane 2) and faster than that recognized by anti–RNA helicase A antibody (Figure 1B, lane 3), but more slowly than the 120-kd protein precipitated by

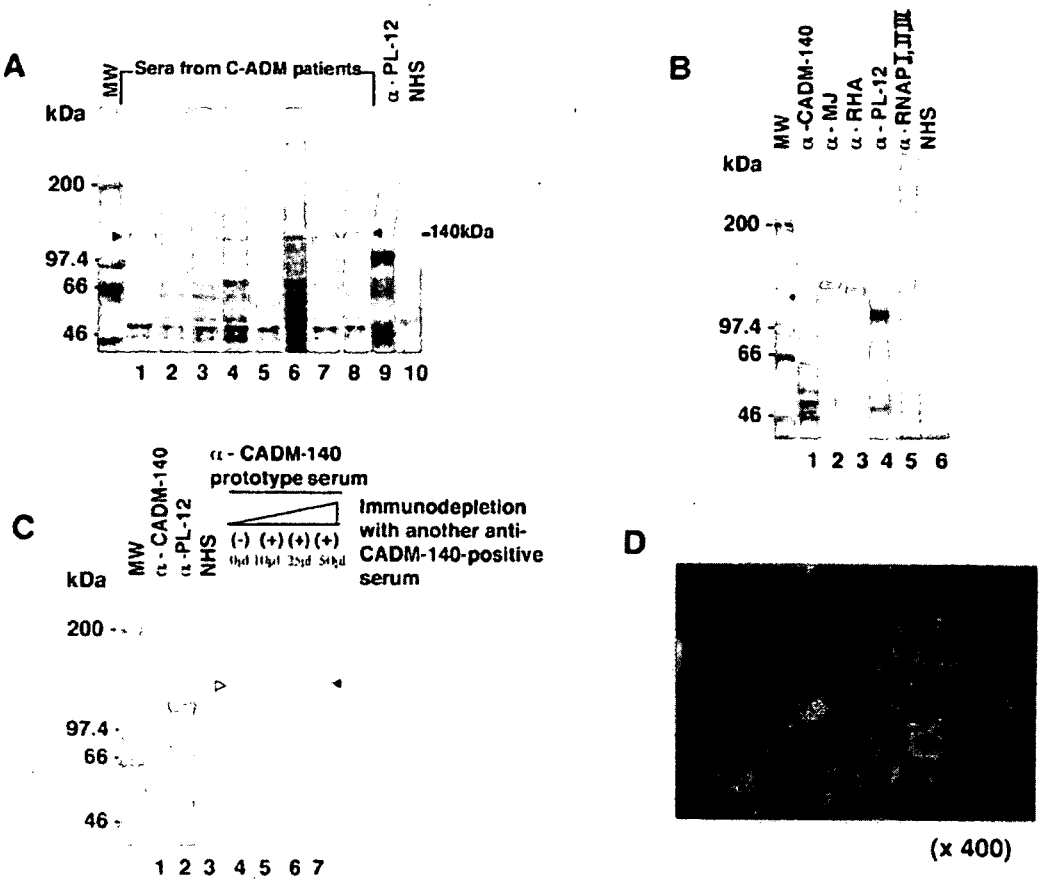

Figure 1. A, Immunoprecipitation of polypeptides with sera from patients with clinically amyopathic dermatomyositis (C-ADM), using ^{35}S-methionine–labeled K562 cell extracts. Lanes 1–8, Sera from C-ADM patients; lane 9, anti–PL-12 serum; lane 10, control normal human serum (NHS). A 140-kd protein was recognized by 8 sera from C-ADM patients (lanes 1–8). B, Immunoprecipitation of polypeptides by the prototype serum and by other known autoantibodies. Lane 1, The prototype anti–CADM-140 serum; lane 2, anti-MJ serum; lane 3, anti–RNA helicase A (RHA) serum; lane 4, anti–PL-12 (alanyl–transfer RNA synthetase) serum; lane 5, anti–RNA polymerase I, II, and III (RNAP I, II, and III) serum; lane 6, control NHS. Anti–CADM-140 serum immunoprecipitated an ~140-kd polypeptide that was easily distinguished from that of other known antibodies. C, Immunodepletion studies. Sera used for immunoprecipitation were as follows: lane 1, anti–CADM-140; lane 2, anti–PL-12; lane 3, control NHS; lanes 4–7, immunoprecipitation with anti–CADM-140 serum after absorption by another anti–CADM-140–positive serum in a dose-dependent manner. Arrows in A and C denote the 140-kd polypeptide. The sizes of the molecular weight markers are indicated to the left in A–C. D, Immunofluorescence pattern of HEp-2 cells stained with anti–CADM-140 serum. A granular or reticular cytoplasmic staining pattern on HEp-2 cells was observed. (Original magnification × 400.)

anti–PL-12 antibody (Figure 1B, lane 4). These results clearly indicate that the 140-kd polypeptide immunoprecipitated by the prototype serum was different from the proteins immunoprecipitated by these other known antibodies. We designated this new autoantibody specificity as anti–CADM-140.

The prototype serum depleted extracts of the 140-kd polypeptide in a dose-dependent manner (Figure 1C, lanes 4–7), and the polypeptide recognized by the prototype serum was no longer immunoprecipitated in these extracts (Figure 1C, lane 7). In contrast, the depletion of radiolabeled K562 cell extracts with the use of autoantibodies of different immunologic specificities did not affect the levels of the anti–CADM-140–specific antigen (results not shown). When sera positive for anti–CADM-140 antibodies were assessed in indirect IF studies, a granular or reticular cytoplasmic staining pattern was observed (Figure 1D).

Table 1. The frequencies of myositis-specific, myositis-associated, and anti–CADM-140 antibodies in patients with connective tissue diseases and IPF*

Autoantibodies	PM (n = 61)	DM (n = 42)		RA (n = 50)	SLE (n = 46)	MCTD/OL (n = 27)	Systemic sclerosis (n = 22)	Sjögren's syndrome (n = 7)	IPF (n = 43)
		Classic DM (n = 27)	C-ADM (n = 15)						
Myositis-specific									
Anti-ARS (anti-Jo-1)	10 (16)	6 (22)	0	0	0	0	0	0	0
Anti-ARS (non–anti-Jo-1)	10 (16)	2 (7)	0	0	0	1 (4)	0	0	4 (9)
Anti-SRP	5 (8)	0	0	0	0	0	0	0	0
Anti–Mi-2	0	2 (7)	0	0	0	0	0	0	0
Myositis-associated									
Anti-SSA/Ro	3 (5)	3 (11)	2 (14)	8 (16)	15 (33)	6 (22)	1 (5)	5 (71)	1 (2)
Anti–U1 RNP	2 (3)	2 (7)	0	1 (2)	18 (39)	23 (85)	2 (9)	0	0
Anti–CADM-140	0	0	8 (53)	0	0	0	0	0	0

* Values are the number (%) of patients. Anti-PM/Scl and other myositis-associated autoantibodies were not detected in any of the sera tested. PM = polymyositis; DM = dermatomyositis; C-ADM = clinically amyopathic dermatomyositis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; MCTD/OL = mixed connective tissue disease/overlap syndrome; IPF = idiopathic pulmonary fibrosis; anti-ARS = anti–aminoacyl-transfer RNA synthetase; anti-SRP = anti–signal recognition particle.

Disease specificity of the anti–CADM-140 antibodies. The frequencies of myositis-specific antibodies, myositis-associated antibodies, and anti–CADM-140 antibodies are summarized in Table 1. Myositis-specific antibodies are found in most patients with myositis, whereas myositis-associated antibodies are frequently found in patients without myositis (12). Among the patients with CTDs or IPF, myositis-specific antibodies (33 with anti-ARS, 5 with anti-SRP, 2 with anti–Mi-2) and myositis-associated antibodies (44 with anti-SSA/Ro, 48 with anti–U1 RNP, none with anti-PM/Scl or other myositis-associated antibodies) were detected. Anti–CADM-140 autoantibodies were found in 19% of sera from patients with DM (especially in 53% with the C-ADM subtype), but were not detected in patients with other CTDs or IPF.

