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Immunogenetic Features in 120 Japanese Patients with Idiopathic Inflammatory Myopathy

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ABSTRACT. *Objective.* To examine the role of HLA-DRB1 and tumor necrosis factor (TNF) promoter genotypes in the development and the autoantibody profiles of idiopathic inflammatory myopathy (IIM) in Japanese patients.

Methods. HLA-DRB1 and TNF promoter genotypes were determined, and serum antinuclear autoantibodies were identified in 120 adult Japanese patients with IIM [72 with dermatomyositis (DM), 30 with polymyositis (PM), 18 with myositis overlapping with other collagen vascular diseases], as well as in 265 controls.

Results. Forty-two patients (35%) were positive for myositis-specific autoantibodies (MSA), including 37 (31%) for anti-aminoacyl-tRNA synthetase (ARS) autoantibodies. Allele carrier frequency of HLA-DRB1*0803 was increased in the patients with IIM [$p = 0.02$, corrected p (pc) NS, 23% vs 14%, odds ratio (OR) = 1.9 (95% confidence interval, CI = 1.1–3.2)], with PM [$p = 0.006$, pc NS, 33%, OR 3.1 (95% CI 1.3–7.1)], and with anti-ARS autoantibodies [27%, $p = 0.04$, OR 2.3 (95% CI 1.0–5.1)] compared with controls. DRB1*0405 was increased in patients with anti-ARS autoantibodies compared with controls [41% vs 25%, $p = 0.04$, pc NS, OR 2.1 (95% CI 1.0–4.3)]. TNF promoter genotype was associated with the presence of interstitial lung disease (ILD). The carriage of a TNF- α haplotype formed by -1031C, -863A, and -857C was increased in the patients with ILD versus those without ILD [33% vs 18%, $p = 0.05$, pc NS, OR 2.3 (95% CI 0.94–5.5)].

Conclusion. HLA-DRB1 alleles were associated with development of IIM and MSA in a Japanese population. (J Rheumatol 2004;31:1768–74)

Key Indexing Terms:

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We previously reported the association of HLA class I and class II alleles with idiopathic inflammatory myopathy (IIM) by analyzing 84 Japanese patients¹. Our previous study did not examine myositis-specific autoantibodies [MSA; anti-aminoacyl-tRNA synthetases (ARS), anti-signal recognition particle (SRP)] or myositis associated autoantibodies (MAA; anti-RNP, Ro, La, Ku) except for anti-Jo-1 autoantibody.

MSA appear to define clinical features, prognosis, and response to treatment². In Caucasian patients, IIM in the presence of MSA is reportedly associated with HLA-DRB1*0301³. Although HLA-DRB1*0301 is common (around 20%) in the Caucasian population, it is very rare (0.1–0.2%) in Japanese⁴. There have been only a few small studies analyzing HLA alleles of patients with IIM among Japanese^{1,5,6}. There have been no reports of an association of HLA alleles with MSA in Japanese.

In our previous study, HLA-DRB1*08 alleles were significantly increased in the Japanese patients with IIM compared with controls¹. However, the actual mechanism by which HLA regulates susceptibility to myositis remains to be defined. Tumor necrosis factor- α (TNF- α), encoded within the MHC, is a potent proinflammatory cytokine with

a wide range of activities. It plays a critical role in the pathogenesis of inflammatory or autoimmune diseases⁷. A recent report showed blockade of TNF may be beneficial for dermatomyositis (DM) and polymyositis (PM)⁸.

Among the single nucleotide polymorphisms (SNP) of the 5'-flanking region of the TNF gene, the -308A allele has been shown to be associated with high promoter activity⁹ as well as susceptibility/severity to juvenile¹⁰ and adult¹¹ IIM. However, the frequency of -308A allele is very low in the Japanese population (less than 3%)¹².

New SNP, which may affect transcriptional activity, were recently reported at position -1031(T/C), -863(C/A), and -857(C/A) in the 5'-flanking region of TNF, and each substitution was found in a substantial proportion of the Japanese population¹². Four major haplotypes were identified, namely, TCC, TCT, CAC, and CCC, which we tentatively designated TNF-U01, U02, U03, and U04, respectively¹³. The associations of TNF-U03 with Crohn's disease¹⁴ and TNF-U01 with asthma¹⁵ have been reported in the Japanese.

In the present study, we aimed to confirm our previous findings of an association of HLA-DRB1*08 alleles with Japanese IIM patients by analyzing a larger number of patients, and to examine whether the polymorphisms of HLA-DRB1 and TNF promoter contribute to susceptibility and autoantibody production among Japanese with IIM.

MATERIALS AND METHODS

Patients. One hundred twenty Japanese patients with IIM seen from September 1994 to December 2001 were studied: 86 at Institute of Rheumatology; 7 at Department of Neurology, Tokyo Women's Medical University (Tokyo); 19 at Kitasato University School of Medicine (Sagamihara, Kanagawa); 6 at Taga General Hospital (Hitachi, Ibaraki); and 2 at Tokyo Metropolitan Otsuka Hospital (Tokyo). The mean \pm standard deviation (SD) age was 47.9 ± 14.8 years. Thirty of the patients were men and 90 were women. Among these patients, 72 and 30 were classified as having dermatomyositis (DM) and polymyositis (PM), respectively, using criteria proposed by Bohan and Peter^{16,17}. Among them, 52, 36, and 14 patients were diagnosed as definite, probable, and possible DM/PM, respectively. The mean \pm SD age of DM and PM patients was 47.1 ± 14.4 years and 51.8 ± 13.7 years, respectively. Among 72 DM and 30 PM patients, 19 and 9 were men, respectively. Heliotrope and Gottron rash were used to classify the patients as DM. Three patients with DM also had malignancy at the time of diagnosis (uterine carcinoma, malignant lymphoma, and lung cancer).

Eighteen patients had myositis overlapping with other collagen vascular diseases. They fulfilled both sets of Bohan and Peter criteria for myositis^{16,17}, as well as criteria for primary collagen diseases. Among them, 6, 8, and 4 patients were diagnosed as definite, probable, and possible DM/PM, respectively. Twelve of the patients met the American College of Rheumatology (ACR) preliminary classification criteria for systemic sclerosis¹⁸, 4 met the ACR criteria for systemic lupus erythematosus (SLE)¹⁹, and 2 met the ACR criteria for rheumatoid arthritis²⁰. The mean \pm SD age of overlap disease patients was 44.4 ± 17.1 years. Among 18 overlap disease patients, 2 were men and 16 were women.

Among the patients, 64 (56%) were diagnosed as having interstitial lung disease (ILD). ILD was defined by the presence of pulmonary fibrosis seen by chest radiography and/or computed tomography. Information regarding ILD was not available for 5 patients including one patient with anti-ARS autoantibody.

After patients gave informed consent, blood samples were obtained.

Controls. The control group consisted of 265 healthy laboratory personnel and students, 148 of the controls were men, 116 were women, and one was unknown. All controls were unrelated Japanese living in the Tokyo area. HLA-DRB1 and TNF promoter genotypes of the control group have been reported²¹. The central part of Japan has been shown to be relatively homogeneous with respect to genetic background²², permitting the case-control approach to be employed in this study.

Autoantibody studies. We analyzed the plasma samples obtained from September 1994 to December 2001, which had been stored at -20°C until analysis. Autoantibodies were identified by RNA immunoprecipitation technique using HeLa cell extract as an antigen source²³.

Genomic DNA. Genomic DNA from patients and healthy individuals was purified from peripheral blood leukocytes using a standard phenol-chloroform extraction procedure or the QIAamp blood kit (Qiagen, Hilden, Germany).

HLA typing. The HLA-DRB1 genotype was determined using a polymerase chain reaction (PCR) restriction fragment length polymorphism method²⁴.

TNF promoter genotyping. TNF promoter allele formed by SNP at -1031, -863, and -857 was determined using the sequence-specific oligonucleotide probing followed by melting-curve analysis in a real-time PCR machine (LightCyclerTM, Roche Diagnostics, Mannheim, Germany) based on fluorescence resonance energy transfer technology. The detailed genotyping method is described in a previous report²⁵.

Statistical analysis. For the comparison of genotypes, allele carrier frequencies (homozygotes and heterozygotes combined) were compared. Statistical significance of the differences between groups was determined by chi-square analysis or Fisher's exact probability test. Corrected *p* (*pc*) values were obtained by multiplying the observed *p* values by the number of alleles examined; namely, 23 for HLA-DRB1, 12 for HLA-DR (serologic specificity), and 4 for TNF. The odds ratio (OR) with 95% confidence interval (95% CI) was calculated. To determine the sex differences in genetics, each allele carrier frequency was compared among patients and controls in each sex.

RESULTS

Autoantibody frequencies in different myositis syndromes. Frequencies of MSA and MAA in different forms of myositis are shown in Table 1. Among 120 patients with IIM, 42 (35%) were positive for MSA, including 37 (31%) for anti-ARS and 5 (4%) for anti-SRP autoantibody. Of the anti-ARS autoantibodies, anti-Jo-1 autoantibody was the most common (15%), and anti-EJ (9%) autoantibody was the second. Anti-SRP autoantibody was found exclusively in patients with PM.

MSA occurred more commonly in patients with PM (63%) than in those with DM [26%, $p = 0.0004$, OR 4.8 (95% CI 1.9–12.0)] or in patients with overlap disease [22%, $p = 0.006$, OR 6.0 (95% CI 1.6–23.0)]. Anti-ARS autoantibodies were more common in PM (47%) versus DM [26%, $p = 0.046$, OR 2.4 (95% CI 1.0–5.9)]. Anti-Jo-1 was present in 27% of patients with PM versus 11% with DM [$p = 0.049$, OR 2.9 (95% CI 0.98–8.7)]. Six DM patients, 4 PM, and one with overlap disease were positive for anti-EJ autoantibody.

Among 36 patients with anti-ARS autoantibodies, 28 (78%) had complicating ILD, while 36 (46%) of the remaining 79 antibody-negative patients had this pulmonary complication [$p = 0.001$, OR 4.2 (95% CI 1.7–10.3)].

