Table 4 Association of SNP −169C→T with AITD and SLE

		Genotype				Recessive-trait comparison		
Disease	*n*	CC	CT	TT	Allele C frequency	OR (95% c.i.)	χ^2	*P*
GD	351	72	179	100	0.46	1.79 (1.34–2.39)	15.7	0.000074
HT	158	30	74	54	0.42	1.62 (1.07–2.47)	5.2	0.022
AITD total	509	102	253	154	0.45	1.74 (1.35–2.24)	18.5	0.000017
SLE	564	100	259	205	0.41	1.49 (1.16–1.92)	9.8	0.0017
RA* + AITD + SLE	2,437	438	1,167	832	0.42	1.52 (1.29–1.79)	24.2	0.00000084
Control	2,037	257	995	785	0.37			

*Rheumatoid arthritis represents sum of three sets (n = 1,364). c.i., confidence interval; GD, Graves' disease; HT, Hashimoto's thyroiditis; OR, odds ratio; RA, rheumatoid arthritis.

expression of *FCRL3* is higher in individuals with the disease-susceptible haplotype and suggest that higher expression of *FCRL3* is a potential cause and component of the pathological mechanism(s) leading to rheumatoid arthritis.

Expression of *FCRL3* mRNA

We then quantified *FCRL3* expression in multiple tissues using TaqMan methods. Expression of *FCRL3* transcripts was high in the spleen and tonsils (**Fig. 3a**), which are secondary lymphoid organs. We observed lower expression in thymus and bone marrow. In human blood fractions, $CD19^+$ cells, which represent the B-cell population, had the greatest *FCRL3* expression among peripheral blood mononuclear cells. $CD4^+$ and $CD8^+$ cells had less expression (**Fig. 3b**). We next examined the effect of B-cell stimulation on *FCRL3* expression. We cultured peripheral blood B cells from a healthy donor for 4 h using known B-cell stimulants and then quantified *FCRL3* mRNA (**Fig. 3c**). Expression of *FCRL3* was increased by antibody to CD40 and lipopolysaccharide (LPS).

We then investigated expression of *FCRL3* transcripts in synovial tissue using *in situ* hybridization methods. T and B cells are the key players with regard to inflammation in synovial tissue, producing proinflammatory cytokines and autoantibodies that might be pathogenic[1]. These cells show three distinct histological patterns: diffuse infiltration, clustering in aggregates and follicles with germinal-center reaction[35,36]. We observed aggregations of T and B cells in paraffin-embedded synovial sections from individuals with rheumatoid arthritis, using immunostaining with antibodies to CD3 and CD20, respectively (**Fig. 3d,e**). *In situ* hybridization assay with serial sections detected *FCRL3* mRNA in aggregated lymphocytes (**Fig. 3f,g**). Although strict differentiation between B and T cells was difficult, at least some aggregated B cells were positive, with strong expression of *FCRL3* mRNA. Synovium from two other individuals with rheumatoid arthritis had similar lymphocyte aggregates and *FCRL3* expression (**Supplementary Fig. 2** online).

SNP association with autoantibody and *HLA-DRB1* status

Because we suspected that higher *FCRL3* expression led to B-cell abnormalities in rheumatoid arthritis, we examined associations in individuals with rheumatoid arthritis between genotype and two rheumatoid arthritis–related autoantibodies: rheumatoid factor (RF) and antibody to cyclic citrullinated peptide (CCP). RF is a well-known autoantibody for the Fc region of IgG, and titers correlate with rheumatoid arthritis disease activity[37]. Antibody to CCP recognizes peptides containing citrulline and is detected in rheumatoid arthritis with extremely high specificity[38,39]. RF titer in individuals with rheumatoid arthritis was significantly positively correlated with the number of susceptible alleles (R^2 = 0.049, P = 0.0065; **Table 3**). The positive ratio of antibody to CCP in individuals with rheumatoid arthritis also differed significantly among genotypes ($P < 0.05$) and correlated with number of susceptible alleles.

Because genetic interactions between HLA and non-HLA loci have been described in susceptibility for rheumatoid arthritis and other autoimmune diseases[26,40], we compared genotype distributions for SNP −169C→T among three rheumatoid arthritis subgroups stratified by number of *HLA-DRB1* shared-epitope alleles. We previously genotyped *HLA-DRB1* in our population and observed significant associations between rheumatoid arthritis susceptibility and shared-epitope alleles[4]. Allele frequency of the rheumatoid arthritis–susceptibility allele −169C was significantly higher in the subgroup with two copies of shared-epitope alleles (0.49, n = 113) than in the subgroup with no shared-epitope alleles (0.39, n = 215; $P < 0.05$).

Replication study of association in three autoimmunities

To confirm associations between the *FCRL3* variant and rheumatoid arthritis susceptibility, we carried out a replication study (540 individuals with rheumatoid arthritis, 636 controls). We compared allele frequency and found a significant association between fcrl3_3 (−169C→T) and rheumatoid arthritis susceptibility (allele frequency was 0.40 in individuals with rheumatoid arthritis versus 0.46 in controls; P = 0.041; **Supplementary Table 2** online). We noted no significant differences between two cohorts that consisted of the replication samples. These results further confirmed the association of the fcrl3_3 −169C allele with rheumatoid arthritis susceptibility in Japanese individuals.

Because this region is associated with multiple autoimmune diseases, and because several variants are involved in multiple autoimmunities, we investigated associations between SNP −169C→T and two other autoimmune diseases: AITD and SLE. We recruited 509 Japanese individuals with AITD (351 with Graves' disease and 158 with Hashimoto's thyroiditis) and 564 Japanese individuals with SLE and compared them with 2,037 Japanese controls. In addition, we combined AITD, SLE and rheumatoid arthritis cases as subjects with an autoimmune phenotype and tested for associations with the SNP. Individual diseases, as well as combination of two AITDs and combination of AITD, SLE and rheumatoid arthritis, were significantly associated with the SNP (odds ratio = 1.52, P = 0.00000084 in Japanese for a recessive model between all four autoimmunities considered in aggregate and controls; **Table 4**). As rheumatoid arthritis–specific autoantibodies were correlated with the number of susceptible alleles, antibody to DNA titer was higher in individuals with SLE with genotype −169C/C than in subjects with other genotypes (294.1 IU ml^{-1} versus 145.5 IU ml^{-1}; n = 120; P = 0.026 by Student's t-test), a conclusion not further established by regression analysis (P = 0.12).

DISCUSSION

LD mapping of 1q21–23 in Japanese subjects identified multiple LD blocks in the region, and one block containing *FCRL3* was associated with rheumatoid arthritis. This association was replicated in a second Japanese case-control set. The rheumatoid arthritis–associated allele was also associated with increased risk of other autoimmune disorders, such as AITD (Graves' disease and Hashimoto's thyroiditis) and SLE. Recent reports on autoimmune disease–associated polymorphisms show that some disease-susceptible variants are limited to specific ethnic groups[12] whereas others are widely dispersed but significantly associated with disease in only specific ethnic groups[41,42]. We evaluated four-SNP haplotypes in *FCRL3* in African American, European American and Asian (Korean and Japanese) subjects and found weaker LD in African Americans than in other groups and substantial differences in allelic frequency among the groups (**Supplementary Table 3** online).

Although the evidence presented here for *FCRL3* being an autoimmune disease–susceptibility gene is powerful, additional autoimmune disease–related genes probably exist in this region. For example, 1q23 is a good candidate locus for SLE susceptibility[6], particularly involving the association of the classical FcγR genes with SLE susceptibility in the Japanese population[19], although those variants are not in LD with SNP −169C→T in our Japanese subjects ($\Delta < 0.05$, **Fig. 1a**). Multiple SLE susceptibility genes are also homologous to human 1q23 in mouse models of SLE[43].