Clinical features of C-ADM patients with anti–CADM-140. Clinical findings were compared between DM patients (including those with C-ADM) with anti–CADM-140 autoantibodies and those without anti–CADM-140 autoantibodies (Table 2). There were no significant differences in the frequencies of skin symptoms. However, the frequency of rapidly progressive ILD was significantly increased in anti–CADM-140–positive patients compared with that in anti–CADM-140–negative patients (50% versus 6%; $P = 0.008$). No myositis-specific antibodies were found in patients with anti–CADM-140; nevertheless, there was no significant

Table 2. Comparison of clinical features in anti–CADM-140–positive versus anti–CADM-140–negative patients with dermatomyositis

Feature	Anti–CADM-140–positive (n = 8)	Anti–CADM-140–negative (n = 34)	P
Age at onset, mean ± SD years	44.5 ± 12.7	46.5 ± 15.7	NS
No. male/no. female	2/6	8/26	NS
Gottron's sign or papules	75	88	NS
Heliotrope rash	50	53	NS
Muscle weakness	0	76	0.02
Elevation of CK	25	74	0.03
Fever	25	50	NS
Raynaud's phenomenon	13	24	NS
Arthritis	50	71	NS
Rapidly progressive ILD	50	6	0.008
Malignancy	0	18	NS
MSAs	0	29	NS
MAAs	13	18	NS

* Except where indicated otherwise, values are the percent of patients. NS = not significant; CK = creatine kinase; ILD = interstitial lung disease; MSAs = myositis-specific autoantibodies; MAAs = myositis-associated autoantibodies.

difference in the frequency of these autoantibodies in comparison with the anti–CADM-140–negative group.

None of the 8 patients with anti–CADM-140–positive sera were treated with steroids or other immunosuppressive medications prior to being assessed for C-ADM. All of these patients had Gottron's sign or papules, or periorbital heliotrope erythema and skin biopsy specimens yielding results compatible with DM. None of these patients had muscle weakness. CK levels were in the normal range in 6 patients (75%) and slightly elevated in the remaining 2 patients. Of the 6 patients assessed for the muscle enzyme aldolase, levels were normal in 2 patients. Of the 2 patients who underwent muscle MRI, neither showed findings compatible with a diagnosis of myopathy. Four patients had a muscle biopsy, and 2 of the muscle specimens exhibited mild infiltration of inflammatory cells, but there was no evidence of necrosis of muscle fibers, variation in fiber size, regeneration, or phagocytosis. Of the 7 patients with ILD (88%), 4 developed rapidly progressive disease.

DISCUSSION

We have identified novel autoantibodies (anti–CADM-140 autoantibodies) to an ~140-kd polypeptide in patients with DM. Anti–CADM-140 antibodies were detected specifically in patients with DM, especially those with C-ADM. In addition, anti–CADM-140 antibodies were associated with rapidly progressive ILD.

It has been reported that amyopathic DM may be accompanied by rapidly progressive ILD, especially in Japanese patients and other Asian patients (7). In contrast, rapidly progressive ILD was shown to be rare in patients with amyopathic DM in a North American population (13). In our series, 5 of 15 patients with C-ADM (33%) (4 of whom had anti–CADM-140 antibodies) had rapidly progressive ILD during their clinical course. Rapidly progressive ILD was more frequent in our series compared with that reported previously in North American populations (13). Although the number of patients that we studied was very limited, it remains possible that racial differences are the reason for this discrepancy, because other clinical studies of Japanese patients also demonstrated findings similar to ours (7).

Furthermore, in a recent preliminary report, using immunoprecipitation and immunoblotting of HeLa cell extracts, Targoff et al documented the presence of antibodies to a 155-kd protein and/or Se protein in patients with C-ADM (8). Thirteen of 18 C-ADM sera possessed an anti–155-kd polypeptide antibody, and 6 also immunoprecipitated a 95-kd polypeptide (anti-Se antibody). In contrast, Oddis et al identified the anti-MJ antibody, which was also found to recognize a 140-kd polypeptide, in patients with juvenile DM (14,15). We have been able to conclude that anti–CADM-140 is distinctively different from anti-MJ, because the molecular weights of the immunoprecipitated polypeptides are different. Moreover, the clinical features of anti-MJ are quite different from those associated with anti–CADM-140. Anti-MJ is detected mainly in juvenile DM, has been observed in the US and Argentina, and is clinically characterized by severe DM with a chronic and polycyclic course, sometimes accompanied by vasculitis (14). In order to elucidate the racial differences in the frequency of these antibodies, the examination of a larger number of patients from several different populations is required.

Our results have thus demonstrated the presence of anti–CADM-140 autoantibodies in patients with C-ADM, and these were found to be associated with rapidly progressive ILD. Further studies of this novel autoantibody specificity may provide insight into the pathogenic mechanisms of C-ADM accompanied by rapidly progressive ILD.

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Amelioration of experimental arthritis by a calpain-inhibitory compound: regulation of cytokine production by E-64-d *in vivo* and *in vitro*

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Abstract

Calpain, a calcium-dependent cysteine proteinase, has been reported to participate in the pathophysiology of rheumatoid arthritis (RA). The aim of this study is to investigate the therapeutic efficacy of calpain-inhibitory compounds in an animal model of RA and to clarify the underlying mechanisms *in vivo* and *in vitro*. Arthritis was induced in BALB/c mice with anti-type II collagen mAbs and LPS, and the mice were treated intra-peritoneally with a high dose (9 mg kg $^{-1}$ per day) or low dose (3 mg kg $^{-1}$ per day) of E-64-d (a membrane-permeable cysteine proteinase inhibitor) or control diluent. As a result, a high dose of E-64-d significantly alleviated the clinical arthritis and the histopathological findings, compared with the control diluent, although a low dose of E-64-d did not have a significant effect. Next, we evaluated the effects of E-64-d on cytokine mRNA expression at the inflamed joints by quantitative reverse transcription–PCR. High dose of E-64-d significantly decreased IL-6 and IL-1β mRNA levels at the inflamed joints. The regulatory effects of E-64-d on cytokine production were also confirmed *in vitro*, using a synovial cell line (E11) and crude synoviocytes derived from RA patients. These results suggest the key roles of calpain in the pathophysiology of arthritis and that calpain-inhibitory compounds might be applicable to the treatment of arthritic diseases such as RA.

Introduction

Rheumatoid arthritis (RA) is a chronic refractory disease, whose main symptom is polyarthritis characterized by autonomous synovial proliferation and osteocartilaginous destruction. Although the pathogenesis of RA remains to be elucidated, studies have indicated the contribution of proteinases, cytokines and auto-antibodies to the progression of the disease. First, over-expression of proteinases such as calpains (1–3), cathepsins (4) and matrix metalloproteinases (MMPs) (5) at the disease-affected joints has been reported and assumed to contribute to the osteocartilaginous destruction. Second, cytokines are now widely recognized to play major roles in RA, since an increasing number of favorable outcomes has been reported in trials to treat RA with biological agents against tumor necrosis factor-α (TNF-α) (6), IL-1 and IL-6. Third, the involvement of various auto-antibodies such as antibodies to citrullinated proteins (7), glucose-6-phosphate isomerase (8) and follistatin-related protein (FRP) (9–11) has been reported in the pathophysiology of RA. For example, we have reported that anti-FRP antibodies were detected in synovial fluids of RA patients (9), and demonstrated that recombinant FRP inhibited the production of MMP-1 and MMP-3 in cultured synoviocytes (10) and ameliorated an animal model of RA (11).

We and another group have identified a new auto-antibody against calpastatin (12, 13), which was detected significantly more frequently in RA patients than in patients with other

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rheumatic diseases or in healthy controls (12, 14, 15). Recently, it was reported that sensitivity and specificity of anti-calpastatin antibodies were 83 and 96% for RA, respectively (14). The positivity of anti-calpastatin antibodies was correlated with serological markers of the disease activity or a recent-onset subset in RA patients (15–17).