Table 1. Autoantibody frequencies in Japanese patients with IIM. Values are the number (%) of patients with autoantibodies.

Autoantibody	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18
Myositis-specific				
Anti-aminoacyl-tRNA synthetases	37 (31)	19 (26)	14 (47)*	4 (22)
Anti-Jo-1	18 (15)	8 (11)	8 (27)**	2 (11)
Anti-EJ	11 (9)	6 (8)	4 (13)	1 (6)
Anti-PL-7	4 (3)	2 (3)	2 (7)	0
Anti-PL-12	3 (3)	3 (4)	0	0
Anti-OJ	1 (1)	0	0	1 (6)
Anti-SRP	5 (4)	0	5 (17)	0
Any of above	42 (35)	19 (26)	19 (63)†	4 (22)
Myositis-associated				
Anti-tRNA	1 (1)	0	0	1 (6)
Ro (SSA)	21 (18)	11 (15)	8 (27)	2 (11)
U1-RNP	11 (9)	1 (1)	1 (3)	9 (50)††
U2-RNP	1 (1)	0	0	1 (6)
La (SSB)	1 (1)	1 (1)	0	0
Ku	1 (1)	0	0	1 (6)

IIM: idiopathic inflammatory myopathy; DM: dermatomyositis; PM: polymyositis; Overlap: overlap disease patients. * $p = 0.046$, OR 2.4 (95% CI 1.0–5.9) vs DM; ** $p = 0.049$, OR 2.9 (95% CI 0.98–8.7) vs DM; † $p = 0.0004$, OR 4.8 (95% CI 1.9–12.0) vs DM, $p = 0.006$, OR 6.0 (95% CI 1.6–23.0) vs Overlap; †† $p = 4 \times 10^{-9}$, OR 71 (95% CI 8.0–627.6) vs DM, $p = 0.0001$, OR 29 (95% CI 3.2–261.0) vs PM.

Among myositis-associated autoantibodies, anti-Ro (SSA) was most commonly found in myositis patients (18%), while its frequency did not differ significantly among the 3 groups. In contrast, anti-U1 RNP autoantibody frequency was significantly increased in overlap disease patients (50%) compared with DM [$p = 4.4 \times 10^{-9}$, OR 71 (95% CI 8.0–627.6)] or PM [$p = 0.0001$, OR 29 (95% CI 3.2–261.0)]. All patients with anti-U1 RNP autoantibody were women.

HLA-DRB1 alleles in patients with myositis. Among all IIM patients, allele carrier frequency of HLA-DRB1*0803 was significantly increased [$p = 0.02$, pc NS, OR 1.9 (95% CI 1.1–3.2)] as compared with controls (Table 2). When DRB1*0802 and 0803 were combined, the association with all IIM remained significant [$p = 0.02$, pc NS, OR 1.8 (95% CI 1.1–3.0)]. DRB1*1302 was significantly decreased in all IIM patients [$p = 0.01$, pc NS, OR 0.4 (95% CI 0.21–0.85)] compared with control patients (Table 2).

Carrier frequency of DRB1*0101 was significantly increased in patients with overlap disease [$p = 0.002$, pc NS, OR 4.6 (95% CI 1.6–13.3)] compared with control patients (Table 2). Although DRB1*0803 appeared increased in both DM and PM groups compared with controls (Table 2), the difference was significant only in the PM group [$p = 0.006$, pc NS, OR 3.1 (95% CI 1.3–7.1)]. When DRB1*0802 and *0803 alleles were combined, a significant increase was observed in PM compared with controls [$p = 0.02$, pc NS, OR 2.5 (95% CI 1.2–5.6)]. The associations of HLA-DRB1*08 or *0803 with IIM or PM, which we previously

reported¹, were confirmed in this study with a larger number of patients.

Carrier frequency of the HLA-DRB1 first hypervariable region sequence (9EYSTS13), shared by DR3, DR5, DR6, and DR8 alleles, reported as candidate epitope for IIM²⁶, was not different between all IIM patients and controls.

HLA-DRB1 alleles in autoantibody subsets of myositis. Carrier frequency of DRB1*0405 was increased in patients with anti-ARS autoantibodies compared with controls [$p = 0.04$, pc NS, OR 2.1 (95% CI 1.0–4.3)] (Table 2). HLA-DRB1*0802 [$p = 0.047$, pc NS, OR 2.6 (95% CI 0.98–7.2)] and *0803 [$p = 0.04$, pc NS, OR 2.3 (95% CI 1.0–5.1)] were increased in the patients with anti-ARS autoantibody compared with controls (Table 2). The frequency of HLA-DRB1*1502 was significantly decreased in patients with anti-ARS autoantibody compared with controls [$p = 0.03$, pc NS, OR 0.22 (95% CI 0.05–0.96)] and MSA negative patients [$p = 0.007$, pc NS, OR 0.16 (95% CI 0.034–0.70)] (Table 2). The frequency of 9EYSTS13 carriers was not significantly different among MSA negative patients, the patients with anti-ARS autoantibodies, and controls (Table 2).

TNF promoter haplotypes in myositis patients and autoantibody subsets of myositis. Four TNF haplotypes (U01, U02, U03, and U04) were present (Table 3). No significant differences in TNF haplotype distribution were observed between IIM patients and controls, or among patients with PM, DM, or overlap disease.

HLA-DRB1 and TNF promoter genotypes with and without

Table 2. Carrier frequencies of HLA-DRB1 alleles in Japanese IIM patients and controls. Values are the number (%) of subjects carrying each HLA-DRB1 allele (homozygotes and heterozygotes combined).

HLA-DRB1	Clinical Subsets				Antibody Subsets		Controls, n = 265
	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18	MSA (-), n = 78	ARS (+), n = 37	
*0101	21 (18)	12 (17)	3 (10)	6 (33)***	15 (19)	6 (16)	26 (10)
*0401	1 (1)	1 (1)	0	0	1 (1)	0	6 (2)
*0403	6 (5)	3 (4)	2 (7)	1 (6)	3 (4)	3 (8)	13 (5)
*0405	35 (29)	24 (33)	6 (20)	5 (28)	19 (24)	15 (41) [#]	65 (25)
*0406	7 (6)	5 (7)	1 (3)	1 (6)	5 (6)	2 (5)	19 (7)
*0407	0	0	0	0	0	0	6 (2)
*0410	4 (3)	2 (3)	2 (7)	0	3 (4)	1 (3)	5 (2)
*0802	11 (9)	8 (11)	2 (7)	1 (6)	5 (6)	6 (16) [‡]	18 (7)
0803	28 (23)	14 (19)	10 (33) [†]	4 (22)	16 (21)	10 (27) ^{§§}	37 (14)
*0901	29 (24)	15 (21)	6 (20)	8 (44)	19 (24)	10 (27)	77 (29)
*1101	1 (1)	0	1 (3)	0	0	1 (3)	5 (2)
*1201	5 (4)	5 (7)	0	0	2 (3)	3 (8)	19 (7)
*1202	5 (4)	3 (4)	2 (7)	0	3 (4)	2 (5)	11 (4)
*1302	11 (9)**	8 (11)	3 (10)	0	8 (10)	2 (5)	51 (19)
*1401	8 (7)	7 (10)	1 (3)	0	7 (9)	1 (3)	13 (5)
*1403	5 (4)	4 (6)	1 (3)	0	4 (5)	1 (3)	10 (4)
*1405	4 (3)	2 (3)	1 (3)	0	2 (3)	1 (3)	17 (6)
*1406	2 (2)	2 (3)	0	0	2 (3)	0	7 (3)
*1501	18 (15)	8 (11)	6 (20)	4 (22)	12 (15)	4 (11)	29 (11)
*1502	26 (22)	17 (24)	5 (17)	4 (22)	21 (27)	2 (5) ^{##}	54 (20)
*1602	4 (3)	1 (1)	2 (7)	1 (6)	3 (4)	1 (3)	8 (3)
Others	2 (2)	0	0	0	2 (3)	0	13 (5)
9EYSTS13	70 (58)	46 (64)	20 (67)	5 (28) ^{††}	43 (55)	24 (65)	161 (61)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody. * $p = 0.02$, p_c NS, OR 1.9 (95% CI 1.1–3.2) vs controls; ** $p = 0.01$, p_c NS, OR 0.4 (95% CI 0.21–0.85); *** $p = 0.002$, p_c NS, OR 4.6 (95% CI 1.6–13.3) vs controls; [†] $p = 0.006$, p_c NS, OR 3.1 (95% CI 1.3–7.1) vs controls; ^{††} $p = 0.006$, OR 0.25 (95% CI 0.086–0.72) vs controls; $p = 0.0006$, OR 0.22 (95% CI 0.07–0.68) vs DM; $p = 0.009$, OR 0.19 (95% CI 0.053–0.69) vs PM; [#] $p = 0.04$, p_c NS, OR 2.1 (95% CI 1.0–4.3) vs controls; [‡] $p = 0.047$, p_c NS, OR 2.6 (95% CI 0.98–7.2) vs controls; ^{§§} $p = 0.04$, p_c NS, OR 2.3 (95% CI 1.0–5.1) vs controls; ^{##} $p = 0.03$, p_c NS, OR 0.22 (95% CI 0.05–0.96) vs controls; $p = 0.007$, p_c NS, OR 0.16 (95% CI 0.034–0.70) vs MSA negative patients.

Table 3. Carrier frequency of TNF promoter genotypes in Japanese patients with IIM and controls. Values are number (%) of patients carrying each TNF allele.

TNF	Clinical Subsets				Antibody Subsets		Controls, n = 265
	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18	MSA (-), n = 77	ARS (+), n = 37	
U01	103 (86)	60 (83)	26 (87)	17 (94)	68 (88)	29 (78)	232 (88)
U02	40 (33)	26 (36)	9 (30)	5 (28)	23 (30)	14 (38)	83 (31)
U03	30 (25)	20 (28)	6 (20)	4 (22)	19 (25)	11 (30)	69 (26)
U04	7 (6)	3 (4)	2 (7)	2 (11)	3 (4)	2 (5)	14 (5)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody.