Further evaluation of polymorphism associations showed that a SNP in the promoter region of *FCRL3* alters expression of *FCRL3* through NF-κB binding. Because higher expression of *FCRL3* was observed in individuals with susceptible alleles, and augmented autoantibody production was associated with the susceptible genotype, important steps in the sequence of events leading to autoimmunity must proceed through *FCRL3*. That the susceptible allele is associated with *HLA-DRB1* in rheumatoid arthritis is consistent with *FCRL3* functioning in the context of HLA class II restriction, which is usually seen in the interaction between T cells and antigen-presenting cells, including B cells. Moreover, together with the dominant expression of *FCRL3* on B cells and the importance of B cells suggested by a recent clinical trial of B cell–depleting therapy[44], the present findings might provide a genetic basis for B-cell abnormality in autoimmunity.

Although the precise function of *FCRL3* is unknown, its predicted molecular structure suggests that it is a membranous protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif and an immunoreceptor-tyrosine inhibitory motif[14]. An *in vitro* study showing the binding of tyrosine kinases syk and ZAP70 to the immunoreceptor-tyrosine activation motif region and of tyrosine phosphatases SHP-1 and SHP-2 to the immunoreceptor-tyrosine inhibitory motif region[17] supports the proposed signaling function of *FCRL3*. In a previous study examining *in situ* hybridization in human tonsil, *FCRL3* was expressed in the germinal center, with particularly high expression in the light zone[16], suggesting that *FCRL3* functions predominantly in centrocytes. The present finding that CD40 stimulation, which is important in germinal-center formation[45], upregulates *FCRL3* expression in B cells could indicate that *FCRL3* is specifically expressed in germinal-center centrocytes under the influence of CD40 signals. In the light zone, centrocytes undergo clonal selection and affinity maturation regulated by positive and negative signals from antigen receptors and coreceptors[46]. High expression of *FCRL3* and augmented autoantibody production in individuals with the disease-susceptible genotype is consistent with the idea that *FCRL3* influences the fate of B cells and augments the emergence of self-reactive cells in the germinal center.

In addition to its role in lymphoid tissues, expression of *FCRL3* in synovial tissue might explain the pathological connection between *FCRL3* variants and rheumatoid arthritis. *FCRL3* is strongly expressed in aggregated lymphocytes. Although our synovial samples showed only T-cell–B-cell aggregates, lymphocytes in rheumatoid arthritis synovial tissue are known to form a germinal center–like structure, called an ectopic germinal center, where T cell–dependent antibody production and affinity maturation occur[36]. Ectopic germinal-center formation also occurs in tissues from individuals with AITD and SLE, and *FCRL3* might be involved in pathological autoimmune reaction in these disease-specific ectopic lymphocyte aggregates.

Considering that augmented expression of *FCRL3* is associated with susceptibility to autoimmune disorders, and that *FCRL3* expression is regulated in B cells in the secondary lymphoid organ and is detected in lymphocytes of disease-specific tissues, *FCRL3* probably functions in immunity and potentially pathogenic in autoimmune disorders.

METHODS

Subjects. We enrolled three independent cohorts of individuals with rheumatoid arthritis (n = 830, 217 and 323), a cohort of individuals with SLE (n = 564) and a cohort of individuals with AITD (n = 509) comprising Graves' disease (n = 351) and Hashimoto's thyroiditis (n = 158) through several medical institutes in Japan. We recruited four independent cohorts of unaffected control subjects (n = 658, 262, 374 and 752) at various sites in Japan. All subjects were Japanese. Individuals with rheumatoid arthritis (84.2% women; age 59.0 ± 12.3 years (mean ± s.d.); 75.0% RF-positive) satisfied the revised criteria of the American Rheumatism Association for rheumatoid arthritis[47]. Individuals with SLE satisfied the criteria of the American College of Rheumatology for SLE[48]. Diagnosis of AITD was established on the basis of clinical findings and results of routine examinations for circulating thyroid hormone and thyroid-stimulating hormone concentrations, serum levels of antibodies against thyroglobulin, thyroid microsomes and thyroid-stimulating hormone receptors, ultrasonography, $^{[99m]}TCO_4^-$ (or $[^{123}I]$) uptake and thyroid scintigraphy.

We evaluated LD at 1q21–23 in the first control cohort compared with the first rheumatoid arthritis cohort to identify the rheumatoid arthritis–associated LD block and SNPs. The second and third rheumatoid arthritis and control cohorts were used for replication testing of results from the first cohorts. We tested Graves' disease; Hashimoto's thyroiditis; SLE; the combination of the two AITDs; and the combination of rheumatoid arthritis, SLE and the two AITDs for associations using the total pool of controls. We enrolled control subjects from three other ethnic groups, Korean (n = 100), African American (n = 120) and European American (n = 120), for evaluation of *FCRL3* haplotypes. We sampled synovial tissues from individuals with rheumatoid arthritis who underwent arthroplastic surgery. All subjects provided informed consent to participate in the study, as approved by the ethical committee of the SNP Research Center, RIKEN.

SNPs and genotyping. We identified SNPs in exons and 5′ and 3′ flanking regions of *FCRL1*, *FCRL2*, *FCRL3* and *FCRLψ4* by direct sequencing of DNA from 24 individuals. We selected other SNPs from the JSNP and Assay-On-Demand SNP databases (Applied Biosystems). We genotyped SNPs using Invader and TaqMan assays[41] as indicated by the manufacturers. Probe sets for the Invader assay were designed and synthesized by Third Wave Technologies, and those for the TaqMan assay were obtained from Applied Biosystems. When assessing the results of SNP genotyping, we generally excluded successful call rates <0.95 and values of $P < 0.01$ obtained by Hardy-Weinberg equilibrium testing in control subjects. The error rate of Invader assay was 0.0023, which was estimated by 11,092 assays in two replicates using 118 randomly selected SNPs (internal control data).

Luciferase assay. We cloned the promoter fragment of three haplotypes corresponding to nt −523 to +203 of *FCRL3* into the pGL3-Basic vector (Promega). We generated oligonucleotides using the allelic sequences of nt −189 to −160 of *FCRL3*. We cloned a single copy or four tandem copies of

these oligonucleotides into pGL3-Promoter vector (Promega). We grew Raji cells (RCB1647; RIKEN Cell Bank) in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics. We electroporated (230 V and 975 μF) 1×10^7 cells with 5 pmol of constructs and 1 pmol of pRL-TK vector (internal control for transfection efficiency) in a 0.4-cm gap cuvette. After 48 h, we collected cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

EMSA. We carried out EMSA and preparation of nuclear extract from Raji cells as previously described[49]. We labeled oligonucleotides −169T and −169C with digoxigenin -11-ddUTP using the DIG gel-shift kit (Roche). We incubated 5 μg of nuclear extract with 40 fmol of digoxigenin-labeled nucleotide for 25 min at room temperature. For competition experiments, we preincubated nuclear extract with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. For supershift assays, we incubated 4 μg of antibodies to p50, p52, p65, RelB or cRel and rabbit IgG (control antibody; Santa Cruz Biotechnology) for 15 min at room temperature after incubation of the labeled probe. We separated protein-DNA complexes on a nondenaturing 6% polyacrylamide gel in 0.5× Tris-Borate-EDTA buffer. We transferred the gel to a nitrocellulose membrane and detected signals using an LAS-3000 luminoimage analyzer (Fujifilm).