Calpastatin, an endogenous inhibitor of calpain, is a ubiquitous 70-kDa protein, comprising five domains, L, I, II, III and IV (18). Calpain, a Ca^{2+}-dependent cysteine proteinase, is a ubiquitous enzyme that has two subtypes, μ- and m-calpain, both of which have two subunits of 80 and 28 kDa (18). Calpain exhibits proteinase activity toward a wide range of substrates, such as cytoskeletal proteins, kinases, phosphatases, membrane-associated proteins and transcription factors (18). Since it cleaves the substrates at a limited number of specific sites for their activation or inactivation, calpain is regarded as a 'biomodulator' rather than a digestive enzyme (19). For example, calpain activates IL-1α or protein kinase C by cleaving it (20, 21). Through the modulation of these substrates, calpain is supposed to contribute to the pathophysiology of RA. It is worth mentioning that calpain is more highly expressed in the synovial membranes of RA patients than in those of healthy controls (2), and similarly, calpain is detected in the synovial membranes of mice with collagen (CL)-induced arthritis, while not detected in those of non-manipulated mice (22).

E-64-d (synonyms: loxistatin, EST and Ep453), a membrane-permeable cysteine proteinase inhibitor, has been used in order to inhibit calpain *in vivo* and *in vitro* (23, 24). In the present study, we examined the therapeutic efficacy of two calpain-inhibitory compounds, E-64-d and recombinant calpastatin (rCS), in mice with anti-type II CL antibody-induced arthritis. We also analyzed the underlying mechanisms *in vivo* and *in vitro*, using the mice with mAb-induced arthritis and a fibroblast-like synovial cell line (E11) or crude synoviocytes of a RA patient.

Methods

Reagents

E-64 [molecular weight (MW) 375.4, non-membrane permeable] and E-64-d (MW 342.4, membrane permeable) were purchased from Amresco (Solon, OH, USA) and Peptide Institute (Osaka, Japan), respectively. *Escherichia coli*-expressed recombinant human calpastatin (14 kDa, identical to domain I) was from Takara Shuzo (Shiga, Japan) and filtrated through an endotoxin cut filter (Zetapore-dispo 020SP, Cuno, Meriden, CT, USA) before use. Phorbol 12-myristate 13-acetate (PMA) and ionomycin (IM) were from Wako Chemicals (Richmond, VA, USA) and EMD Biosciences (La Jolla, CA, USA), respectively.

Induction and treatment of murine arthritis

Institutional bioethical approval (no. 02004, Kyoto University Graduate School of Medicine) was acquired prior to the animal experiments. Arthritis was induced in 7-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) with a mixture of anti-type II CL mAbs (Arthrogen-CIA mAb, Chondrex, Redmond, WA, USA) and LPS, according to the method of Terato *et al.* (25). Briefly, 2 mg per body of mAb mixture was injected intra-peritoneally (i.p.) into each mouse (day −2), and 48 h later, 50 μg per body of LPS was injected i.p. in order to enhance the arthritis (day 0). To prevent the development of the disease, a high dose (9 mg kg^{-1} per day) or low dose (3 mg kg^{-1} per day) of E-64-d, rCS (9 mg kg^{-1} per day) or control diluent (sterilized saline containing 10% dimethylsulfoxide) was administered i.p. to the disease-affected mice ($N = 8, 6, 9$ and 9, respectively) for 10 days from the day before the injection of mAbs (days −3 to 6).

Clinical and histopathological assessment of murine arthritis

The severity of the arthritis was evaluated macroscopically for every paw with a scoring system as follows: 0 = intact, 1 = mild swelling, 2 = severe swelling and 3 = deformed or ankylosed (11), although no deformity or ankylosis was observed in the present study. The sum of the four paws gave a clinical arthritis score, ranging 0–12. The assessments were performed in a blind-test fashion. For histopathological evaluation, the mice were sacrificed on the 19th day after the LPS injection. The right hind paw was resected from each body, fixed with 10% formaldehyde, decalcified in 10% EDTA, embedded in paraffin and stained with hematoxylin and eosin. The histopathological severity was evaluated for the findings of cell infiltration, pannus formation and bone erosion with a scoring system as follows: 0 = no signs, 1 = slight, 2 = moderate and 3 = remarkable. The sum of the three items gave a histopathological arthritis score, ranging 0–9.

Quantification of cytokine mRNA levels at inflamed joints

To analyze the time course of cytokine mRNA expression, the arthritis was induced in 40 BALB/c mice, and 5 mice each were sacrificed 0, 2, 4, 8, 24, 48, 72 or 96 h after the LPS injection. To examine the effects of E-64-d on the cytokine mRNA expression, 32 BALB/c mice affected by the arthritis were treated i.p. with a high dose (9 mg kg^{-1} per day) of E-64-d or control diluent from the day before the injection of mAbs (day −3), until they were sacrificed at the 4th or 48th h after the LPS injection ($N = 8$; given the same treatment and sacrificed at a time). Then the four paws of each mouse were resected from the body and homogenized altogether in 6 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and the cDNA was synthesized by random hexamer priming with the TaqMan reverse transcription (RT) reagents (Applied Biosystems, Foster City, CA, USA). The amount of cytokine mRNA was determined with a quantitative real-time RT–PCR system (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Briefly, the cDNA was amplified with the primers and probes for the targeted cytokine (IL-1α, IL-1β, IL-6 or TNF-α) mRNA and control 18S rRNA, which were available commercially (Applied Biosystems). The data were analyzed by the Sequence Detector software (Applied Biosystems). *Ct* values (cycle numbers to the threshold) for each of the targeted mRNA were normalized by subtracting *Ct* values for 18S rRNA (ΔCt), i.e. the quantities of the targeted mRNA were normalized by the quantities of control 18S rRNA. The normalized mRNA quantities (proportional to $2^{-\Delta Ct}$) were

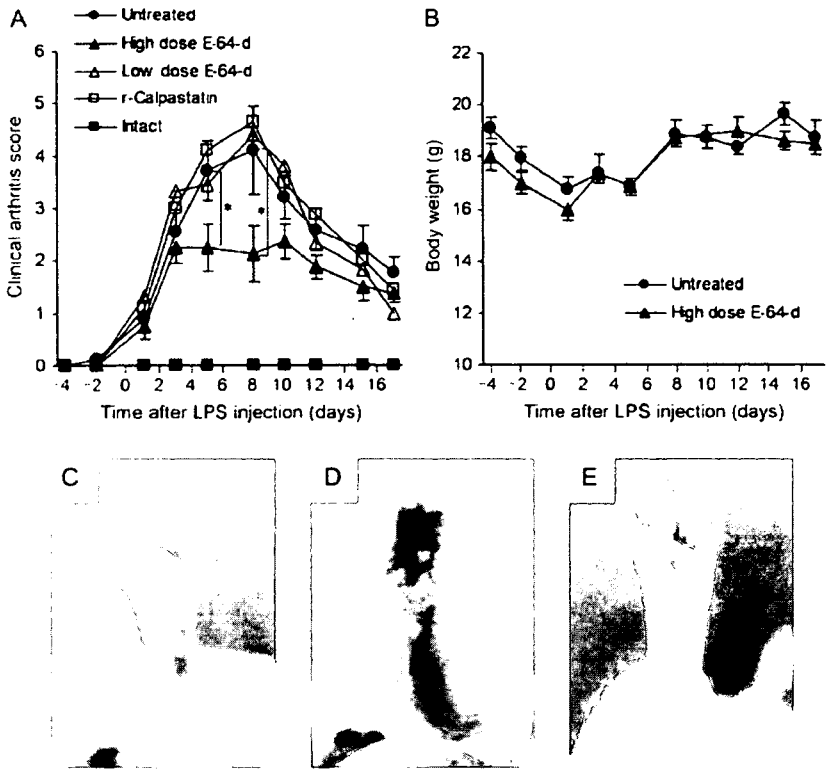

Fig. 1. Effects of calpain inhibitors on mice with anti-type II CL antibody-induced arthritis. Arthritis was induced in 7-week-old BALB/c mice with anti-type II CL mAbs (day -2) and LPS (day 0). To alleviate the arthritis, a high dose (9 mg kg^{-1} per day) or low dose (3 mg kg^{-1} per day) of E-64-d, rCS (9 mg kg^{-1} per day) or control diluent was administered for 10 days (days -3 to 6). (A and B) Time courses of the clinical arthritis scores and body weight of the mice, which were treated or not treated with calpain inhibitors. SEMs are indicated for the untreated group and the high-dose E-64-d group. $^{*}P < 0.05$ between the untreated group and the high-dose E-64-d group. (C–E) Representative macroscopic images of the hind paws photographed on day 8. (C) A non-manipulated mouse. (D) A disease-affected mouse, whose hind paws are swollen severely. (E) A disease-affected mouse that was treated with a high dose of E-64-d, whose hind paws are less affected than in (D).

compared among the experimental groups (Δ-ΔCt method). We fixed the mRNA quantities at the fourth hour after the LPS injection in the control group as 100%, and relative mRNA quantities were calculated.