ILD. No significant association was observed between HLA-DRB1 and the presence or absence of ILD (Table 4). When the TNF promoter was examined, carrier frequency of TNF-U03 was found to be increased in patients with ILD as compared with those without ILD [33% vs 18%, $p = 0.05$, p_c NS, OR 2.3 (95% CI 0.94–5.5)].

Analysis of sex effect. The sex distributions were not significantly different among DM, PM, and overlap disease

patients. To examine the sex-specific effects, the frequencies of HLA-DRB1 and TNF alleles were compared between patients and controls in each sex separately (Table 5). When the HLA-DRB1 carrier frequencies were compared, DRB1*0101 was increased in female patients with IIM compared with female controls [$p = 0.05$, p_c NS, OR 2.1 (95% CI 0.99–4.4)]. DRB1*0405 was increased in male IIM patients [$p = 0.02$, p_c NS, OR 2.6 (95% CI 1.1–5.8)] and

Table 4. Carrier frequencies of *HLA-DRB1* and *TNF* promoter genotypes in Japanese IIM patients with and without interstitial lung disease (ILD). Values are the number (%) of patients.

Genotype	ILD (+), n = 64	ILD (-), n = 51	p	OR (95% CI)
<i>HLA-DRB1*0405</i>	20 (31)	11 (22)	NS	—
<i>HLA-DRB1*0803</i>	14 (22)	13 (25)	NS	—
<i>TNF-U01</i>	55 (86)	44 (86)	NS	—
<i>TNF-U02</i>	19 (30)	17 (33)	NS	—
<i>TNF-U03</i>	21 (33)	9 (18)	0.05	2.3 (0.94–5.5)
<i>TNF-U04</i>	2 (3)	4 (8)	NS	—

NS: not significant.

male IIM patients without MSA [$p = 0.05$, p_c NS, OR 2.5 (95% CI 0.98–6.5)] compared with male controls. *DRB1*0803* was increased in both male and female IIM patients compared with individual control subjects. The carrier frequency was significantly increased in male patients with IIM without MSA [$p = 0.045$, p_c NS, OR 2.7 (95% CI 0.99–7.5)] and female IIM patients with anti-ARS autoantibody [$p = 0.04$, p_c NS, OR 2.8 (95% CI 1.0–7.5)] compared with individual controls. The distributions of *TNF* haplotypes were not significantly different between patients and controls even when male and female patients were analyzed separately (data not shown).

DISCUSSION

The number of patients analyzed in our study is the largest among the Japanese IIM studies examining autoantibodies and *HLA-DRB1*. The distribution of autoantibody subsets in our series was similar to that in a previous Japanese study examining 91 IIM patients²⁷. Anti-EJ was the second most common MSA and seemed to be more common in our series than in those reported in Caucasians⁵. Anti-EJ was associated with PM as well as DM in our series, whereas others found that this autoantibody was associated with DM^{28,29}. Ohson, et al also reported that 4 out of 13 Japanese patients with anti-ARS autoantibodies had anti-EJ, and anti-EJ was the second most common anti-ARS autoantibody. We did not examine anti-Mi-2 autoantibody in this study because

this autoantibody has been reported to be very rare (0%) in Japanese IIM patients⁵.

In this study, our previous findings of an association of *HLA-DRB1*08* and **0803* with Japanese IIM ($n = 84$)¹ were confirmed by analyzing a larger number of patients ($n = 120$). We also found significant associations of *HLA-DRB1*08* and **0803* with anti-ARS autoantibodies. Since the most common MSA are directed against ARS, these 2 results are consistent. The genetic risk factors for IIM and MSA appear to be different between Japanese (*DR8, DRB1*0803*) and Caucasians (*DR3, DRB1*0301*)³.

DR8 alleles are reportedly increased in African-American IIM patients⁵, Hispanic SLE patients³⁰, and Korean autoimmune thyroiditis patients³¹. Both SLE³⁰ and autoimmune thyroiditis³² have been reported to be associated with *DR3* in Caucasians. Thus, *DR8* alleles might be related to the susceptibility to IIM, SLE, and autoimmune thyroiditis in the ethnic groups in which *DR3* alleles are rare. It was suggested that *DR8* gene was generated by a gene contraction event in a primordial *DR52* haplotype (*DR3, DR11, to DR14*)³³. *DR3* and *DR8* nucleotide sequences have been reported to be similar at introns 4 and 5³⁴ as well as the 5' end³³. These shared sequences between *DR3* and *DR8* may be related to the pathogenesis of IIM.

Arnett, et al⁵ reported that *DR2* alleles (*DRB1*15* alleles) were decreased in Caucasian and African-American patients with PM. Also, we found that *DRB1*1502* was significantly decreased in Japanese patients with anti-ARS autoantibodies compared with controls. Thus, Japanese patients with IIM may share some common features of *DRB1* alleles with other ethnic groups.

We found an association between *DRB1*0405* and anti-ARS autoantibodies in Japanese IIM patients. And the frequency of *HLA-DRB1*0405* seemed to be increased in DM patients compared with controls; however, the difference was not statistically significant. Horiki, et al reported a significant association between Japanese PM patients with ILD and *DRB1*0405*⁶; however, our current study was not able to confirm this. *HLA-DRB1*0405* was associated with anti-ARS autoantibodies in our present study. Since 28 (78%) patients with anti-ARS autoantibodies had complicating ILD, it may be possible that the association reported

Table 5. Carrier frequencies of *HLA-DRB1* alleles in Japanese IIM patients and controls by sex. Values are the number (%) of patients.

<i>HLA-DRB1</i>	Male				Female			
	All IIM, n = 30	MSA (-), n = 21	ARS (+), n = 8	Controls, n = 148	All IIM, n = 90	MSA (-), n = 57	ARS (+), n = 29	Controls, n = 116
0101	1 (3)	0	1 (13)	12 (8)	20 (33)	15 (26)	5 (17)	14 (12)
*0405	13 (43)**	9 (43)***	4 (50)	34 (23)	22 (24)	9 (16)	11 (38)	31 (27)
*0803	9 (30)	7 (33) [†]	2 (25)	23 (16)	19 (21)	9 (16)	8 (28) ^{††}	14 (12)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody. * $p = 0.05$, p_c NS, OR 2.1 (95% CI 0.99–4.4); ** $p = 0.02$, p_c NS, OR 2.6 (95% CI 1.1–5.8); *** $p = 0.05$, p_c NS, OR 2.5 (95% CI 0.98–6.5); [†] $p = 0.045$, p_c NS, OR 2.7 (95% CI 0.99–7.5); ^{††} $p = 0.04$, p_c NS, OR 2.8 (95% CI 1.0–7.5).

by Horiki, *et al* was primarily due to the association with anti-ARS autoantibodies. In Japanese, DRB1*0405 allele is associated with rheumatoid arthritis³⁵, Crohn's disease¹⁴, arthritis, and rheumatoid factor in systemic sclerosis³⁶. These results suggest a similar genetic background among these autoimmune diseases in Japanese.

Since the HLA-DQA1*0501 or *0401 allele was reported to be commonly increased in Caucasian and African-American patients with IIM, while different DRB1 alleles were associated with the disease in each ethnic population, Arnett, *et al* suggested that susceptibility to IIM may be localized in the DQA1 locus⁵. However, we¹ and others^{5,6} did not observe that the susceptibility to IIM was primarily associated with the DQA1 locus in the Japanese. We also examined distribution of MSA in 83 IIM patients for whom DQA1 alleles were analyzed in our previous study and found no significant association of MSA with DQA1 alleles (data not shown).

We found an association of TNF-U03 with ILD in this study. Previous studies concerning the promoter activity of TNF-U03 are conflicting, i.e., increased¹², unchanged³⁷, or decreased³⁸. TNF is considered to have a disease-promoting effect for ILD^{39,40} and some reports suggest that blockade of TNF may be beneficial for ILD⁴¹. TNF-U03 was recently reported to show a higher binding to the transcriptional factor OCT-1, compared with the common TNF-U01 allele⁴².

Our results suggest that the association of HLA-DRB1*0101 and HLA-DRB1*0405 with IIM may be influenced by sex. Although previous IIM studies have not showed any sex differences in genetics, we⁴³ and others^{44,45} have reported significant sex influences on modifier loci in other autoimmune diseases. Our data suggest that the association of some loci with IIM may be influenced by sex.

We confirmed in this study our previous findings of immunogenetic features of Japanese patients with IIM. Further studies of non-HLA as well as HLA genes are needed for determining the genetic contribution of the susceptibility and the pathogenesis of IIM.

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Ameliorative Effects of Follistatin-Related Protein/TSC-36/FSTL1 on Joint Inflammation in a Mouse Model of Arthritis

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Objective. To clarify the *in vivo* function of follistatin-related protein (FRP)/TSC-36/FSTL1 in rheumatoid arthritis (RA), we investigated the roles of FRP in a mouse model of arthritis.

Methods. Arthritis was induced in BALB/c mice by injecting anti-type II collagen monoclonal antibody and lipopolysaccharide. Mice were treated with daily intraperitoneal injections of 20 µg of recombinant FRP. Development of arthritis was assessed by the clinical score and footpad swelling. Histologic examination of affected paws was performed on day 21 after the onset of arthritis. The gene expression profiles of affected paws in FRP-treated and untreated mice were compared using commercially available complementary DNA (cDNA) arrays. The difference in gene expression was confirmed by real-time quantitative reverse transcription-polymerase chain reaction.

Results. Treatment with recombinant FRP showed significant amelioration of the arthritis severity. Histologic analyses confirmed this finding and revealed the alleviation of cellular infiltration into the synovium as well as cartilage damage. The significant decrease in the amount of urinary deoxyypyridinoline also indicated

the ameliorative effect of FRP on joint destruction. Moreover, cDNA array analysis of the gene expression profile in FRP-treated arthritic lesions revealed a reduced expression of the *c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9* genes, some of which are thought to be associated with synovial inflammation and joint destruction.