RNA extraction and cDNA preparation. We collected peripheral blood from healthy volunteers to obtain $CD19^+$ lymphocytes. We separated polymorphonuclear cells by differential centrifugation using Lymphoprep resolving solution (AXIS-FIELD). We isolated $CD19^+$ lymphocytes using the MACS system with CD19 microbeads (Miltenyi Biotec) and confirmed that cell purity was >95% using flow cytometry. We stimulated cells with antibodies to CD40 (Cymbus Biotechnology) or IgM (Jackson Immunoresearch), with Il-4 (eBioscience), with APRIL (PeproTech), with BAFF (PeproTech) or with LPS (Sigma) for 4 h. We isolated total RNA using RNeasy Mini Kit (Qiagen). We quantified RNA in other normal tissues using Premium Total RNA (Clontech). We reverse-transcribed total RNA using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems) in accordance with the instructions of the manufacturer.

Quantification of *FCRL3* expression using real-time RT-PCR. We carried out real-time quantitative PCR using an ABI PRISM 7900 (Applied Biosystems) and Assay-on-Demand TaqMan probe and primers (Hs00364720_m1 for *FCRL3*) in accordance with the manufacturer's instructions. We generated a standard curve from the amplification data for *FCRL3* primers using a dilution series of total RNA from Raji cells as templates and normalized data to GUS level.

ASTQ. We carried out ASTQ as previously described[34] with some modifications. We prepared cDNA from B cells as described above. We amplified both cDNA and genomic DNA by PCR for 37 cycles using primers specific for exon 2 of *FCRL3* (**Supplementary Table 4** online) and for an additional cycle using forward primer with Alexa Fluor 488 label at the 5′ end. Products were directly digested using *Eag*I by incubation at 37 °C for 12 h. We monitored full digestion by the inclusion of PCR products from +358G/G homozygotes. We then separated digested products on a 12.5% polyacrylamide gel and quantified them using an LAS-3000 analyzer.

***In situ* hybridization and immunohistochemistry.** We carried out *in situ* hybridization as previously described[50]. We obtained probes from PCR products using the sequence of *FCRL3* (nt 2052–2490, comprising the intracellular unique region that is poorly conserved among members of this family). An additional probe of the 5′ untranslated sequence yielded similar results. We also examined control probes, which yielded no specific hybridization (data not shown). We used antibodies to CD3 (clone PS-1, Nichirei) and CD20 (clone L26, Zymed) for immunohistochemistry with an ABC Elite kit (Vector Labs) in accordance with the manufacturer's instructions. No specific staining was detected using mouse isotype IgG (data not shown).

Measurement of autoantibodies. We measured RF in sera of individuals with rheumatoid arthritis using latex-enhanced immunonephelometric assay. We measured antibody to DNA in sera of individuals with SLE by radioimmunoassay. Individuals with rheumatoid arthritis (n = 147, 81.1% women; age 63.9 ± 10.6 years (mean ± s.d.); 87.8% RF-positive; mean Steinbrocker radiographic stage 3.2) or SLE (n = 120, 92.6% women; age 36.6 ± 12.7 years (mean ± s.d.)) were part of the cohorts or from a single medical institute, respectively. For each individual, we used the maximum value of RF and antibody to DNA measured during the treatment period in the medical center or outpatient clinic. We detected antibody to CCP at a single time point using enzyme-linked immunosorbent assay, as previously described[38].

Statistical analysis. We calculated LD index Δ (ref. 28) and drew **Figure 1a** using Excel software (Microsoft). We estimated haplotype frequencies using HAPLOTYPER software. We applied the χ^2 test for contingency table tests for associations between allele-genotype distribution and phenotypes. *FCRL3* expression in B cells and autoantibody production were regressed on the number of susceptible alleles (coded 0, 1 and 2). All other statistical analyses, unless otherwise stated, were done using STATISTICA software (StatSoft).

URLs. The JSNP database is available at http://snp.ims.u-tokyo.ac.jp/index.html. TRANSFAC is available at http://www.gene-regulation.com/. HAPLOTYPER is available at http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm.

GenBank accession number. *FCRL3* mRNA, NM_052939.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Corrigendum: Sarcoidosis is associated with a truncating splice site mutation in the gene *BTNL2*

R Valentonyte, J Hampe, K Huse, P Rosenstiel, M Albrecht, A Stenzel, M Nagy, K I Gaede, A Franke, R Haesler, A Koch, T Lengauer, D Seegert, N Reiling, S Ehlers, E Schwinger, M Platzer, M Krawczak, J Müller-Quernheim, M Schürmann & S Schreiber
Nat. Genet. 37, 357–364 (2005).

In the version of Supplementary Table 3 initially published online, the nomenclature of DQB and DPB alleles was partly incorrect. The errors have now been corrected and Supplementary Table 3 has been replaced. Neither the stratified analyses that highlighted the independence of the BTNL2 effect from the two HLA loci (Supplementary Table 3 online) nor any other conclusions of the manuscript were affected by these mistakes.

Corrigendum: Frequent somatic mutations of the transcription factor ATBF1 in human prostate cancer

X Sun, H F Frierson, C Chen, C Li, Q Ran, K B Otto, B L Cantarel, R L Vessella, A C Gao, J Petros, Y Miura, J W Simons & J-T Dong
Nat. Genet. 37, 407–412 (2005).

The name of the seventh author was incorrect. The correct name is Brandi L Cantarel.

Erratum: A functional variant in *FCRL3*, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities

Y Kochi, R Yamada, A Suzuki, J B Harley, S Shirasawa, T Sawada, S-C Bae, S Tokuhiro, X Chang, A Sekine, A Takahashi, T Tsunoda, Y Ohnishi, K M Kaufman, C P Kang, C Kang, S Otsubo, W Yumura, A Mimori, T Koike, Y Nakamura, T Sasazuki & K Yamamoto
Nat. Genet. 37, 478–485 (2005).

In the replication study confirming the association between fcrl3_3 (–169C→T) and rheumatoid arthritis susceptibility, the allele frequency of fcrl3_3 in controls is 0.36.

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SHORT COMMUNICATION

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Ethnic differences in allele frequency of autoimmune-disease-associated SNPs

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Abstract Several multiple, large-scale, genetic studies on autoimmune-disease-associated SNPs have been reported recently: peptidylarginine deiminase type 4 (*PADI4*) in rheumatoid arthritis (RA); solute carrier family 22 members 4 and 5 (*SLC22A4* and *5*) in RA and Crohn's disease (CD); programmed cell death 1 (*PDCD1*) in systemic lupus erythematosus (SLE), type 1 diabetes mellitus (T1D), and RA; and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) in T1D, RA, and SLE. Because these reports on association were not always evaluated in multiple ethnic groups and because ethnic difference in allele frequency of the variants has been also reported, we investigated allele frequencies of nine SNPs in four autoimmune-disease-associated loci in Caucasian, African-descent, and Japanese populations. Although SNPs in *PADI4* had similar allele frequency among three groups [maximal difference 11%; ($P > 0.05$)], the other three loci revealed statistically significant allele frequency differences (maximal difference 39% ($P < 0.00001$), 13% ($P < 0.00001$), and 8% ($P < 0.00001$) in *SLC22A4*, *PDCD1*, and *PTPN22*, respectively). Of note, three SNPs in the three loci that had allele frequency more than 8% in the Caucasian population were either not polymorphic at all or extremely rare in the Japanese population. Our data suggest that ethnic variations of polymorphisms should be evaluated in detail, and differences should be incorporated into investigations of susceptibility variants for common diseases.