Cell line and synoviocytes

E11, a fibroblast-like synovial cell line established from knee joint tissues of a RA patient (26), was kindly provided by Y. Tanaka (University of Occupational and Environmental Health, Kitakyushu, Japan). Crude synoviocytes were obtained from joint tissues of a RA patient who fulfilled the American College of Rheumatology criteria, as described elsewhere (9). Briefly, the synovial membrane was cut into small pieces and digested with 0.25% collagenase (type S-1, Nitta Gelatin, Osaka, Japan). E11 cells and the crude synoviocytes were cultured in RPMI 1640 medium containing 10% FCS, 100 IU ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2 incubator. The crude synoviocytes were subjected to the assays on the third passage. The following *in vitro* assays were triplicated.

Cytokine production assay

E11 cells (1×10^4 per 200 μl per well) were seeded in a 96-well microplate and incubated for 12 h. After the supernatants were exchanged for new medium, the cells were treated with various concentrations of E-64-d, E-64, rCS or control (2% dimethylsulfoxide) for 24 h in the presence or absence of 10 ng ml^{-1} PMA plus 750 ng ml^{-1} IM. Then, the supernatant cytokines were quantified by ELISA (Cytoscreen ELISA Kit, Biosource, Camarillo, CA, USA). To quantify the intracellular cytokines, E11 cells (1×10^5 ml^{-1} per well) were seeded in a 24-well microplate, incubated for 12 h and treated with each of the calpain inhibitors for 24 h. Then, the supernatants were removed, and the cells were lysed with 500 μl of a solution containing 0.1% Triton X-100, 20 mM HEPES–NaOH (pH 7.9), 300 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.5 mM phenylmethylsulfonylfluoride and 0.5 mM dithiothreitol. After centrifugation at 6000 × *g* for 5 min, the supernatant cytokines were quantified by ELISA.

Cell proliferation assay

A colorimetric cell viability assay was performed with a water-soluble tetrazolium salt-1 (WST-1) solution (Cell Counting Kits, Dojindo Laboratories, Kumamoto, Japan). Briefly, E11 cells (1×10^4 per 200 μl per well) were seeded in a 96-well microplate, incubated for 12 h and treated with each of the calpain inhibitors or 2.5% sodium azide for 20 h. Then, the

WST-1 solution (10 µl per well) was added, and the culture was continued for an additional 4 h. The number of viable cells was estimated by measuring the optical density at 450 nm (corrected by subtracting the optical density at 600 nm) with a microplate reader (model 550, Bio-Rad, Hercules, CA, USA).

Statistical analysis

Cell counts, mRNA levels and cytokine concentrations were compared among the experimental groups by the unpaired Student's *t*-test, whereas clinical and histopathological arthritis scores were compared by the Mann–Whitney *U*-test. *P*-values <0.05 were considered significant.

Results

Efficacy of calpain inhibitors in murine arthritis

To examine the therapeutic effects of calpain inhibitors on arthritis *in vivo*, experimental arthritis was induced with anti-type II CL mAbs and LPS (25) in the BALB/c strain, which is highly susceptible to this system (11, 27). Similar to a previous report (11), signs of arthritis emerged around 48 h after the LPS injection, peaked at around the eightth day (Fig. 1D) and remitted gradually (Fig. 1A). To prevent the development of the disease, a high dose or low dose of E-64-d or rCS was administered. In the macroscopic examination, the high-dose E-64-d group exhibited significantly lower clinical arthritis scores than the untreated group, while the low-dose E-64-d group and the rCS-treated group showed no improvements (Fig. 1A). The paws of the mice treated with a high dose of E-64-d were less affected than those of the untreated mice (Fig. 1D and E). In the microscopic evaluation, the joints of the mice treated with a high dose of E-64-d showed lighter findings than those of the untreated mice, which exhibited typical arthritic findings such as inflammatory cell infiltration, pannus formation and bone erosion (Fig. 2C and D). The histopathological score for cell infiltration and the total histopathological arthritis score in the high-dose E-64-d group were significantly lower than those in the untreated group (Fig. 2A). E-64-d was considered to be safe since there were no significant differences in macroscopic findings or body weight between the high-dose E-64-d group and the untreated group, although both groups of mice lost weight transiently after the LPS injection (Fig. 1B).

Effects of E-64-d on cytokine expression in vivo

We examined the time courses of IL-1α, IL-1β, IL-6 and TNF-α mRNA expression at the inflamed joints of the disease-affected mice by quantitative RT-PCR. The IL-6 and IL-1β mRNA levels showed prominent surges around 4 h after the LPS injection, then dropped and gradually increased again from around the 48th to the 72nd h (Fig. 3A and B, solid lines). When we examined the effects of LPS alone, the second peaks were not observed (Fig. 3A and B, broken lines). Compared with the IL-6 and IL-1β mRNA, the TNF-α and IL-1α mRNA levels showed different patterns, increasing around the fourth hour and remaining elevated for ~24 h, and then gradually decreasing (Fig. 3C and D). Next, we administered E-64-d to the mice and examined its effects on the cytokine mRNA expression. At the fourth hour, none of the cytokine mRNA

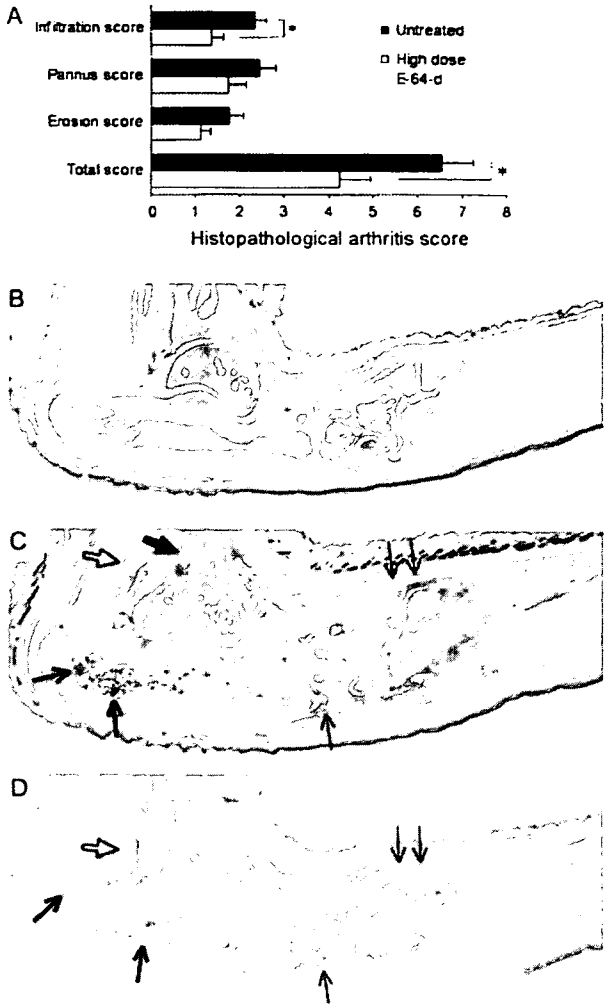

Fig. 2. Effects of calpain inhibitors on histopathological findings of the inflamed joints. The hind paws were sampled on the 19th day after the LPS injection, and hematoxylin- and eosin-stained specimens were prepared. (A) Histopathological arthritis scores are compared between the untreated group and the high-dose (9 mg kg^{-1} per day) E-64-d group. (B–D) Representative microscopic images of the joints. (B) A non-manipulated mouse. (C) A disease-affected mouse, exhibiting typical arthritic findings such as bone erosion (filled arrow), pannus formation (opened arrow), inflammatory cell infiltration (thick arrows) and synovial proliferation (thin arrows). (D) A disease-affected mouse that was treated with a high dose of E-64-d, exhibiting lighter findings than in (C).

levels was affected by E-64-d (Fig. 4). At the 48th h, the IL-6 and IL-1β mRNA levels were significantly decreased by E-64-d, compared with those of the untreated mice (Fig. 4A and B).