Conclusion. These findings from *in vivo* experiments suggest that FRP could be one of the key molecules in the treatment of inflammatory joint diseases such as RA.

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by destructive polyarthritis, and its etiology remains to be elucidated. In the pathogenesis of RA, an antigen-driven immune response involving synovial T cells and B cells is thought to play a central role in persistent joint inflammation (1). Therefore, several self antigens, such as type II collagen (CII), heat-shock protein 60, calpastatin, human cartilage glycoprotein 39, and glucose-6-phosphate isomerase, have been reported to be candidate target antigens of pathogenic autoantibodies in RA (2-6).

In an effort to find such antigens, we have cloned novel target proteins of autoantibodies from RA synovium with the use of RA synovial fluid IgG probes by an expression cloning method; one of these clones was follistatin-related protein (FRP) (7). FRP was originally isolated as the product of TSC-36, one of the genes found to be up-regulated by transforming growth factor β1 (TGFβ1) in the mouse osteoblastic cell line MC3T3-E1 (8). Rat and human homologs have been cloned from glioma cell lines (9).

FRP is a secreted glycoprotein, and it is expressed in all organs, except for peripheral blood leukocytes (7). FRP shares a characteristic structural module (the FS domain) with follistatin, an inhibitor of activin

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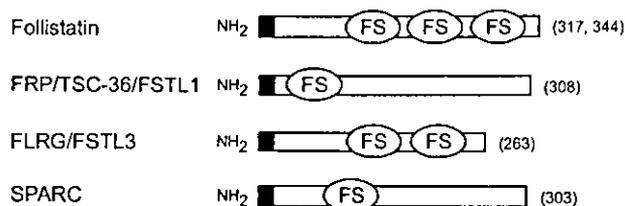


Figure 1. Schematic representation of follistatin and related FS domain-bearing proteins. Follistatin and follistatin-related gene protein (FLRG)/follistatin-like 3 (FSTL3) have more than 2 FS domains in the COOH terminus. Follistatin-related protein (FRP)/TSC-36/FSTL1 contains a single follistatin domain in the NH₂ terminus. Numbers in parentheses represent amino acid residues in the human proteins. Signal peptides are shown in the solid portions of the bars.

(10) (Figure 1). In addition to follistatin and FRP, other FS domain-bearing proteins, such as secreted protein, acidic and rich in cysteine (SPARC), SC1/hevin, testican, QR1, and follistatin-related gene protein (FLRG), have been cloned, and together, they compose the follistatin family (11). FLRG is a translated product of follistatin-related gene (*FLRG*), which was isolated from a B cell leukemia cell line (12). FRP and FLRG are different proteins (12,13) and are listed in the OMIM database under the names follistatin-like 1 (FSTL1) and follistatin-like 3 (FSTL3), respectively (13,14). Follistatin and FLRG have activin-binding activity and inhibit activin-mediated gene transcription (15). Other follistatin family proteins, including FRP, have not been demonstrated to bind activin or related TGF β family proteins. SPARC, the best-studied of the group, is known to regulate cell-matrix interactions and thereby regulate tissue remodeling and homeostasis (11). FRP has been reported to play some roles in cell-cycle inhibition and to have negative regulatory effects on growth in human lung cancer cells (8,16). However, the exact physiologic function of FRP remains to be clarified.

We previously reported that serum autoantibodies to FRP were detected in 30% of patients with RA, and their appearance correlated with disease activity (7). Recently, our *in vitro* experiments demonstrated that FRP suppressed the production of matrix metalloproteinase 1 (MMP-1), MMP-3, and prostaglandin E₂ in synovial cells derived from patients with RA, and suggested that FRP could act as a joint defensive factor in RA (17).

To examine the *in vivo* effect of FRP on arthropathy in inflammatory joint diseases such as RA, recombinant FRP was administered in a mouse model of arthritis. In this experiment, we induced arthritis in mice with the use of anti-CII monoclonal antibodies and

lipopolysaccharide (LPS) in order to achieve high reproducibility (18). We then examined the macroscopic and microscopic effects of FRP on arthritis. To obtain insight into the FRP signaling pathway, we analyzed the gene expression profiles of affected joint tissues using complementary DNA (cDNA) arrays.

MATERIALS AND METHODS

Recombinant human FRP. *Escherichia coli*-expressed glutathione S-transferase (GST)-FRP was prepared as previously described (7). The GST tag was removed from GST-FRP using a GSTrap FF column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the protein was purified by an anion-exchange chromatography method with a Mono Q HR column (Amersham Pharmacia Biotech). Finally, the protein was filtered with a Zetapore Dispo filter (CUNO, Meriden, CT) to remove endotoxins.

Induction and evaluation of arthritis. Arthritis was induced in 7-8-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) by intraperitoneal injection with 500 μ l of phosphate buffered saline (PBS) containing 4 monoclonal antibodies to CII (500 μ g of D1, D8, A2, and F10, respectively; Chondrex, Redmond, WA). After 72 hours, the mice were injected intraperitoneally with 100 μ l of PBS containing 50 μ g of LPS (Sigma, St. Louis, MO). The mice were maintained under specific pathogen-free conditions.

The severity of arthritis in each paw was estimated in a blinded manner. Paws were assessed by both visual inspection and measurement of limb swelling with calipers. Each paw was scored on a scale of 0-3, as previously described (19), where 0 = no swelling, 1 = swelling of finger joints or mild swelling of the ankle or wrist joint, 2 = severe inflammation of the entire paw, and 3 = deformity or ankylosis. The severity of arthritis in each mouse was the sum of the scores in the 4 paws (maximum possible score 12). Institutional approval of the bioethics of the study was obtained prior to these experiments (approval no. 02004).

Treatment with FRP. The mice were randomly assigned to 1 of 2 treatment groups before the onset of arthritis: FRP treatment or PBS treatment (control). The mice in the FRP treatment group were injected intraperitoneally with 20 μ g (1 mg/kg) of recombinant human FRP daily for 10 days, starting 1 hour before injection of the monoclonal antibody. The mice in the PBS treatment group were injected intraperitoneally with 250 μ l of PBS daily for 10 days.

Histopathologic evaluation. Pairs of arthritic hind paws from representative mice in each group ($n = 6$ animals, 12 paws) were removed at the end of the experiment (day 21), fixed in 10% buffered formalin, decalcified in 10% formic acid, and then embedded in paraffin. Serial sagittal sections of whole hind paws were subjected to hematoxylin and eosin staining for histologic analyses and to Safranin O and fast green/iron hematoxylin staining for estimation of the loss of cartilaginous proteoglycan. Synovial lesions were scored on a scale of 0-3, where 0 = normal synovium, 1 = mild synovial hyperplasia and/or mild synovial infiltration, 2 = moderate synovial hyperplasia and/or moderate synovial infiltration, and 3 = severe synovial hyperplasia and/or severe synovial infiltra-

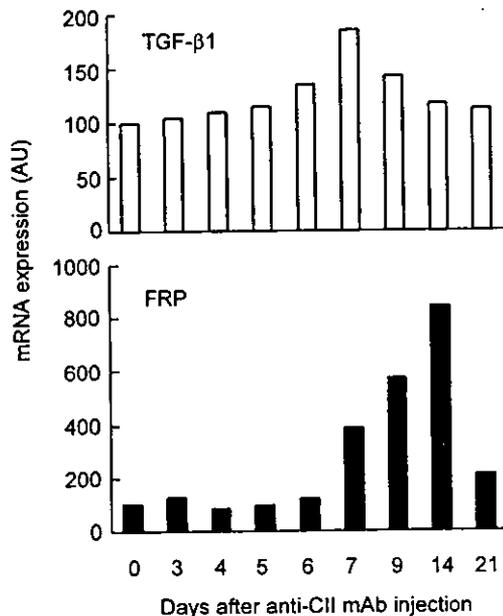


Figure 2. Changes in expression levels of transforming growth factor $\beta 1$ (TGF $\beta 1$) and follistatin-related protein (FRP) mRNA during the development of arthritis. Levels of TGF $\beta 1$ and FRP mRNA expression (in arbitrary units [AU]) were quantified by real-time reverse transcription–polymerase chain reaction. Anti-CII = anti-type II collagen; mAb = monoclonal antibody.

tion. Cartilage depletion was identified by the presence of diminished Safranin O staining of the matrix and was scored on a scale of 0–3, where 0 = no cartilage destruction (full staining with Safranin O), 1 = localized cartilage erosions, 2 = more extended cartilage erosions, and 3 = severe cartilage erosions (depletion of entire cartilage). Histologic analyses were performed in a blinded manner.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) for TGF $\beta 1$ and FRP messenger RNA (mRNA) expression. Groups of mice were killed on days 0, 3, 4, 5, 6, 7, 9, 14, and 21, and total RNA was obtained from 6 arthritic hind paws in each group by mixing with TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Five micrograms of total RNA was subjected to RT using the SuperScript First Strand Synthesis system (Invitrogen, Carlsbad, CA). Each reaction was run in duplicate using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for TGF $\beta 1$, 5'-AGA-TCT-CCC-TCG-GAC-CTG-CT-3' and 5'-GGG-TCT-CCC-AAG-GAA-AGG-TA-3'; for FRP, 5'-GGA-AGA-GAG-CGT-GAA-GGA-CA-3' and 5'-CGG-AGG-CTC-AAA-GAA-AGG-AT-3'; and for GAPDH, 5'-CGC-TGA-GTA-CGT-CGT-GGA-GT-3' and 5'-GGT-GCT-AAG-CAG-TTG-GTG-GT-3'. Amplification reactions, data acquisition, and analysis were performed with

the ABI Prism 7900 HT instrument (PE Applied Biosystems), and relative levels of gene expression were normalized based on those of the GAPDH gene.