Keywords SNP · Ethnicity · Autoimmune disease · PADI4 · SLC22A4 · SLC22A5 · PTPN22 · PDCD1

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Introduction

Autoimmune diseases are common disorders, affecting more than 5% of the world population. They share aspects of aberrant immunological tolerance toward self-antigens, and all are known to have genetic components. Several multiple, large-scale, genetic studies on autoimmune-disease-associated SNPs have been reported recently: peptidylarginine deiminase type 4 (*PADI4*) in rheumatoid arthritis (RA) (Suzuki et al. 2003); solute carrier family 22 members 4 and 5 (*SLC22A4* and *5*) in RA (Tokuhiro et al. 2003) and Crohn's disease (CD) (Peltekova et al. 2004); programmed cell death 1 (*PDCD1*) in systemic lupus erythematosus (SLE) (Prokunina et al. 2002), type 1 diabetes mellitus (T1D) (Nielsen et al. 2003), and RA (Prokunina et al. 2004); and *PTPN22* in T1D (Bottini et al. 2004), RA (Begovich et al. 2004), and SLE (Kyogoku et al. 2004).

Interestingly, the allele frequencies of some of these autoimmune-disease-associated SNPs vary substantially in different ethnic groups, as reported for CD-associated SNPs in the Caucasian and Japanese populations (Yamazaki et al. 2004). Information on ethnic differences in allele frequency of disease-associated variants is important for better understanding of the pathologic mechanisms of polymorphisms. We therefore investigated the allele frequency of nine SNPs in four autoimmune-disease-associated genes/loci in Caucasian, African-descent, and Japanese populations.

Subjects and methods

Japanese volunteers were recruited, and their informed consent to the study was obtained, as required by our ethical committee and as previously described (Suzuki et al. 2003). Human variation panels for African American and Caucasian samples were obtained from Coriell Cell Repositories. We genotyped 376 Japanese,

94 African American, and 94 Caucasian subjects for the following nine SNPs in four loci: padi_89, 90, 92, and 104 in *PADI4* (Suzuki et al. 2003); slc2F1 and slc2F2 (Tokuhiro et al. 2003) and L503F in *SLC22A4* (Peltekova et al. 2004); G-207C in *SLC22A5* (Peltekova et al. 2004); PD-1.3A in *PDCD1* (Prokunina et al. 2002), and R620W in *PTPN22* (Bottini et al. 2004). Japanese data for the four SNPs in *PADI4* were identical to those reported previously (Suzuki et al. 2003). Genotyping was performed using the Invader assay, TaqMan assay, or direct sequencing, and haplotype frequency was inferred with an EM algorithm and/or Haplotyper (Niu et al. 2002). The statistical significance of differences in allele frequency was determined (*P* value in Fisher's exact test), and the genetic distance indicated by differences in allele frequency was quantitated as F_{ST}.

Results

Allele frequencies of the nine SNPs in the three ethnic groups are shown in Table 1. The largest difference in allele frequency among ethnic groups was 39% (L503F, C versus J). Three pairwise comparisons Caucasian versus Japanese (C versus J), African American versus Japanese (A versus J), and African American versus Caucasian (A versus C), were performed for the allele frequency of each SNP and CD-susceptible haplotype in the SLC22A4/5 locus. Of the nine SNPs in four loci, a significant difference in allele frequency ($P < 0.01$) was observed: five SNPs in three loci were significant in the C versus J comparison, five SNPs in three loci in the A versus J comparison, and two SNPs in two loci in the A versus C comparison. The F_{ST} values of more than 0.1 were obtained for four SNPs in two loci: one SNP in one locus was significant in the C versus J comparison, two SNPs in one locus in the A versus J comparison, and two SNPs in two loci in the A versus C comparison.

Discussion

The allele frequencies of the four SNPs in *PADI4* do not differ among the three ethnic groups, as indicated by a low F_{ST} value and an insignificant *P* value. However, SNPs in all the other three loci had allele frequencies that were significantly different in at least one of the comparisons among the three ethnic groups. The most remarkable difference was observed for the L503F SNP and haplotypes containing the SNP in *SLC22A4/5* in a comparison between the Caucasian and Japanese populations. The SNP and the corresponding haplotypes were much rarer in the Japanese population and were also rare in African Americans. Although other differ-

Table 1 Allele frequency of autoimmune-disease-associated variants in three ethnic groups

Gene name/ variant name	Caucasian	Japanese	African American	Caucasian versus Japanese		African American versus Japanese		African American versus Caucasian	
				P value	F_{ST}	*P* value	F_{ST}	*P* value	F_{ST}
PADI4									
padi_89	0.38 (0.40[a])	0.40[j]	0.49	0.86	0.00034	0.10	0.0079	0.078	0.12
padi_90	0.42 (0.41[a])	0.40[j]	0.46	0.86	0.00052	0.51	0.0037	0.88	0.0014
padi_92	0.38 (0.42[a])	0.39[j]	0.48	0.84	0.000053	0.023	0.0090	0.31	0.010
padi_104	0.31 (0.31[a])	0.33[j]	0.35	0.66	0.0056	0.70	0.00065	0.73	0.0024
SLC22A4									
slc2F1	0.09 (0.07[b])	0.29	0.04	<0.00001*	0.064	<0.00001*	0.12	0.14	0.012
slc2F2	0.09 (0.05[b])	0.29	0.04	<0.00001*	0.066	<0.00001*	0.12	0.14	0.012
L503F (C1672T)	0.39 (0.43[b])	0.001 (0.00[k])	0.04	<0.00001*	0.24	<0.00001*	0.018	<0.00001*	0.19
SLC22A4/A5									
Haplotype TC	0.39 (0.42[c])	0.00 (0.00[k])	0.02	<0.00001*	0.24	<0.00001*	0.011	<0.00001*	0.037
PDCD1									
PD-1.3A	0.13 (0.07[d]) 0.13 (0.07[e]) 0.13 (0.07[f])	0.00	0.03	<0.00001*	0.068	0.00040*	0.014	0.0039*	0.036
PTPN22									
R620W	0.08 (0.09[g]) 0.08 (0.09[h]) 0.08 (0.12[i])	0.00	0.02	<0.00001*	0.040	0.0032*	0.011	0.16	0.016

Data from other groups shown in *parenthesis*:
[a]British data derived from Barton et al. (2004)
[b]Canadian data derived from Newman et al. (2005)
[c]European data derived from Peltekova et al.(2004)
[d]Swedish data derived from Prokunina et al. (2004) and Prokunina et al. (2002)
[e]European American data derived from Prokunina et al. (2004)
[f]Danish data derived from Nielsen et al. (2003)
[g]North American data derived from Begovich et al. (2004)
[h]North American data derived from Kyogoku et al. (2004)
[i]North Amerian data derived from Bottini et al. (2004)
[j]Japanese data from our group, reported by Suzuki et al. (2003)
[k]Japanese data derived from Yamazaki et al. (2004)
* *P* value calculated by Fisher's exact test: $P < 0.01$

ences in allele frequency less outstanding than L503F in *SLC22A4/5*, it was of note that PD-1.3A in *PDCD1* and R620W in *PTPN22* were only polymorphic in Caucasians and in the African-descent population, as for L503F in *SLC22A4/5*. Populations of African descent, Caucasians, and Japanese (an ethnic subgroup from far-east islands), should have different population histories of mutation, migration, isolation, and genetic drift, and there is no doubt that there should be substantial differences in allele frequencies among these ethnic groups. However, an explanation of the remarkable difference seen for L503F in *SLC22A4/5* and others appears to require an additional mechanism. We note that Stefansson et al. (2005) reported that a 900-kb inversion polymorphism is frequently present in Caucasians but is rare in Africans and almost absent in East Asians, similar to the L503F SNP, and that prevalence of the variation in Caucasians was due to a positive effect in reproduction.