Effects of calpain inhibitors on cytokine production in vitro

To examine the effects of the calpain inhibitors *in vitro*, we used a fibroblast-like synovial cell line (E11) established from knee joint tissues of a RA patient (26). After E11 cells were treated with E-64-d, E-64 or rCS, the supernatant IL-6 was quantified by ELISA. We found that E11 cells spontaneously secreted

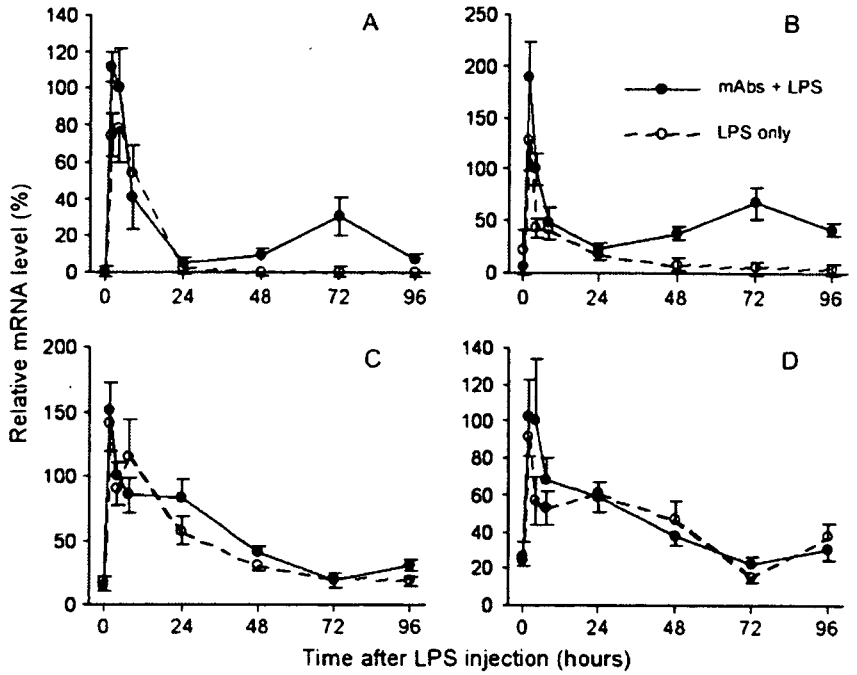

Fig. 3. Time courses of cytokine mRNA expression at the inflamed joints of mice with anti-type II CL antibody-induced arthritis. The arthritis was induced in 40 BALB/c mice, and 5 mice each were sacrificed 0, 2, 4, 8, 24, 48, 72 or 96 h after the LPS injection (solid lines). Other 40 mice were treated with LPS alone, and sacrificed similarly (broken lines). The mRNA levels of IL-6 (A), IL-1β (B), TNF-α (C) and IL-1α (D) at the joints were determined by quantitative RT-PCR. The quantities of the targeted mRNA were normalized by the quantities of control 18S rRNA. The normalized mRNA quantities were compared among the experimental groups. We fixed the mRNA quantities at the fourth hour in the mAbs + LPS group as 100%, and relative mRNA quantities were indicated. Values are means ± SEM.

considerable levels of IL-6 and that E-64-d significantly inhibited the production of IL-6 in a dose-dependent manner (Fig. 5A and B). The significantly effective concentration of E-64-d was 10–30 μg ml^{-1}, consistent with those of other reports (24, 28). The production of IL-6 in E11 cells was markedly enhanced by PMA plus IM, and E-64-d also decreased the production of IL-6 on stimulation with PMA plus IM (Fig. 5C and D). In contrast to E-64-d, E-64 and rCS showed only marginal effects (Fig. 5A–C). Although the MW of rCS (14 kDa) is much larger than that of E-64 (375.4 kDa) or E-64-d (342.4 kDa), even a high dose (4000 μg ml^{-1}) of rCS, equivalent to 100 μg ml^{-1} of E-64-d in molar concentration, did not affect the production of IL-6 in E11 cells (supernatant IL-6 levels were 432 ± 78 and 403 ± 37 pg ml^{-1} in the high-dose rCS-treated and the untreated E11 cells, respectively). Next, since IL-1α, IL-1β and TNF-α could not be detected in the supernatants of E11 cells, we examined intracellular levels of the cytokines by ELISA. Among the three cytokines, IL-1α (mature form) could be detected in E11 cells. E-64-d decreased the production of IL-1α in E11 cells significantly in a dose-dependent manner (Fig. 6B), whereas E-64 and rCS did not show significant effects (Fig. 6A). Suppression of the production of IL-1α by E-64-d was also observed on stimulation with PMA plus IM (Fig. 6C and D).

Since the inhibitory effects of E-64-d on cytokine production might have been due to its direct cytotoxicity, we examined the effects of E-64-d on the viability of E11 cells by a colorimetric WST-1 assay (29). As shown in Fig. 5(E and F), the three calpain inhibitors had neither cytotoxic nor proliferative effects on E11 cells. Finally, we examined the effects of the calpain inhibitors using crude synoviocytes, which were obtained from joint tissues of a disease-active RA patient. Consistent with the results for E11 cells, the crude RA synoviocytes spontaneously secreted IL-6, which was significantly decreased by E-64-d in a dose-dependent manner, while E-64 and rCS did not show significant effects (Fig. 7).

Discussion

We and another group have found a new auto-antibody against calpastatin in RA patients (12, 13). Calpain, the target molecule of calpastatin, is a Ca^{2+}-dependent cysteine proteinase that cleaves a broad range of substrates (18), and is considered to participate in the pathogenesis of RA through the modulation of its substrates. First, calpain converts pro-IL-1α into its mature form (20). Second, it degrades inhibitor κB (IκB), leading to the activation of nuclear factor-κB (NF-κB) (30, 31). Third, it activates neutrophils and promotes granular exocytosis (21, 32).

To clarify the role of calpain in the pathophysiology of RA, we treated an animal model of RA with calpain-inhibitory compounds and examined their biological effects. We found that the administration of a high dose of E-64-d significantly ameliorated the clinical arthritis and the histopathological findings in murine mAb-induced arthritis (Figs 1 and 2). Cuzzocrea *et al.* have previously demonstrated that CL-induced

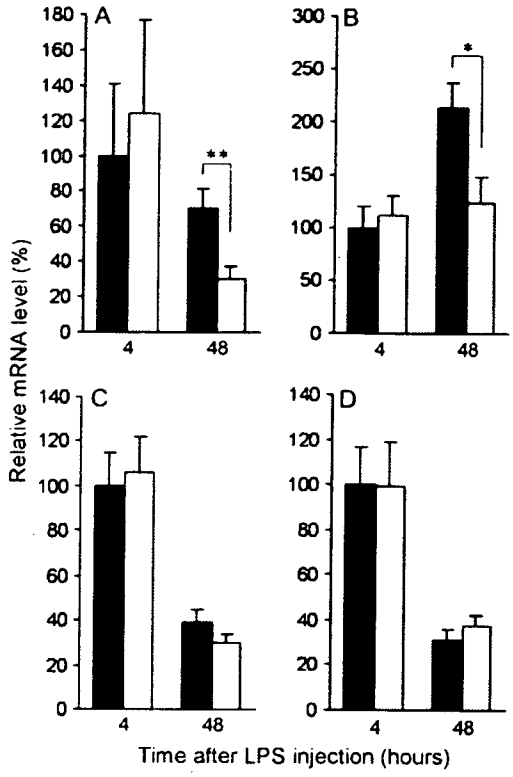

Fig. 4. Effects of E-64-d on cytokine mRNA expression at the inflamed joints of mice. The disease-affected mice were treated with a high dose (9 mg kg^{-1} per day) of E-64-d (white bars) or control diluent (black bars), until they were sacrificed at the 4th or 48th h after the LPS injection. The mRNA levels of IL-6 (A), IL-1β (B), TNF-α (C) and IL-1α (D) at the joints were determined by quantitative RT-PCR. We fixed the mRNA quantities at the fourth hour in the control group as 100%, and relative mRNA quantities were indicated. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

arthritis was successfully treated with calpain inhibitor I (*N*-acetyl-leucyl-leucyl-norleucinal) in rats and that the administration of calpain inhibitor I reduced the levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the inflamed joints (33). Since calpain can activate NF-κB through the degradation of IκB (30, 31), the therapeutic efficacy of calpain inhibitor I was assumed to be brought about by the inhibition of iNOS and COX-2, whose gene expression is induced by NF-κB (34). The contribution of calpain to the activation of NF-κB has been clearly demonstrated by the work of Chen *et al.*, in which the transduction of the *calpastatin* gene into a macrophage-like cell line inhibited the degradation of IκB and the activation of NF-κB in the cells (31).