Urinary deoxyypyridinoline levels. Mouse urine was collected on day 9, and urinary levels of deoxyypyridinoline were measured with an Osteolinks DPD enzyme-linked immunosorbent assay kit (Metra Biosystems, Mountain View, CA).

Complementary DNA array analysis. Arthritic mice treated with FRP (20 μ g/day) and untreated mice were killed on day 10, and total RNA was prepared from 3 hind paws by mixture in each group as described above. Moreover, mRNA was purified from the total RNA with a MagExtractor mRNA kit (Toyobo, Osaka, Japan). The Gene Navigator cDNA Array system (Toyobo) was used in this analysis. The cDNA array consisted of 561 species of immunologically related mouse cDNA fragments (20). (The Microsoft Excel file can be downloaded from <http://www.toyobo.co.jp/seihin/xr/product/mouse%20immunology.xls>) The biotin-labeled probes were prepared from the sample mRNA with biotin-16-dUTP with the Gene Navigator cDNA Amplification system (Toyobo). Signals produced by the hybridized probe–avidin–biotinylated alkaline phosphatase complex and substrate (Vistra ECF substrate; Amersham Pharmacia Biotech) were detected with the Imaging High Chemifluorescence detection kit (Toyobo). The chemifluorescence image of the cDNA array filter was analyzed using ImaGene software version 4.2 (Takara Bio, Otsu, Japan). The net signal intensity of genes was calculated from the measured raw signal intensity minus that of the background. Expression of individual genes was evaluated by their net signal intensity ratio compared with the standard house-keeping gene.

Confirmation of differentially expressed genes by quantitative RT-PCR. Expression levels of mRNA were estimated by real-time quantitative PCR using SYBR Green I. We obtained cDNA from the hind paws of FRP-treated ($n = 3$) and PBS-treated ($n = 3$) mice killed on day 10 as described above. Each reaction was run in triplicate using SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for c-Fos, 5'-CTA-TGC-AGC-AGA-CTG-GGA-GC-3' and 5'-ACA-GCC-TGG-TGT-GTT-TCA-CG-3'; for Ets-2, 5'-CTT-CCA-AAA-GGA-GCA-ACG-AC-3' and 5'-GTC-CTG-GCT-GAT-GGA-ACA-GT-3'; for interleukin-6 (IL-6), 5'-CAG-AGG-ATA-CCA-CTC-CCA-ACA-3' and 5'-CCA-GTT-TGG-TAG-CAT-CCA-TC-3'; for MMP-3, 5'-CGA-TGG-ACA-GAG-GAT-GTC-AC-3' and 5'-CCC-TCG-TAT-AGC-CCA-GAA-CT-3'; for MMP-9, 5'-ACT-TTC-CCT-TCA-CCT-TCG-AG-3' and 5'-GAA-CAG-GCT-GTA-CCC-TTG-GT-3'; and for GAPDH, 5'-CGC-TGA-GTA-CGT-CGT-GGA-GT-3' and 5'-GGT-GCT-AAG-CAG-TTG-GTG-GT-3'. The amplification reactions, data acquisition, and analysis were performed with the ABI Prism 7900 HT instrument, and relative levels of gene expression were normalized against those of the GAPDH gene.

Statistical analysis. Differences between experimental groups were tested using the Mann-Whitney U test and Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

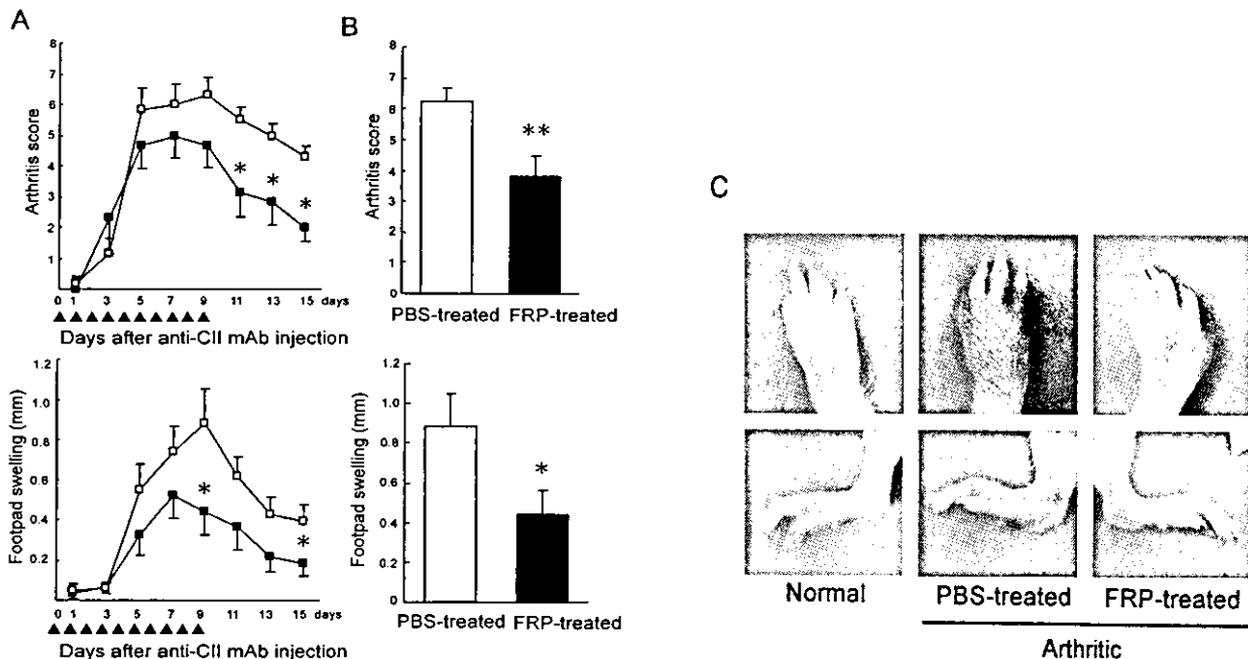


Figure 3. Amelioration of disease in arthritic mice treated with recombinant follistatin-related protein (FRP). **A**, Disease progress estimated by the arthritis score and footpad swelling ($n = 6$ mice per group). Anti-type II collagen (anti-CII) monoclonal antibodies (mAb) were injected on day 0, and 50 μg of lipopolysaccharide was injected on day 2. Recombinant FRP (■) or phosphate buffered saline (PBS; vehicle control) (□) was administered daily for 10 days, beginning on day 0 (arrowheads). **B**, The arthritis score and footpad swelling in both the PBS-treated ($n = 12$) and the FRP-treated ($n = 11$) groups were evaluated at the peak of arthritis (day 9). Bars show the mean and SEM. * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. **C**, Photographs showing representative paws of mice from each group on day 9.

RESULTS

First, to assess the regulation of FRP and its synovial inducer $\text{TGF}\beta 1$ in arthritis, we examined time course changes in their mRNA expression levels in arthritic paws from monoclonal antibody-injected mice (Figure 2). Depending on arthritis activity, both $\text{TGF}\beta 1$ and FRP were elevated temporarily. The elevation of $\text{TGF}\beta 1$ preceded up-regulation of FRP. Interestingly, after $\text{TGF}\beta 1$ began to decrease, FRP continued to rise for a time. Thus, some molecule besides $\text{TGF}\beta 1$ may regulate FRP expression.

To investigate the antiarthritic properties of FRP in vivo, we studied its effect on mouse arthritis using anti-CII antibodies and LPS. In this mouse model, severe arthritis occurs about 24 hours after LPS injection and persists for more than 3 weeks (18). As shown in Figure 3, FRP treatment demonstrated a significant ameliorative effect on the arthritis by reducing the clinical score and preventing the development of footpad swelling. On day 9, we could not detect a significant difference in the arthritis score in the small number of

mice shown in Figure 3A ($n = 6$), but a significant difference was detected in the larger sample shown in Figure 3B ($n = 11$). Macroscopically, redness and swelling of the fore paws and hind paws were milder in the FRP-treated mice than in the PBS-treated mice (Figure 3C). The 10 daily 20- μg doses of FRP produced no significant change in body weight and no macroscopically abnormal findings in FRP-treated mice.

Tarsocrural joints from normal mice had no synovial inflammation or cartilage erosion (Figure 4a). Joints from PBS-treated mice with an arthritis score of 2 had severe synovial inflammation, cartilage erosion, and bone resorption (Figure 4b), while joints from FRP-treated mice with an arthritis score of 1 had mild synovial inflammation and no cartilage or bone erosion (Figure 4c). The weak staining by Safranin O in the articular cartilage of the PBS-treated mice indicated a loss of proteoglycans from the cartilage matrix (Figure 4e). In contrast, Safranin O staining of articular cartilage from FRP-treated mice was equivalent to that in cartilage from normal mice (Figures 4f and d). Histologic

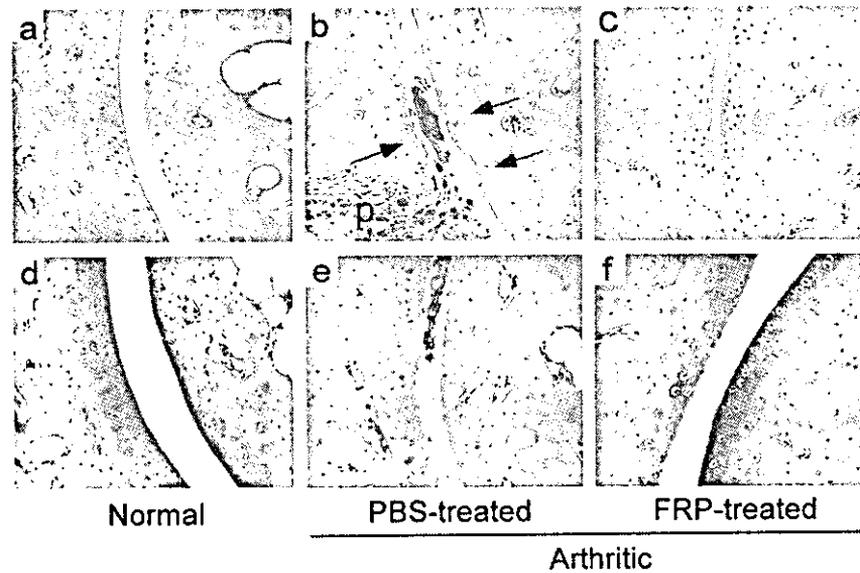


Figure 4. Histologic analysis of tarsocrural joints from normal and arthritic BALB/c mice. All specimens from normal, phosphate buffered saline (PBS)-treated, and follistatin-related protein (FRP)-treated mice were collected on day 21 after monoclonal antibody injection and stained with either hematoxylin and eosin (a, b, and c) or Safranin O (d, e, and f). a and d, Joints from normal mice exhibit normal histologic features, with intact articular cartilage and no evidence of inflammation or erosion. b and e, Affected joints from PBS-treated mice show severe synovitis, pannus formation (p), and cartilage/bone erosion (arrows). Absence of Safranin O staining indicates depletion of proteoglycans. c and f, In contrast, synovitis, cartilage/bone erosion, and loss of Safranin O staining of the cartilage were mild in FRP-treated arthritic mice. (Original magnification $\times 200$.)

scores assessed on day 21 were significantly lower in the FRP-treated mice than in the PBS-treated mice. This confirmed the ameliorative effects of FRP on joint inflammation and cartilage destruction (Figure 5). Thus, FRP seemed to prevent cartilage breakdown in arthritis.