Beside the population genetics perspective, the difference in allele frequency among ethnic groups is important for association studies. Ioannidis et al. (2004) recently summarized multiple meta-analyses on common disease-susceptible polymorphisms from the perspective of ethnic variability. They concluded that the relative risk produced by susceptible variants seemed to be more similar among various ethnic groups compared with variability in their allele frequency. These findings would be correct in general but may not be applicable for polymorphisms that are common for some ethnic groups but absent in other groups. Ethnic uniformity and diversity in the identity of common disease-susceptible variants and their genetic contribution need to be extensively investigated before ethnic variations of polymorphisms are understood. Therefore, the details and differences in ethnic variations need to be incorporated into investigations of susceptibility variants for common diseases.

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Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis ☆

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Abstract

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, but its autoimmune mechanisms are not clearly understood. Recently, anti-citrullinated peptide antibodies have been specifically observed in sera of RA patients. Furthermore, we identified RA-susceptible variant in a gene encoding citrullinating enzyme, peptidylarginine deiminase type 4 (PADI4). Therefore, we hypothesized that proteins which are modified in RA synovium by PADI4 act as autoantigens. Subsequently, we obtained human collagen type I (huCI) as one of the autoantigens using a RA synoviocyte cDNA library by immunoscreening. We also investigated that the levels of anti-citrullinated huCI were significantly higher in RA patient sera than in normal control sera with high specificity (99%) and positively correlated with the levels of anti-cyclic citrullinated peptide (anti-CCP) antibodies. We concluded that huCI is a novel substrate protein of PADIs and that citrullinated huCI is a candidate autoantigen of RA.

Keywords: Autoantibodies; Peptidylarginine deiminases; Rheumatoid arthritis; Human type I collagen; Human type II collagen

Rheumatoid arthritis (RA) is a major autoimmune disease and affects ~1% of the world population. Many autoantibodies have been found in sera of RA patients, including antibodies against rheumatoid factor (RF). However, the antibodies that are most specific to RA are autoantibodies against citrulline-containing proteins or peptides. These are the so-called anti-citrullinated protein antibodies, including APF (anti-perinuclear factor), AKA (anti-keratin antibodies), AFA (anti-filaggrin antibodies), and anti-Sa. Anti-CCP antibody is an anti-citrullinated protein antibody that is reasonably sensitive (41–88%) and extremely specific (~98%) for RA, and has been used in clinical applications with high reliability [4,23,24]. The anti-CCP antibody system is also suited to the diagnosis of early RA [33]. Although the highly reliable anti-CCP antibody assay system (second generation) is designed to detect antibodies that recognize a mixture of synthetic peptides containing citrulline, the precise sequences of those peptides are not known. To clarify the pathologic mechanism of anti-citrullinated peptide antibodies in RA, it is important to identify native citrullinated peptides that are recognized by RA sera. Native citrulline-containing peptides are only produced by enzymatic conversion of peptidylarginine to citrulline, because citrulline is a non-coded amino acid in vivo. The enzymes involved in this conversion are

☆ *Abbreviations:* RA, rheumatoid arthritis; PADI, peptidylarginine deiminase; huCI, human collagen type I; huCII, human collagen type II; anti-MC, anti-modified citrulline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; pNPP, *p*-nitrophenyl phosphate.

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peptidylarginine deiminases (PADIs). Five PADI isozymes have been detected in humans, and two of them (PADI2 and PADI4) have been detected in RA synovial tissue [35]. Previously, we found that the PADI4 gene is associated with RA [28]. These facts strongly suggest that citrullination of self proteins (particularly by PADI4) and production of autoantibodies against those citrullinated proteins play pathologic roles in RA. Consequently, identification of the substrates of PADIs is important for investigation of autoimmunity in RA.

PADIs are enzymes that participate in post-translational modification of proteins by catalyzing citrullination of arginine residues. PADIs have five mammalian isoforms, which are expressed in different tissues and different stages of development [37]. All PADIs are dependent on calcium ions for activity. Arginine residues in a variety of proteins are modified by PADIs, but free L-arginine is not a substrate of PADIs [30]. Recently, citrullinated histone was reported to have an ability to antagonize transcriptional induction by regulating histone Arg methylation levels [8,38,41]. Although the physiologic role of peptidylcitrulline is unknown, it has been reported that citrullination is related to several diseases including autoimmune diseases [35,37,40].

Many substrates that are citrullinated by PADIs have been identified; e.g., histones, nucleophosmin/B23 [12], keratins [25], filaggrin [32], vimentin [2,34], myelin basic protein (MBP) [42], and fibrin(ogen) [18]. Not all of those proteins are expressed in RA-specific tissues, such as synovial tissue or joints, but all are recognized by RA autoantibodies. Also, there appears to be cross-reaction with anti-citrullinated antibodies. On the other hand, citrullinated fibrin(ogen) has been detected in synovial membranes of RA patients, and is recognized by RA autoantibodies [18]. However, it is unclear whether citrullinated fibrin(ogen) or other citrullinated peptides are pathogenic autoantigens of anti-citrullinated antibodies in RA. Therefore, we designed a survey to identify further candidate citrullinated proteins that are important in the pathology of RA autoimmunity.

To identify novel substrates of PADIs, which are candidate autoantigens of anti-CCP and anti-citrullinated peptide antibodies, we targeted proteins expressed in RA synovium. Because the anti-CCP antibody assay is designed to detect peptide epitopes, we also focused on citrulline-containing peptides rather than structures or various modifications of proteins. In order to survey proteins expressed by RA synovium regardless of their expression level, we adopted a λ-ZAP library of cDNA from RA synoviocytes as the origin of our proteins, and expressed proteins were citrullinated in vitro.

As a result of immunoscreening, we identified human collagen type I (huCI) peptides as a substrate of PADIs. Collagens, including CI, are structural proteins found in connective tissues of animals, and comprise the main extracellular support system. Type II collagen (CII)-induced arthritis (CIA) is a widely used model of arthritis [7,16,27,39]. Anti-CII antibodies were also commonly found in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. However, there have been no reports of a pathogenic role of CI in RA, although CI is distributed in bone, tendon, vascular tissues, synovial tissues, and skin (where it has functions related to its unique mechanical properties). In the present study, we identified human collagen type I (huCI) peptides as a candidate native substrate of PADIs. We also demonstrated that anti-citrullinated huCI peptide antibody is specific to RA, and that anti-citrullinated huCI peptide antibody appears to share some diagnostic features with anti-CCP antibody, although there are some distinguishing features.

Materials and methods

Human sera and synovial tissues. Human serum was obtained from three groups of subjects: 117 patients diagnosed with RA according to the criteria of the American Rheumatism Association [1]; 47 healthy blood donors (control); 37 patients with non-RA diseases (systemic lupus erythematosus, 19 patients; Sjogren's syndrome, 4 patients; Bechet's disease, 2 patients; other rheumatic disease, 12 patients). All non-RA patient serum and healthy control serum were anti-CCP negative. Mean age of the RA patients was 61.1 years (range, 33–81 years), and 66% of RA cases were positive for rheumatoid factor. No relationship was observed between age and anti-CCP antibody ($R^2 = 0.00001$). Mean age of non-RA patients and the healthy controls was 45.7 years (range, 20–82) and 40.5 years (range, 23–84), respectively. Informed consent was obtained from all subjects.

Preparation of PADIs. His-tagged human PADI4 (hPADI4) was expressed in *Escherichia coli*, BL21-SI, and was purified using a HiTrap protein purification system (Amersham Biosciences, Piscataway, NJ), as previously described [21]. Rabbit PADI2 (rPADI2) was obtained from Sigma. We used hPADI4 or rPADI2 in assays for in vitro citrullination of substrates according to assay condition requirements, after adjusting their enzymatic activity unit for artificial substrates, as described elsewhere [21].