IL-6 is recognized as an important factor in the pathophysiology of RA since it enhances the proliferation and differentiation of T and B cells, and IL-6 levels in synovial fluids correlate with the disease activity of RA patients (35). However, the relationship between calpain and cytokines such as IL-6 has not been well described. In the present study, we demonstrated that the expression of IL-6 mRNA at the inflamed joints of mice was decreased by the administration of E-64-d (Fig. 4A) and that the production of IL-6 in cultured synoviocytes was inhibited by E-64-d (Figs 5 and 7). Since the gene expression of IL-6 can be enhanced by NF-κB (36, 37), one of the reasons why E-64-d inhibited IL-6 might be its negative effect on NF-κB. However, Kagari *et al.* have reported that IL-6 was not as responsible for the progression of this experimental arthritis as TNF-α and IL-1β were (27). There may be other mechanisms by which E-64-d ameliorated the arthritis.

In the present study, we employed anti-type II CL antibody-induced arthritis, a kind of antibody-transfer model of autoimmune diseases, since the reproducibility of the disease is high (25) and cytokine levels change sharply in this model (27). In our analysis of the time course of cytokine mRNA expression at the affected joints (Fig. 3), the IL-6 and IL-1β mRNA levels showed elevations twice at around 4 and 48–72 h after the LPS injection (Fig. 3A and B, solid lines), whereas the TNF-α and IL-1α mRNA levels showed early peaks and declined gradually (Fig. 3C and D). When we examined the effects of LPS alone, the second peaks of the IL-6 and IL-1β mRNA levels were not observed (Fig. 3A and B, broken lines). These results suggest that the peaks at the fourth hour in all the cytokines, which were not affected by E-64-d (Fig. 4), are related to the direct effects of LPS, and the second elevations of IL-6 and IL-1β mRNA levels around the 48th–72nd h, which were significantly decreased by E-64-d (Fig. 4A and B), are associated with the local immunologic reactions induced by anti-type II CL mAbs. The results also imply that calpain may be responsible for the antibody-induced chronic local inflammatory reactions, but not for the LPS-induced acute systemic inflammation.

IL-1, which stimulates the degradation of cartilage matrices (28), is also an important mediator of RA. Interestingly, calpain is crucial for the activation of IL-1α since it processes pro-IL-1α into its mature form (20). This is confirmed by Fig. 6, which shows that E-64-d inhibited the production of mature IL-1α in E11 cells. Therefore, one of the mechanisms by which E-64-d ameliorated murine mAb-induced arthritis might be the suppression of conversion of IL-1α. Although we could not observe the negative effects of E-64-d on IL-1α mRNA expression *in vivo* (Fig. 4D), it is not inconsistent because the conversion of IL-1α occurs downstream of the mRNA transcription.

In the histopathological evaluation, the score for cell infiltration was significantly decreased by the administration of E-64-d (Fig. 2A). Calpain contributes to the migration of fibroblasts through the degradation of cytoskeletal proteins such as talin (38). Calpain is also associated with the spreading and polarization of neutrophils by regulating Rho-family GTPases (39). The chemotactic migration of cultured fibroblasts was inhibited by E-64-d (40), whereas the chemotactic movement of neutrophils was enhanced by calpain inhibitor I (39). Although the two results seem to be opposite, they at least make sure that calpain plays important roles in cellular migration. Taken together, these mechanisms can be applied to the ameliorative effects of E-64-d on murine mAb-induced arthritis.

Since an excessive amount of calpain is produced in synovial fluids of RA patients (1, 2) and calpain can degrade cartilage matrices including proteoglycans (41), the contribution of calpain to the pathogenesis of RA has been assumed to occur in the extracellular space. However, we found that

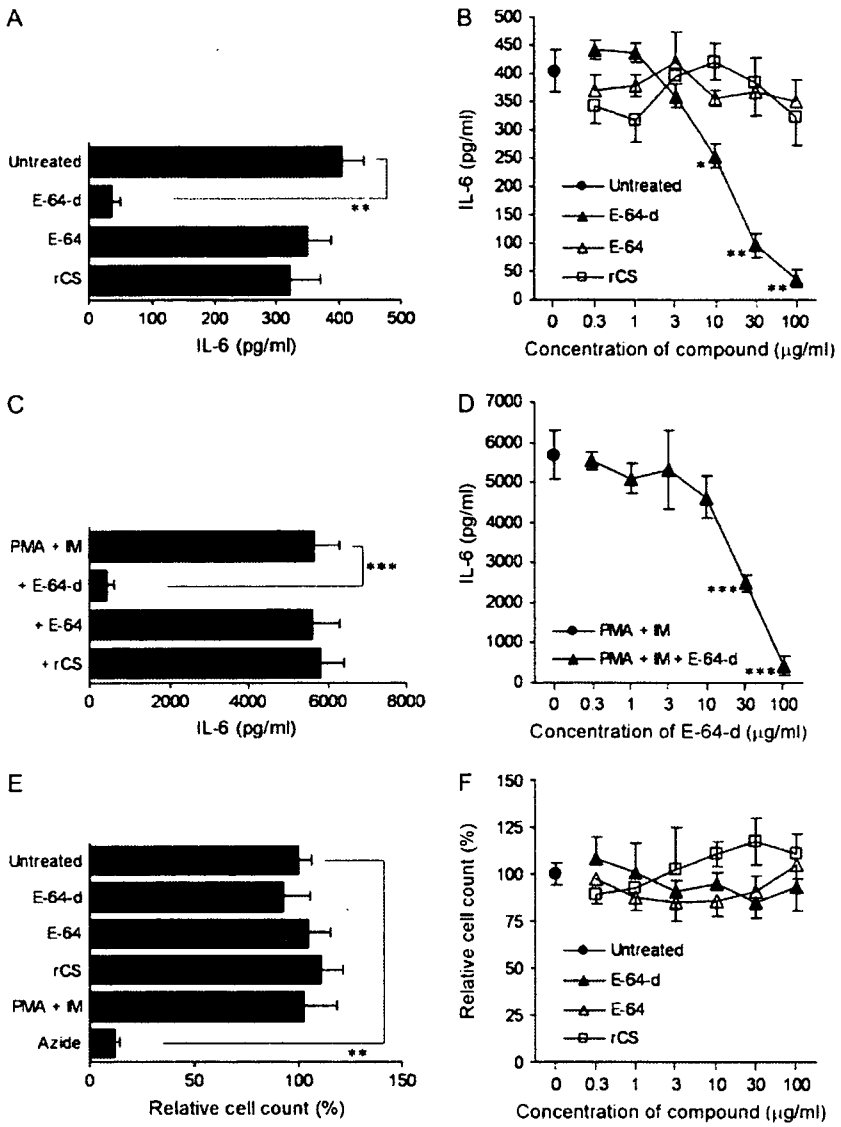

Fig. 5. Effects of calpain inhibitors on IL-6 production in E11 cells. After E11 cells were incubated with the indicated calpain inhibitors for 24 h in the presence (C and D) or absence (A and B) of 10 ng ml^{-1} PMA plus 750 ng ml^{-1} IM, the supernatant IL-6 was quantified by ELISA. (E and F) Effects of calpain inhibitors on viability of E11 cells. After E11 cells were cultured with the indicated calpain inhibitors for 24 h, a colorimetric cell viability assay was performed with WST-1 solution. Relative cell counts among the groups are indicated. In (A, C and E), concentrations of E-64-d, E-64 and rCS were 100 μg ml^{-1}. (B, D and F) Analysis of dose dependency. Values are means ± SEM. $*P < 0.05$, $**P < 0.01$ versus untreated, $***P < 0.01$ versus PMA + IM treated.