Because deoxyypyridinoline is one of the common markers of bone resorption, we measured urinary levels

of deoxyypyridinoline on day 9 in arthritic mice treated with recombinant FRP or PBS. The urinary levels of deoxyypyridinoline were markedly higher in the arthritic mice than in the normal mice. However, those in the FRP-treated arthritic mice were significantly lower than those in the PBS-treated mice (Figure 6). This suggested that FRP worked against the bone destruction in arthritis.

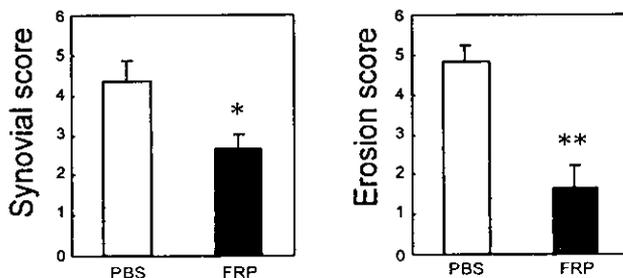


Figure 5. Comparison of histologic scores in phosphate buffered saline (PBS)-treated and follistatin-related protein (FRP)-treated mice on day 21. Bars show the mean and SEM ($n = 6$ mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test.

To identify the genes that are affected by FRP administration in this mouse model of arthritis, we examined the gene expression profiles by cDNA array techniques in joint tissues from normal, PBS-treated, and FRP-treated mice ($n = 3$ per group). We studied the expression of 561 genes blotted on the cDNA array, including 9 housekeeping genes and 2 negative control genes (see Materials and Methods). As a result, we found a change of >1.5 -fold in the expression levels of 170 genes in the FRP-treated group versus the PBS-treated group. Five of these 170 genes (*c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9*) were down-regulated in the FRP-treated group (Figure 7A); these genes have been

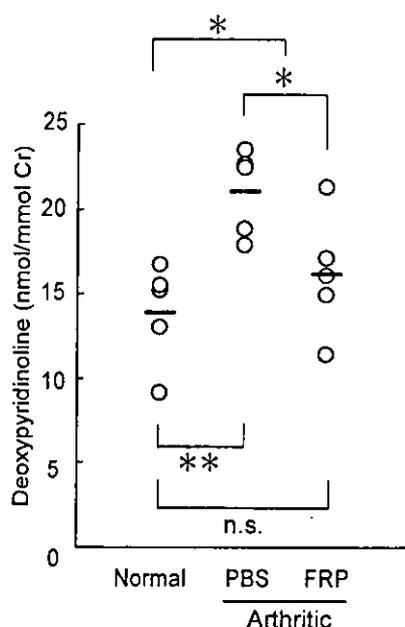


Figure 6. Urinary deoxyypyridinoline levels in normal and arthritic mice. Urinary levels of deoxyypyridinoline (expressed in nmoles/mmoles of creatinine [Cr]) were significantly decreased in the follistatin-related protein (FRP)-treated arthritic mice compared with the phosphate buffered saline (PBS)-treated arthritic mice. Deoxyypyridinoline levels were measured by enzyme-linked immunosorbent assay in samples collected on day 9 ($n = 5$ mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. n.s. = not significant.

reported to be responsible for the destructive joint inflammation in RA (21–24).

To confirm whether the change in the expression of the 5 genes was in fact significant, we performed a real-time RT-PCR analysis using the same cDNA sources as those for the cDNA array. We found that the expression levels of the *c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9* genes were significantly lower in paws from the FRP-treated mice than in the paws from the PBS-treated mice (Figure 7B). Therefore, FRP seemed to exert its preventive effect on the arthritis, in part, by down-regulating disease-promoting genes such as the 5 we identified.

DISCUSSION

In this study, we demonstrated that FRP had therapeutic effects on murine arthritis. Because of its rapid induction and high reproducibility, we used a mouse model of acute, destructive arthritis (resembling human RA) induced by anti-CII monoclonal antibodies and LPS. The expression of endogenous FRP and its synovial inducer TGF β 1 was higher in the arthritic paws of this model than in the normal paws. In addition, the expression of FRP was maintained at a high level even if the arthritis had peaked. A recent study also showed that the expression of FRP was increased at the synovium-bone erosion interface in early and late-phase collagen-

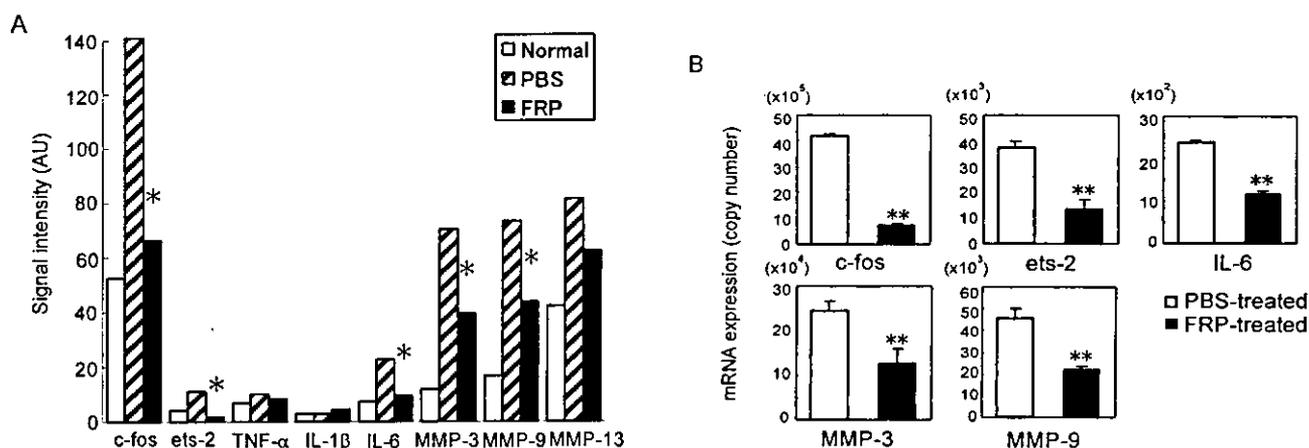


Figure 7. Gene expression in joint tissues of normal, phosphate buffered saline (PBS)-treated, and follistatin-related protein (FRP)-treated mice, as analyzed by cDNA arrays and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). In both experiments, mRNA was extracted from joint tissues on day 10 of the experiment ($n = 3$ mice per group). **A**, The results of cDNA arrays analysis showed that the gene expression levels of *c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9* were lower in the joints of FRP-treated mice than in the joints of PBS-treated mice. * = ratio of signal intensity (in arbitrary units [AU]) in PBS-treated mice to that in FRP-treated mice > 1.5 . TNF- α = tumor necrosis factor α . **B**, Real-time quantitative RT-PCR studies confirmed the significantly reduced levels of gene expression in FRP-treated mouse joints compared with PBS-treated mouse joints (control) for *c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9*. ** = $P < 0.01$, by Student's unpaired *t*-test.

induced arthritis (25). Thus, we presumed that endogenous FRP was induced by TGF β 1 mainly from the synovium as the arthritis developed and may contribute to the regulation of arthritis. Our previous in vitro experiments suggested that FRP could prevent destructive arthritis (17). Therefore, we postulated that abundant exogenous FRP, in addition to feedback-regulated FRP, may improve arthritis.

FRP is a genetically highly conserved protein, having 92% amino acid sequence identity between humans and mice (9), and our previous in vitro study strongly suggested that recombinant human FRP may function in other species (17). Accordingly, we used recombinant human FRP for the present in vivo experiments. Treatment of mice with recombinant FRP just before the onset of arthritis brought about a significant improvement in the clinical parameters of arthritis (i.e., arthritis score and footpad swelling), indicating that FRP had preventive effects on joint inflammation. In addition, the striking benefit of FRP administration was the prevention of joint destruction. Postmortem histologic examination of tarsocrural joints from the FRP-treated mice revealed not only reduced synovial cellular infiltration, but also retained cartilage proteoglycan. Moreover, the reduction in urinary levels of deoxypyridinoline, one of the metabolic markers of bone resorption in RA (26), confirmed that FRP protected joints against destructive arthritis by preventing cartilage degradation and bone resorption. Therefore, FRP has antidestructive as well as antiinflammatory effects, and therapeutic potential in inflammatory joint diseases such as RA.

FRP is considered to be an orphan molecule because its receptor has not yet been identified, and there has been no information about the signaling system of FRP. Thus, to investigate the molecular mechanisms through which FRP treatment ameliorated murine arthritis, we compared the gene expression profiles in FRP-treated and PBS (vehicle)-treated joints. Among 170 genes with significant changes in expression levels, 5 down-regulated genes, *c-fos*, *ets-2*, *IL6*, *MMP3*, and *MMP9*, have been reported to be closely associated with immune response and joint destruction in inflammatory arthritis. Their reduced expression in FRP-treated mice was confirmed by a real-time quantitative RT-PCR method.