Immunoscreening. A human RA synoviocyte cDNA library in λ-ZAP (Stratagene, La Jolla, CA) was used for the immunoscreening of citrullinated proteins. Induction of protein expression was performed according to the manufacturer's instructions. Briefly, the phage library was plated at 5×10^3 pfu/plate on a series of 100-mm NZY agar plates and was incubated at 37 °C for 5 h. After transfer to nitrocellulose membrane, protein expression was induced on the membranes by incubation with 20 mM IPTG at 37 °C for 4 h. After the membranes were washed, citrullination by hPADI4 was performed on a membrane with 0.075 U/ml hPADI4, 100 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT at 37 °C for 1 h. Detection of citrullinated proteins was performed using an anti-MC detection kit (Upstate, Waltham, MA). The positive clones were converted to a pBluescript II SK (+). The sequence of the inserted DNA was determined using an ABI PRISM 3700 Sequencer (Applied Biosystems, Foster City, CA).

Identification of citrullinated sites of human CI by LC/MS/MS. Human CI was citrullinated by rPADI2, which is generally used for analysis of anti-citrullinated antibodies, in a reaction buffer containing 25 mM Tris–HCl (pH 7.6), 20 mM $CaCl_2$, and 5 mM DTT for 12 h at 50 °C. Then, 100 μg huCI, with or without enzyme treatment, was separate by SDS–PAGE, and bands on Coomassie brilliant blue-stained gel were excised and digested with trypsin. The mixture of the digested peptides was analyzed by LC/MS/MS (APROscience, Toku-

shima, Japan). A Q-TOF2 mass spectrometer (Micromass, Manchester, UK) equipped with MAGIC 2002 (Michrom BioResources, Auburn, CA) was also used. Elution of peptides was performed using an acetonitrile gradient in 0.1% formic acid. The MS/MS data were searched against the SWISS-PROT database with protease specificities under consideration of fixed modification (propionamide-Cys) and variable modifications (citrullination of Arg, deamination of Asn and Gln, hydroxylation of Pro, Pyro-glu of N-terminus of Gln, and oxidation of Met) using the Mascot program (Matrix Science, Boston, MA).

Western blotting using human antisera. Citrullinated proteins were separated by SDS–PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad). Membrane strips were probed with human sera (1:50) in blocking buffer (5% skimmed milk in T-TBS). Goat anti-human IgG peroxidase-conjugated antibodies were used for the detection of primary antibody, and peroxidase activity was visualized using ECL Western blotting reagents (Amersham).

Detection of citrullination of collagen type I and type II by ELISA. We coated 96-well ELISA plates (Corning, Corning, NY) with 100 μl of acid-extracted huCI (Becton, Dickinson and Company) and huCII (Collagen Research Center, Tokyo, Japan, or Becton, Dickinson and Company) per well in 0.01 M acetic acid at 5 μg/ml, and performed citrullination by rPADI2 at 50 °C. After washing with T-PBS and adding 0.1% ovalbumin in TBS, the plate was treated with 1% glutaraldehyde in PBS. Then, the plate was washed with 0.2 M Tris–HCl (pH 7.8) and distilled water. The modified citrullinated collagen was detected by anti-MC antibody (Upstate) using the standard method.

Detection of anti-citrullinated antibodies in RA patients and normal sera by ELISA. After coating and deimination as described above, anti-citrullinated collagen antibodies were detected by a modification of a previously reported method [29]. Briefly, citrullinated collagen coated plates were blocked with 5% skimmed milk in T-TBS. Then, 100 μl of each diluted serum was added, followed by incubation for 2 h at room temperature. After washing, 100 μl of 1:20000 dilution of goat $F(ab')^2$ anti-human IgG AP (Biosource, Camarillo, CA) was added, followed by incubation at room temperature for 1 h. Plates were developed at room temperature with 100 μl of pNPP substrate (Sigma) per well, and the absorbance at 405 nm was measured using a Fusion plate reader (PerkinElmer, Boston, MA). Standard control serum was analyzed, to assure standardized conditions for ELISA of anti-citrullinated collagen. All tests were run in triplicate. Testing for significant differences between means was performed using Student's *t* test (Microsoft Excel).

Results

Identification and confirmation of (in vitro) citrullination of proteins from synoviocyte cDNA library

To identify novel self molecules that are citrullinated as targets of RA-specific autoantibody, we immunoscreened ~10^6 pfu lambda of a ZAP cDNA expression library that was constructed using synoviocytes from RA patients. The expressed proteins were citrullinated and expressed proteins that were not treated with the citrullinating enzyme were used as a negative control (Fig. 1A). Three of the positive clones were obtained from a second screening plate and were identical with the huCI α1 gene. To confirm that the huCI was citrullinated, we performed Western blotting (Fig. 1B). We detected citrullination of huCI using ELISA (Figs. 1C and D). Our results indicate that PADIs citrullinated huCI.

After in vitro citrullination of huCI by rPADI2, we performed LC/MS/MS to identify the citrullination site of huCI. The coverage of LC/MS/MS analysis was 45% for huCI α1 precursor and 55% for huCI α2 precursor. We identified 20 citrullination sites of 35 arginine residues in analyzed fragments of huCI α1 and identified 27 citrullination sites of 45 arginine residues in analyzed fragments of huCI α2 (Figs. 1E and F). We did not observe sequence specificity of recognition of targeted arginine residues by the enzyme for conversion from peptidylarginine to peptidylcitrulline. These findings are consistent with those of a previous study [21]. We also confirmed that huCII was citrullinated by PADIs in vitro (data not shown), in addition to their citrullination of huCI.

IgG from RA patients' sera recognized citrullinated human collagen type I in vitro

Reactivity of sera to citrullinated and uncitrullinated huCI was analyzed by Western blotting using 10 RA sera and five normal control sera (Table 1). There was a slight difference in reactivity toward uncitrullinated huCI between sera of RA patients and sera of normal controls. Sera of RA patients were reactive toward the citrullinated huCI.

Additionally, we detected anti-citrullinated huCI by ELISA using serum from a RA patient with a high anti-CCP titer and anti-CCP-negative serum from a healthy control (Fig. 2A). Serum from the RA patient was highly reactive with citrullinated huCI, but serum from the healthy control was not reactive. We also performed time-course analysis (Fig. 2B). The level of anti-citrullinated huCI antibodies detected was increased by citrullination, and was dependent on the enzyme reaction time.

High titers of anti-citrullinated collagen antibody are present in RA sera, but not in normal sera

To investigate whether RA patients, non-RA patients, and normal healthy controls had serum antibodies against citrullinated collagens, ELISA was used to analyze panels of sera from RA patients, non-RA patients, and normal healthy controls.

Next, we measured levels of anti-collagen antibodies in the present subjects. The relative levels of anti-huCII were significantly higher for RA patients than for normal controls ($p = 0.0026$, Student's *t* test; Fig. 3B), and 22 of 56 sera (39%) from RA patients and 1 of 9 sera (11%) from healthy controls were positive for anti-huCII antibodies. However, the relative levels of anti-huCII were significantly higher for non-RA patients than for normal controls ($p = 0.00014$, Student's *t* test; Fig. 3B), and 8 of 13 sera (62%) from non-RA patients were positive for anti-huCII antibodies. In contrast, there