non-membrane-permeable E-64 and rCS did not have significant effects on cytokine production *in vivo* and *in vitro*, compared with lipophilic E-64-d (Figs 1A, 5–7). These results suggest that the intracellular calpain is more strongly related to the pathophysiology of RA than the extracellular calpain. We have previously described that anti-calpastatin antibodies of RA patients were able to block the physiological function of calpastatin *in vitro* (12). Theoretically, the blockade of calpastatin by the auto-antibodies results in the activation of calpain, leading to the above-mentioned induction of various inflammatory mediators. Although the mechanism by which the auto-antibodies can interfere with the intracellular calpain–calpastatin system remains unknown, Menard and el-Amine have pointed out the existence of extracellular calpastatin in RA patients, and speculated that the auto-antibodies can bind to the extracellular calpastatin and indirectly affect the inner balance of calpain and calpastatin (19).

E-64-d has been used in order to inhibit calpain *in vivo* and *in vitro*. Ray *et al.* applied E-64-d for the treatment of spinal cord injury in rats, demonstrating that 1 mg kg^{-1} of intravenous E-64-d inhibited calpain and alleviated neuronal apoptosis at the injured spinal cord (23). Tram *et al.* found that

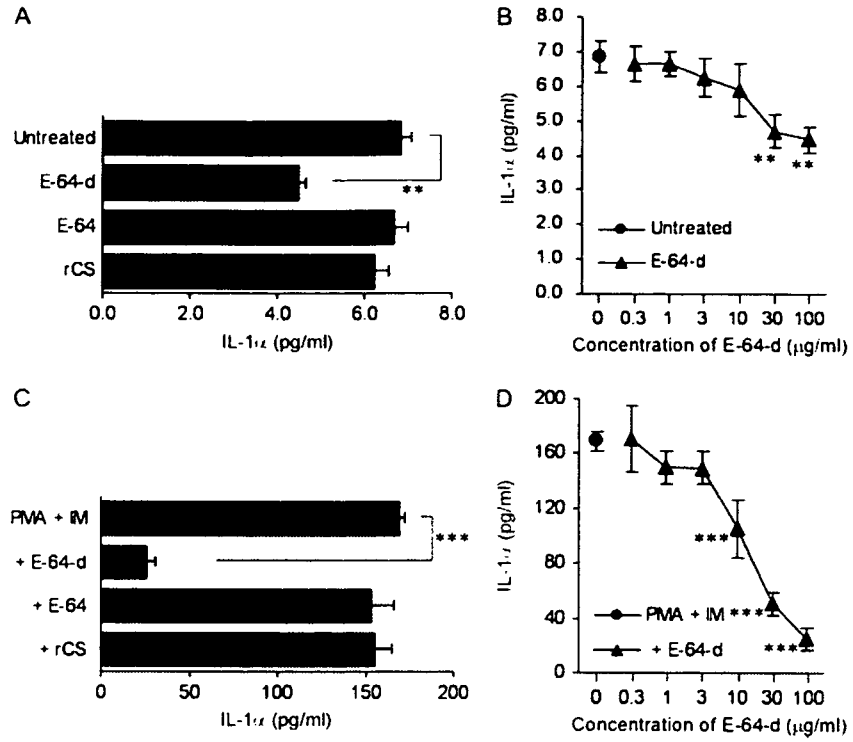

Fig. 6. Effects of calpain inhibitors on IL-1α production in E11 cells. After E11 cells were incubated with the indicated calpain inhibitors for 24 h in the presence (C and D) or absence (A and B) of 10 ng ml^{-1} PMA plus 750 ng ml^{-1} IM, the intracellular IL-1α was extracted and quantified by ELISA. In (A and C), concentrations of E-64-d, E-64 and rCS were 100 μg ml^{-1}. (B and D) Analysis of dose dependency. Values are means ± SEM. $^{**}P < 0.01$ versus untreated, $^{***}P < 0.01$ versus PMA + IM treated.

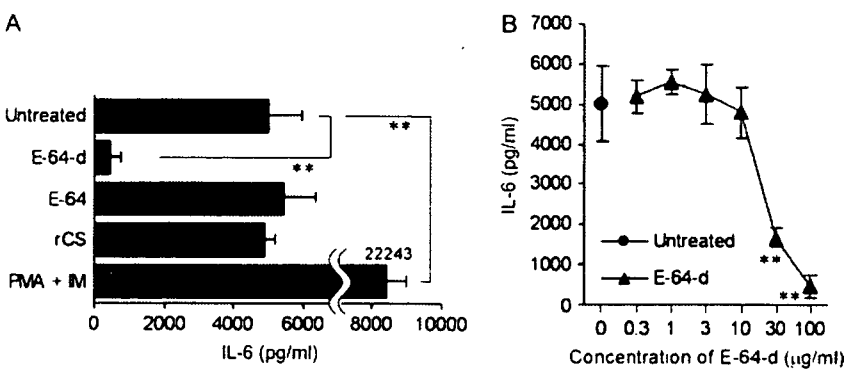

Fig. 7. Effects of calpain inhibitors on IL-6 production in crude RA synoviocytes. Synoviocytes obtained from a disease-active RA patient were incubated with the indicated calpain inhibitors for 24 h, and then the supernatant IL-6 was quantified by ELISA. In (A), concentrations of E-64-d, E-64 and rCS were 100 μg ml^{-1}. (B) Analysis of dose dependency. Values are means ± SEM. $^{**}P < 0.01$ versus untreated.

parathormone-induced contraction of cultured osteoblasts was inhibited by E-64-d but not by non-membrane-permeable E-64-c (24). Since E-64-d and calpain inhibitor I can inhibit not only calpain but also cathepsin (42, 43), it cannot be excluded that the effects of the two compounds on the animal models of arthritis might also be due to the inhibition of cathepsin. To target calpain more selectively, a trial is ongoing to treat cultured synoviocytes or an animal model of RA by transfection of the *calpastatin* gene.

In conclusion, we demonstrated the ameliorative effects of E-64-d, a calpain-inhibitory compound, on an animal model of RA, and presented new evidence for the negative potentiality of E-64-d on cytokine production. These results suggest that calpain contributes to the pathophysiology of arthritis and that

the regulation of the calpain–calpastatin system may be a new strategy to treat arthritic diseases such as RA.

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Abbreviations

CL	collagen
COX-2	cyclooxygenase-2
Ct values	cycle numbers to the threshold
FRP	follistatin-related protein
IκB	inhibitor κB
IM	ionomycin
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneally
MMP	matrix metalloproteinase
MW	molecular weight
NF-κB	nuclear factor-κB
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
rCS	recombinant calpastatin
RT	reverse transcription
TNF-α	tumor necrosis factor-α
WST-1	water-soluble tetrazolium salt-1

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Role of membrane sphingomyelin and ceramide in platform formation for Fas-mediated apoptosis

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Engagement of the Fas receptor (CD95) initiates multiple signaling pathways that lead to apoptosis, such as the formation of death-inducing signaling complex (DISC), activation of caspase cascades, and the generation of the lipid messenger, ceramide. Sphingomyelin (SM) is a major component of lipid rafts, which are specialized structures that enhance the efficiency of membrane receptor signaling and are a main source of ceramide. However, the functions of SM in Fas-mediated apoptosis have yet to be clearly defined, as the responsible genes have not been identified. After cloning a gene responsible for SM synthesis, *SMS1*, we established SM synthase–defective WR19L cells transfected with the human Fas gene (WR/Fas-SM(−)), and cells that have been functionally restored by transfection with *SMS1* (WR/Fas-SMS1). We show that expression of membrane SM enhances Fas-mediated apoptosis through increasing DISC formation, activation of caspases, efficient translocation of Fas into lipid rafts, and subsequent Fas clustering. Furthermore, WR/Fas-SMS1 cells, but not WR/Fas-SM(−) cells, showed a considerable increase in ceramide generation within lipid rafts upon Fas stimulation. These data suggest that a membrane SM is important for Fas clustering through aggregation of lipid rafts, leading to Fas-mediated apoptosis.