The expression of *IL6* was significantly reduced in joints from FRP-treated mice, suggesting that inhibition of IL-6 could be one of the reasons for the antiinflammatory effects of FRP. IL-6 is thought to be one of the proinflammatory cytokines responsible for

disease progression in RA, together with TNF α and IL-1 (23). Thus, FRP could be expected to have beneficial effects on joint inflammation in other animal models of arthritis and still greater effects in human diseases such as RA. However, a recent study indicated that in this mouse model of arthritis induced by anti-CII monoclonal antibodies and LPS, IL-6 was not as responsible for the progression of arthritis as TNF α and IL-1 were (27). There may be other mechanisms by which FRP ameliorates joint inflammation than by antagonism of IL-6.

The inhibitory effect of FRP on *MMP3* and *MMP9* was notable because these MMPs are thought to play a crucial role in the joint destruction of RA, where they are major direct effectors that degrade the extracellular matrix components of the joint structure, such as collagen and proteoglycan (24). Serum levels of MMP-3 in particular have been reported to be correlated with joint damage in RA (28). Synovial down-regulation of MMP-3 by FRP was also observed in our previous in vitro experiments (17). Gene expression of these MMPs is strongly up-regulated by proinflammatory cytokines, such as TNF α and IL-1, in the RA synovium (24), and it is considered that conditions in which the activities of the MMPs overwhelm the activities of the tissue inhibitors of metalloproteinases and MMP inhibitors, the joint destructive process is facilitated, as in RA (29). For these reasons, inhibition of MMPs has been considered as a new therapeutic approach to rheumatoid joint destruction (24). It has been reported that specific inhibition of individual MMPs has almost no effect in the treatment of murine arthritis, possibly due to another MMP compensation (30), but broad-spectrum MMP inhibition was shown to successfully reduce the clinical severity and cartilage destruction in a mouse model of RA (31,32). In our study, FRP inhibited the expression of *MMP3*, *MMP9*, and *MMP13*. The decrease ratio was significant in *MMP3* and *MMP9* (>1.5), but slight in *MMP13* (<1.5). Therefore, we hypothesized that one of the major mechanisms by which FRP prevented joint destruction was the down-regulation of several MMPs, such as MMP-3 and MMP-9.

How does FRP regulate MMP gene transcription? We found that 2 transcription factor genes, *c-fos* and *ets-2*, were down-regulated in FRP-treated joints. Their expression has been associated with a proliferative role in many tissues (21,33). It has been reported that *c-fos* and *ets-2* expression was higher in rheumatoid synovial tissue than that in normal synovial tissue, suggesting that they may play an important role in the progression of RA (22,34,35). The nuclear oncogene *c-fos* and its product c-Fos bind to activator protein 1

sites on matrix-degrading genes, such as *MMP3* and *MMP9*. The gene *ets-2* is one of the Ets family of transcription factors that binds to polyomavirus enhancer 3 (PEA-3) sites (36). Genes involved in the degradation of the extracellular matrix, such as *MMP3* and *MMP9*, contain PEA-3 sites in their regulatory sequences, and the binding of Ets-2 to PEA-3 sites accelerates their transcription (37). Moreover, Ets-2 cooperates with other transcription factors, including c-Fos, to stimulate the transcription of matrix-degrading proteases (37). Therefore, it is likely that FRP negatively regulates *c-fos* and *ets-2* gene expression and, as a result, the transcription of *MMP3* and *MMP9* is suppressed.

In this series of experiments, for the limited amount of available recombinant FRP and the relatively short disease duration in this mouse model of arthritis, we performed preventive therapy for the arthritis by administering FRP just before the onset of disease. However, for further estimation of the therapeutic potential of FRP in arthritis, another series of experiments conducted according to the controlled curative therapy protocol will be required, involving the administration of FRP and control drugs during disease progression in other mouse models of chronic arthritis.

In conclusion, we demonstrated that the progression of murine arthritis can be inhibited by recombinant FRP treatment. Its beneficial effect was the prevention of joint destruction, which seemed to be mainly due to the reduction in MMP activities at the transcription level. In inflammatory joint diseases such as RA, FRP may be one of the key molecules that prevents the disease progression and a candidate as a new therapeutic biologic agent that may block the disease process in a manner different from that of TNF α , IL-1, and IL-6 antagonists.

ACKNOWLEDGMENTS

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Effect of Intermittent Cyclical Etidronate Therapy on Corticosteroid Induced Osteoporosis in Japanese Patients with Connective Tissue Disease: 3 Year Followup

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ABSTRACT. Objective. A 3 year prospective randomized study was conducted to clarify the efficacy of intermittent cyclical etidronate therapy on corticosteroid induced osteoporosis.

Methods. A group of 102 Japanese patients were enrolled, each taking > 7.5 mg of prednisolone daily for at least 90 days. Patients were randomly divided into 2 treatment groups: Group E (etidronate) took 200 mg etidronate disodium per day for 2 weeks with 3.0 g calcium lactate and 0.75 µg alphacalcidol daily; Group C (control) took 3.0 g calcium lactate and 0.75 µg alphacalcidol daily. Outcome measurements included changes from baseline in bone mineral density (BMD) of the lumbar spine and the rate of new vertebral fractures at 48 and 144 weeks.

Results. The mean (± SD) lumbar spine BMD increased 3.7 ± 5.6% (p < 0.01) and 1.5 ± 4.1% (NS) from baseline at 48 weeks and 4.8 ± 6.9% (p < 0.005) and 0.4 ± 5.0% (NS) from baseline at 144 weeks in Group E and Group C, respectively. The improvement of BMD in Group E was significantly greater than in Group C at 144 weeks (p < 0.01). In 3 subgroups, men and premenopausal and postmenopausal women, the postmenopausal women showed the greatest improvement. Mean percentage change in this subgroup was 10.1 ± 8.0% and 1.35 ± 6.4% in Group E and Group C, respectively. We noted that 2 patients in Group C had new vertebral fractures, whereas no fractures were observed in Group E.

Conclusion. These results indicate that intermittent cyclical etidronate therapy is effective for the prevention and treatment of corticosteroid induced osteoporosis in patients with connective tissue diseases. (J Rheumatol 2003;30:2673-9)

Key Indexing Terms:

CORTICOSTEROID INDUCED OSTEOPOROSIS
BONE MINERAL DENSITY

BISPHOSPHONATE
CONNECTIVE TISSUE DISEASES

Osteoporosis is one of the major adverse effects caused by longterm corticosteroid treatment for connective tissue disease. Recently the efficacy of bisphosphonate, activated vitamin D₃, calcitonin, and hormone replacement therapy on corticosteroid induced osteoporosis has been reported¹⁻¹⁵. Among these agents, bisphosphonate therapy has been reported as beneficial in both the prevention and treatment of corticosteroid induced osteoporosis^{3,5,13,14}. Although several studies have reported the effect of etidronate for

corticosteroid induced osteoporosis in North America^{1-4,7,10}, a longterm (> 3 years) observation has never been reported. Further, few studies are available on the effect of etidronate in Japanese patients with corticosteroid induced osteoporosis, and the differences between Japanese and Caucasian populations in the efficacy of etidronate are unknown. In one study, the effects of alendronate, one of the bisphosphonates, in Japanese patients were similar to those seen in Caucasian patients¹⁶. For this reason, we conducted a 3-year prospective randomized study to determine the prevention and treatment efficacy of intermittent cyclical etidronate therapy on corticosteroid induced osteoporosis.

MATERIALS AND METHODS

Patients. Patients were 21 to 73 years of age, had underlying connective tissue diseases (systemic lupus erythematosus, n = 56, rheumatoid arthritis 12, polymyositis/dermatomyositis 10, vasculitis syndrome 9, adult onset Still's disease 8, polymyalgia rheumatica 5, systemic sclerosis 1, Sjögren's syndrome 1), and had taken > 7.5 mg prednisolone daily for at least 90 days. Patients were excluded if they had abnormalities on spinal radiographs that prevented accurate measurements of the lumbar spine bone mineral density (BMD) by dual-energy x-ray absorptiometry (DEXA), had

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severe cardiac disease or renal insufficiency, or had taken medications known to affect bone metabolism within the preceding year. Written informed consent was obtained from all patients.

Study design. In this 3-year prospective randomized study, the patients were classified according to sex and menopausal status (i.e., men, premenopausal women, postmenopausal women). All patients were randomly assigned to one of 2 investigational groups: Group E (etidronate), who took etidronate disodium (200 mg/day; Didronel, Sumitomo Pharmaceuticals, Osaka, Japan) for 2 weeks with 3.0 g calcium lactate and 0.75 µg alphacalcidol (Alfarol, Chugai Pharmaceuticals, Tokyo, Japan) daily for 90 days; or Group C (control), who took 3.0 g calcium lactate and 0.75 µg alphacalcidol daily for 90 days. This cycle was repeated 12 times during the 3-year treatment period. Patients were instructed to take the study drug with water at bedtime.

BMD and radiological measurements. Radiographs of the lateral and anteroposterior lumbar and thoracic spine were taken and evaluated at Keio University Hospital at baseline and at 48 weeks and 144 weeks. All radiographs were evaluated by experienced skeletal physicians who were blinded to treatment assignments. The diagnosis of vertebral fracture and osteoporosis was based on diagnostic criteria defined by the Japanese Society for Bone and Mineral Research in 1996¹⁷.

A vertebral fracture was defined as follows: (1) The ratio of the central height of the vertebra (C) and the anterior height of the vertebra (A) was less than 0.8 or the ratio of C and the posterior height of the vertebra (P) was less than 0.8. (2) The ratio of A and P was less than 0.75. (3) Crush vertebra was defined when the height of the vertebrae was reduced more than 20% in either A, C, or P compared with the adjacent vertebrae.