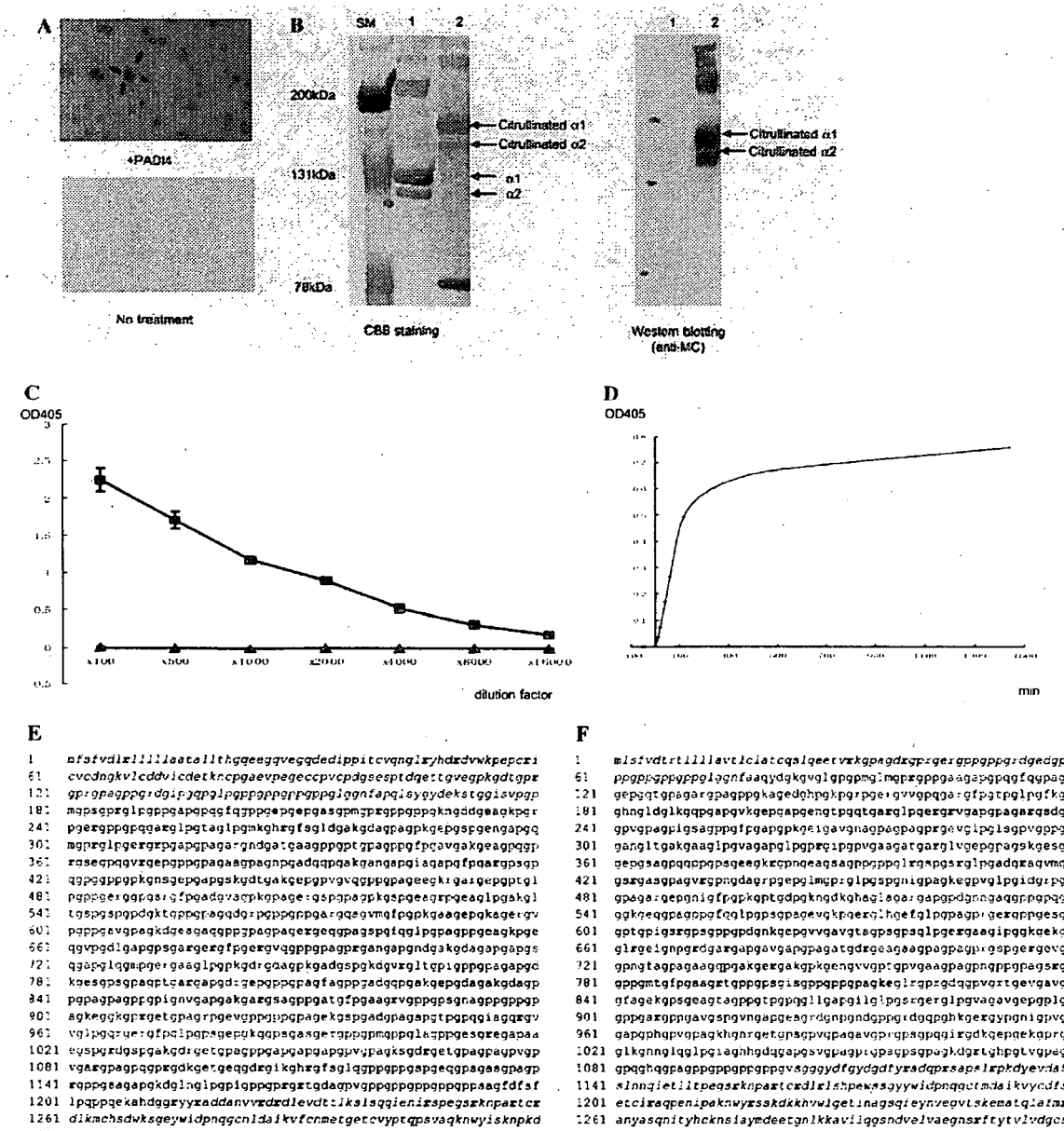

Fig. 1. Identification and confirmation of citrullinated CI modified by PADIs. (A) Secondary immunoscreening using λ-ZAP cDNA expression library, with detection by anti-MC (1:1000). Three selected positive clones were identical: CI. Citrullination of CI by PADI2 and PADI4. (B) Citrullination by PADI2 was confirmed using Western blotting (SM, size marker; lane 1, CI; lane 2, modified CI). (C) The citrullinated CI was detected by ELISA at each dilution rate of anti-MC. Square indicates reaction with PADI2 and triangle indicates reaction with enzyme reaction buffer. (D) Time course of citrullination of human CI by PADI. The reaction mixtures were incubated at 50 °C for 0, 10, 20, 40, 60, 120, 240, 480, and 1440 min, respectively. Anti-MC (1:1000) was used for detection of citrullination by ELISA. (E,F) Amino acid sequence of procollagens (NCBI database, NP_000079 and NP_000080). Procollagen type I α1 (E) and procollagen type I α2 (F) were digested by peptidase, producing mature collagen. Italic letters indicate digested peptide. Colored amino acid sequence indicates peptide fragments that were analyzed by LC/MS/MS. Red-colored residues are highly citrullinated arginine residues, and green-colored residues are arginine residues that are unlikely to be citrullinated. All arginines are shown in bold letters.

was no difference in the level of anti-huCI antibodies among RA patients, non-RA patients, and normal controls (RA versus normal individuals, $p = 0.87$; non-RA versus normal individuals, $p = 0.88$; Student's t test; Fig. 3A). Five of 117 sera (4%) from RA patients, 1 of 46 sera from non-RA patients (2%), and 1 of 37 sera (3%) from healthy controls were positive for anti-huCI antibodies.

Table 1
Summary of Western blotting using antisera from RA patients and normal controls

	Citrullinated CI	Non-citrullinated CI
RA patients ID		
RA_1	+	+
RA_2		
RA_3	+	
RA_5	+	
RA_6	+	+
RA_7	+	
RA_8		
RA_9	+	
RA_10	+	
RA_11		
	7/10 (70%)	2/10 (20%)
Healthy controls ID		
HC_2		
HC_3		
HC_4		
HC_5		
HC_11		+
	0/5 (0%)	1/5 (20%)

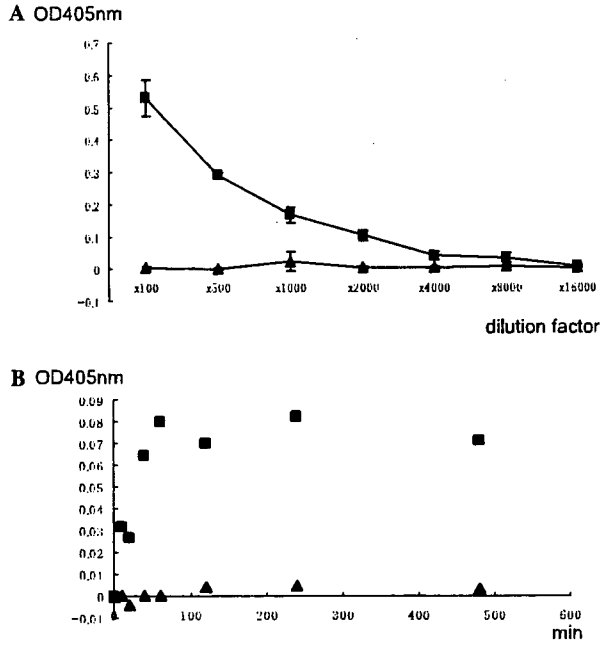

Fig. 2. Detection of citrullinated CI in sera of a RA patient and a normal control. (A) Citrullinated huCI modified by rPADI2 was detected by ELISA, using antisera from a RA patient (square) and a normal control (triangle). (B) Time course was confirmed using serum from the patient in (A).

Also, we measured the relative levels of anti-citrullinated collagen antibodies in RA sera, non-RA sera, and healthy control sera (Figs. 3C and D). The relative levels of anti-citrullinated huCI were significantly higher for RA patients than for normal controls or non-RA patients ($p = 0.000026$, $p = 0.0011$, Student's t test; Fig. 3C). Thirty-eight of 117 sera (32%) from RA patients, none of 37 sera from non-RA patients (0%), and 1 of 47 sera from normal controls (2%) were positive for anti-citrullinated huCI antibodies. In contrast, there was no significant difference in levels of anti-citrullinated huCII between RA patients and either normal controls or non-RA patients. Thirty-seven of 56 sera (66%) from RA patients, 7 of 13 sera from non-RA patients (54%), and none of 9 sera from normal controls (0%) were positive for anti-citrullinated huCII antibodies. In addition, no correlation between age and both of the level of anti-citrullinated CI ($R^2 = 0.0037$) and anti-citrullinated CII ($R^2 = 0.022$) was observed.