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Abbreviations used: aSMase: acid sphingomyelinase, CTx: choleratoxin B; $\Delta\Psi m$, mitochondrial membrane potential; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FasL, Fas ligand; MBP, maltose-binding protein; PI, propidium iodide; SM: sphingomyelin.

Fas, also known as CD95, is a death domain–containing member of the TNFR super family (1). Currently, two distinct pathways that require different initiator caspases (caspase-8, -9, and -10) but converge at activation of executor caspases (caspase-3, -6, and -7) are proposed in Fas-mediated apoptosis signaling (2, 3). In type I cell apoptosis (mitochondrion independent), engagement by Fas ligand (FasL), or anti-Fas antibody (CH11), leads to receptor trimerization and recruitment of the cytoplasmic adaptor protein, Fas-associated death domain (FADD), and procaspase-8 and -10, thus forming a death-inducing signaling complex (DISC). On the other hand, in type II cell apoptosis (mitochondrion dependent), minimal caspase-8 can cleave the Bcl-2 family member Bid. The truncated Bid translocates to mitochondria, where it perturbs the mitochondrial membrane potential ($\Delta\Psi m$) and facilitates the formation of mitochondrial permeability transition pores, resulting in the release of cytochrome *c* (4).

Aggregation and clustering of cell surface receptors on binding to their specific ligands has been reported for a variety of receptors, including the TCR–CD3 complex, B cell receptor, TNFR, epithelial-derived growth factor receptor, CD2, CD44, CD11a/CD18, and Fas,

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and is facilitated by localization of receptor and proximal signaling components within cholesterol, glycosphingolipids, and sphingomyelin (SM)-rich membrane microdomains, known as lipid rafts (5–9). Recently, there is accumulating evidence that rafts are involved in Fas-induced apoptosis through the translocation and clustering of Fas into rafts on stimulation (7, 10, 11). Studies have reported that Fas ligation triggers translocation of the acid sphingomyelinase (aSMase) from an intracellular compartment onto the cell surface, where it hydrolyzes SM to ceramide, and that accumulation of ceramide contributes to transforming small rafts into larger signaling platforms that trap and cluster Fas (7, 10). Rafts have been detected using choleratoxin B (CTx), which binds to ganglioside GM1 colocalized in rafts, and the role of rafts has been evaluated by the disruption of rafts using the cholesterol-chelating reagent methyl-β-cyclodextrin. However, there has been no direct evidence that membrane SM, a major raft component, is involved in raft functions or Fas-mediated apoptosis. Very recently, we and others have succeeded in cloning the human cDNA for SM synthase (*SMS1*; references 12 and 13). Using SM synthesis–deficient cells, and cells in which function has been restored by transfection with the novel *SMS1* gene, we demonstrated that expression of membrane SM enhanced Fas-mediated apoptosis through efficient clustering of Fas itself with a concomitant increase in DISC formation, the activation of caspases, and the loss of $\Delta\Psi$m.

RESULTS

Characterization of SM-deficient and functionally restored cells by transfection with the SM synthase gene

Lysenin is an SM-directed cytolysin purified from the earthworm (14), which binds to membrane SM and induces pore formation in the plasma membrane and subsequent cell death (14). During investigation of the sphingolipid metabolism in SM synthase–defective WR19L mouse lymphoid cells transfected with the human *Fas* gene (WR19L/Fas; reference 15), we established membrane sphigomyelin-deficient cells, WR/Fas-SM(−), which were resistant to lysenin-mediated cell lysis. Recently, we established a functional revertant cell line, designated WR/Fas-SMS1 cells, by transfection of *SMS1* (12). WR/Fas-SMS1 cells exhibited restored SM synthesis assayed by radiolabeling of cellular lipids with [^{14}C]serine (Fig. 1 A) and recovered sensitivity against lysenin-mediated cytolysis (not depicted; reference 12).

Recently, Yamaji-Hasegawa et al. produced a mutant lysenin, which specifically binds to SM without induction of cell death (16). Using the mutant lysenin conjugated with maltose-binding protein (MBP), we examined SM expression on the plasma membrane of WR/Fas-SM(−) and WR/Fas-SMS1 cells by confocal microscopy. Expression of SM detected by lysenin–MBP plus FITC-labeled anti-MBP antibody was positive in WR/Fas-SMS1 but not in WR/Fas-SM(−) cells (Fig. 1 B). Membrane expression of ganglioside GM1 detected by FITC-labeled CTx was observed on both cells (not depicted; reference 12).

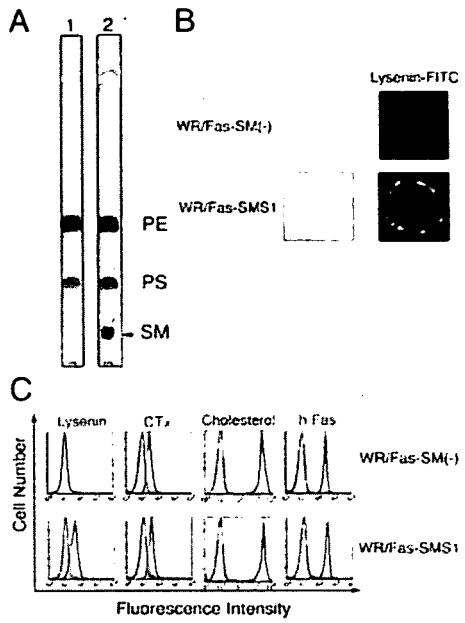

Figure 1. Characterization of WR/Fas-SM(−) and WR/Fas-SMS1 cells. (A) SM synthase activity of WR/Fas-SM(−) (lane 1) and WR/Fas-SMS1 (lane 2) cells. The cellular lipids were labeled with [^{14}C]serine, extracted by the Bligh and Dyer method (reference 53), and assessed by TLC. PE, phosphatidylethanolamine; PS, phosphatidylserine. (B) Analysis of membrane SM expression by confocal microscopy. Cells were stained with lysenin-MBP, and FITC-conjugated anti-mouse IgG mAb, then examined by laser scan confocal microscopy. (C) FACS analysis of membrane sphingolipids. To detect membrane SM, cells were stained with lysenin-MBP (Lysenin), CH11, and FITC-conjugated anti-mouse IgG mAb. Surface expression of ganglioside GM1, cholesterol, and human Fas were analyzed using FITC-conjugated CTx, cholesterol-PEG (cholesterol), or anti-Fas mAB (h-Fas), respectively.

We confirmed the accumulation of SM on the surface of WR/Fas-SM(−) and WR/Fas-SMS1 cells by FACS analysis. Binding of lysenin–MBP was positive in WR/Fas-SMS1 cells but not in WR/Fas-SM(−) cells. We also examined the expression of other lipid components of the plasma membrane, such as ganglioside GM1 and cholesterol, as well as the expression of human Fas. Binding of CTx, which binds the oligosaccharide portion of ganglioside GM1, considered to be a maker of lipid rafts, and binding of fluorescein ester of polyethylene glycol-derivatized cholesterol (fPEG-cholesterol; reference 17), which specifically binds membrane cholesterol, as well as expression of Fas, was detected equally on both cells (Fig. 1 C).

Fas-mediated apoptosis and loss of $\Delta\Psi$m in WR/Fas-SM(−) and WR/Fas-SMS1 cells

Although Itoh et al. have reported that cross-linking of human Fas on WR19L/Fas cells promotes type I cell apoptosis (15), we found that WR/Fas-SM(−) cells are resistant to Fas-mediated apoptosis. Therefore, we used WR/Fas-SM(−) and WR/Fas-SMS1 cells to determine whether membrane SM