The classification of BMD was based on the following criteria: (1) Normal BMD: > 80% of the young adult mean (YAM). (2) Osteopenia: between 70% and 80% of YAM. (3) Osteoporosis: < 70% of YAM.

Based on these criteria, the definition of osteoporosis (i.e., < 70% of YAM) corresponds to the osteoporosis criteria recommended by the World Health Organization (i.e., < -2.5 SD of YAM).

All measurements of BMD were made by DEXA using the XR-36 (Norland Medical Systems, Fort Atkinson, WI, USA). The primary outcome was the difference between the 2 investigational groups at Weeks 48 and 144 as compared with baseline in the mean percentage change of the lumbar spine BMD (L2-L4).

Markers of bone turnover. Serum and urine samples were obtained at baseline and at 24 weeks for measurement of biochemical markers of bone turnover. Alkaline phosphatase (ALP) isozymes were separated on a cellulose-acetate membrane with Tris-barbital buffer. Enzyme staining was carried out on 5-bromo-3-indoxylphosphate p-toluidine salts. Quantitation of bone ALP (BAP) isozyme was performed using a transmission densitometer (EDC; Helena Laboratories, Saitama, Japan) connected to a micro-computer and urinary excretion of deoxypyridinoline (Dpd) (Pyrilinks-D, Quidel Corp., San Diego, CA, USA). All analysis was done at Keio University.

Statistical analysis. An intent-to-treat analysis was performed. The baseline characteristics and homogeneity of the patients' background were compared between the 2 investigational groups by Student t test and Wilcoxon rank-sum test. The main efficacy analysis was the percentage change of BMD from baseline to last measurement. If the measurement of the lumbar spine BMD at Week 144 was not available, the measurement obtained at Week 48 was used in the analysis. The percentage change of BMD of each group was determined by paired t test and comparison of percentage change of BMD between the 2 groups was calculated by Student t test. Significance level was set at 5% and all results were expressed as mean ± standard deviation.

RESULTS

A set of 51 patients were randomly assigned to each group to receive either etidronate + calcium lactate + alphacalcidol

(Group E) or calcium lactate + alphacalcidol (Group C). There were no significant differences in baseline characteristics in either investigational group (Table 1). The cumulative corticosteroid dose in Group E 6 months before the study was 2199 ± 855 mg, and in Group C 1784 ± 655 mg. The number of patients with compression fractures at baseline was 5 (13%) in Group E and 4 (10%) in Group C. This finding was not statistically significant. Twenty-two patients in Group E and 24 patients in Group C withdrew before study completion. Among these patients, 13 patients in Group E and 14 in Group C were dropped from the study due to protocol violations. Two patients in Group E withdrew because of adverse events; one experienced a headache and the other developed a facial rash. Two patients in each group died during the study. These deaths were attributed to progression of their underlying connective tissue disease. The baseline characteristics of those that dropped out were not significantly different from those that continued.

At baseline, lumbar spine BMD in the women ranged from 0.534 to 1.333 g/cm² (mean 0.846 ± 0.166 g/cm²). Among 88 women, 71 (81%) were below the average of age matched normal individuals. Twenty-two (25%) were diagnosed as having osteopenia; 23 (26%) were diagnosed as osteoporosis.

In Group E, the mean (± SD) percentage change in BMD of the lumbar spine significantly increased $3.7 \pm 5.6\%$ ($p < 0.01$) and $4.8 \pm 6.9\%$ ($p < 0.005$) from baseline at 48 weeks and 144 weeks, respectively (Figure 1). The improvement of BMD in Group E was significantly higher than in Group C at 144 weeks ($p < 0.01$). In Group E, the mean percentage change of the lumbar spine BMD at 144 weeks was greater than at 48 weeks, whereas in Group C there was a decrease at 144 weeks compared to 48 weeks.

When classified into 3 subgroups (men, premenopausal women, postmenopausal women) (Figure 2), all subgroups of Group E showed an increase in the mean percentage change in BMD during their course. Among these subgroups, the postmenopausal women of Group E showed the greatest improvement. In the subgroups of premenopausal women and postmenopausal women, the increase of mean percentage change was significantly greater in Group E than in Group C at 144 weeks: $3.8 \pm 6.6\%$ vs $-0.2 \pm 4.8\%$ ($p < 0.01$) and $10.1 \pm 8.0\%$ vs $1.4 \pm 6.4\%$ ($p < 0.05$), respectively.

Analysis of the subgroups of women based on the baseline BMD (osteoporosis + osteopenia, and normal BMD) revealed that Group E women with osteoporosis + osteopenia showed a significant increase of the lumbar spine BMD from baseline. The greatest improvement was seen at 144 weeks. In both subgroups of osteoporosis + osteopenia and normal BMD, the increase of mean (± SD) percentage change was significantly greater in Group E than in Group C at 144 weeks: $5.8 \pm 7.2\%$ vs $0.9 \pm 5.1\%$ ($p < 0.05$) and $4.1 \pm 7.2\%$ vs $-1.7 \pm 5.1\%$ ($p < 0.05$), respectively.

Table 1. Baseline characteristics of patients in Group E and Group C. Values are means \pm SD or percentages.

Characteristics	Group E, n = 51	Group C, n = 51	p
Men, n (%)	4 (10)	5 (13)	NS
Premenopausal women, n (%)	24 (60)	21 (52)	NS
Postmenopausal women, n (%)	12 (30)	14 (35)	NS
Age, yrs			
Men	49 \pm 23	45 \pm 17	NS
Women	42 \pm 13	44 \pm 12	NS
Total corticosteroid dose, mg	2,199 \pm 855	1,784 \pm 655	NS
Lumbar spine BMD, g/cm ²			
Men	0.92 \pm 0.22	0.85 \pm 0.14	NS
Premenopausal women	0.88 \pm 0.19	0.90 \pm 0.14	NS
Postmenopausal women	0.77 \pm 0.12	0.75 \pm 0.15	NS
Vertebral fractures, n (%)	5 (13)	4 (10)	NS

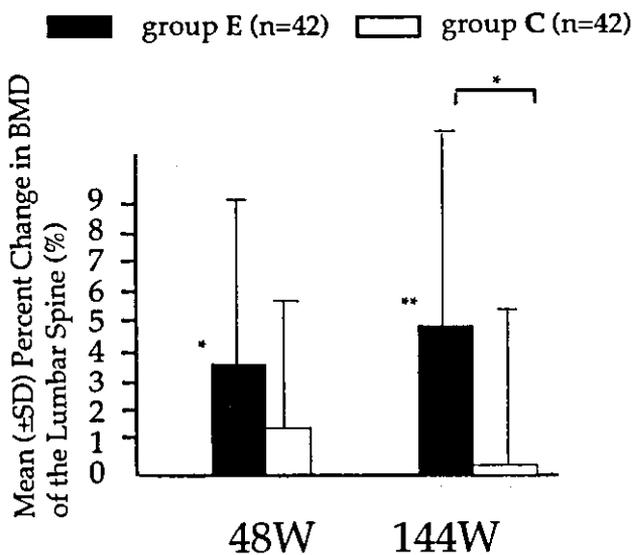


Figure 1. The mean (\pm SD) percentage change in BMD of the lumbar spine between baseline (0 week) and 48 and 144 weeks of Groups E and C. In all patients, the mean (\pm SD) percentage change in BMD of the lumbar spine increased from baseline at 48 weeks and 144 weeks in Group E ($p < 0.01$, $p < 0.005$, respectively). The improvement of BMD in Group E was significantly greater than in Group C at 144 weeks ($p < 0.01$). * $p < 0.01$, ** $p < 0.005$.

Patients taking corticosteroid therapy had various underlying disorders. In analysis of subgroups based on underlying disease, there was no significant difference between groups in mean percentage change in BMD of lumbar spine (data not shown).

The change in BMD of the lumbar spine between baseline and 48 and 144 weeks of the female patients diagnosed with osteoporosis or osteopenia was compared between Group E and Group C. Thirteen of 15 (87%) patients with osteoporosis or osteopenia in Group E showed an increase in BMD of the lumbar spine at 144 weeks. In contrast, 7 of

12 (58%) patients with osteoporosis or osteopenia in Group C showed a decrease. The rate of increase of BMD in Group C was lower than in Group E.

The baseline values for serum and urine markers of bone turnover were similar in the 2 treatment groups (Figure 3). The baseline concentration of BAP was 108 ± 33 IU/l in Group E and 111 ± 57 IU/l in Group C. It had decreased significantly, 22.8% ($p < 0.01$) in Group E and 23.6% in Group C ($p < 0.05$), at 24 weeks. The difference between investigational groups was not significant. The urinary Dpd value was 6.2 ± 2.4 nM/mM Cr in Group E and 6.3 ± 3.1 nM/mM Cr in Group C at baseline. It decreased 11.1% (NS) in Group E and 3.2% (NS) in Group C at 24 weeks from baseline. The difference between investigational groups was not significant.

Five patients (13%) in Group E and 4 (10%) in Group C had vertebral fractures at baseline; and 2 patients in Group C had a total of 3 new vertebral fractures during the study (Table 2). No patient in Group E had new fractures, but the difference between the groups was not statistically significant. The 2 postmenopausal women in Group C had new vertebral fractures at 144 weeks. One patient in Group C had 2 vertebral fractures. Two patients in Group C who had new vertebral fractures showed a decrease of lumbar spine BMD at 48 weeks and 144 weeks. On radiograph, one patient (Case 1) showed new compression fractures in the 6th and 8th thoracic vertebrae and another (Case 2) had a 12th thoracic vertebral fracture (Figure 4).

Two patients in Group E had adverse events: one had a headache and the other developed a facial rash. Although these events were not considered drug related, patients withdrew from the study for their own safety. No patient had gastrointestinal (GI) side effects.

DISCUSSION

We showed that intermittent cyclical etidronate therapy increases the BMD of lumbar spine induced by corticosteroid in Japanese patients with connective tissue diseases.