We investigated correlation between the relative levels of anti-citrullinated collagen antibodies and anti-CCP antibodies (Figs. 4B and D). Correlation between the level of anti-CCP and anti-citrullinated huCI antibodies was higher than the correlation between anti-CCP and anti-citrullinated huCII antibodies. We also compared the level of anti-CCP with that of anti-collagen antibody. Weak correlation or no correlation was observed between anti-CCP and both anti-huCII and anti-huCI (Figs. 4A and C). Furthermore, we found that anti-citrullinated huCI weakly correlated with anti-citrullinated huCII (Fig. 4E). We hypothesize that some of the anti-citrullinated huCI antibody cross-reacted with citrullinated huCII.

Discussion

Citrullinating enzymes [35] and autoantibodies that specifically recognize peptidylcitrulline, including anti-keratin antibody [26], anti-filaggrin autoantibodies [10], anti-Sa [19], and anti-CCP [11,14,29], are associated with RA. Citrullination of self-peptides is strongly suspected to be pathogenic in RA. However, self-peptides that are citrullinated have not been found to be pathologically linked to RA. Because anti-citrullinated antibodies are thought to be locally produced in RA synovium [17,22], we adopted RA synovial tissue as a source of pathologic citrullinated self-peptides. Among five PADI isoforms in humans, PADI2 and PADI4 are present in synovial fluid as well as synovial fluid mononuclear cells [36]. Citrullinated proteins have been detected not only in nuclear and intracellular areas, but also in amorphous deposits and extracellular matrix in RA synovial tissue [18]. Therefore, we widely targeted proteins expressed in synovium including intra- and extracellular proteins, regardless of their expression level. To identify substrates of PADIs as candidate autoantigens, we immunoscreened an expression cDNA phage library of RA synoviocytes. Our method allows

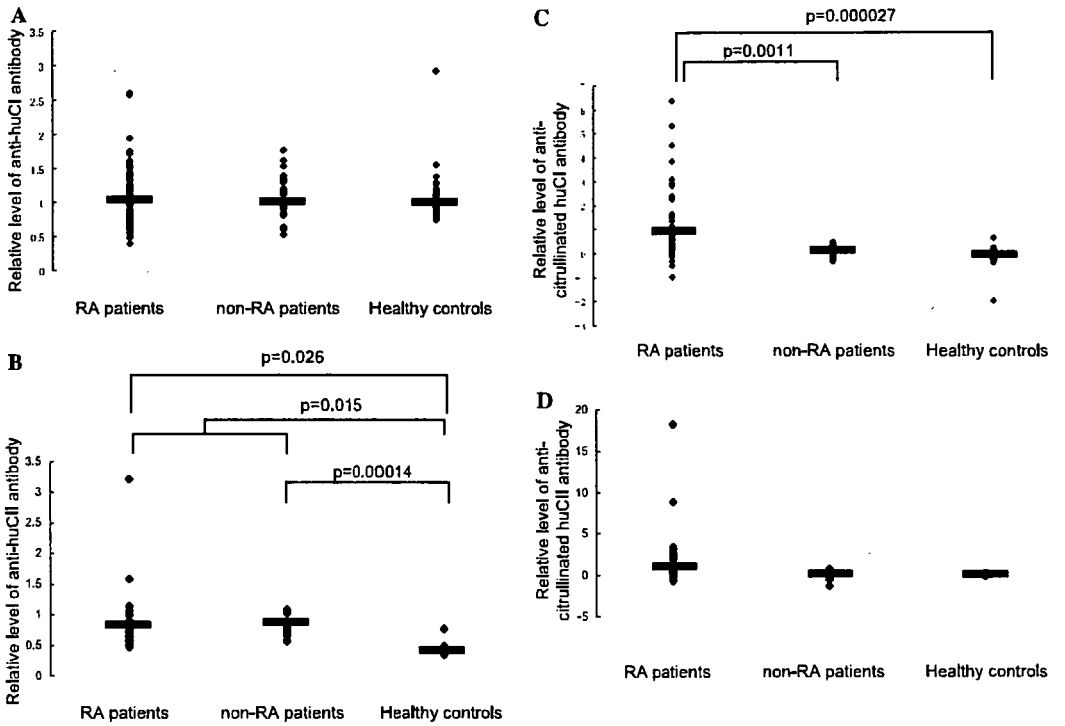

Fig. 3. Measurement of anti-collagen antibodies and anti-citrullinated collagen antibodies in RA patient sera and normal control sera. After coating, citrullination was produced by incubation with PADI2, followed by testing of the titers of anti-huCI antibodies and anti-citrullinated huCI antibodies (A,C, 117 RA sera, 37 non-RA patient sera, and 47 normal control sera). We also tested the titers of anti-huCII antibodies and anti-citrullinated huCII antibodies (B,D, 56 RA sera, 13 non-RA patient sera, and 9 normal control sera). The mean + 2 SD of healthy control values (A, >1.71; B, >0.72; C, >0.58; D, >0.12, respectively) was positive.

detection of insoluble proteins (including extracellular matrix proteins) as well as soluble proteins. This method also detects target proteins expressed at low levels, and is superior to other methods such as 2D-PAGE. One of several clones we identified was huCI peptides, which we examined in conjunction with other collagens known to be relevant to RA [9]. We confirmed citrullination of huCI by Western blotting, ELISA, and LC/MS/MS. We identified many citrullinated sites in huCI.

In the present study, we identified huCI peptide as a candidate substrate of citrullinated autoantigens by immunoscreening and found that anti-citrullinated huCI peptide antibody was specific to RA patients. huCI is one of the collagens that function as structural proteins, all of which have a characteristic triple helix structure with cyclic glycine and a high content of proline and hydroxyproline in their amino acid sequence. Among the collagens, CII has been the most studied, and there is evidence that it plays a pathologic role in RA. CII is major collagen in cartilage, and immunization with CII induces arthritis in mice and rats [6]. Bovine CII is also highly antigenic in transgenic mice that express HLA-DR1(*0101) and (*0401), which are associated with susceptibility to RA [5]. Anti-CII antibodies were observed in both RA (IgG, 41–72.4%) and non-RA (e.g., osteoarthritis and infective arthritis) (IgG, 36–88%) sera [3,31]. In the present study, anti-huCII antibody was also detected (41%) in RA patients. Compared to CII, there have been few reports indicating that CI plays a pathologic role in RA, although CI is widely expressed in bone, tendon, vascular tissues, synovial tissue, and various other tissues. In the present study, we observed no autoantibody recognizing non-citrullinated huCI in RA or control sera. In RA subjects, we observed that a marked increase in autoantibody positivity was associated with citrullination of huCI, but not with citrullination of huCII.

Although the present data indicate that anti-citrullinated huCI antibody is an RA-specific autoantibody, there are several issues that remain unresolved. First, collagen molecules form a triple helix with post-translational modification and their tertiary structure is believed to be a determinant of epitopes [13,15,20], although epitopes of anti-CCP antibodies are modified peptides. Second, it is not known how peptidylcitrullination alters antigenicity and breaks immunologic tolerance. The present findings, obtained by peptide-based immunoscreening and confirmation of recognition of citrullinated acid-extracted huCI molecules, provide a basis for further investigation to clarify the mechanisms of the roles of anti-citrullinated peptide antibodies in RA.

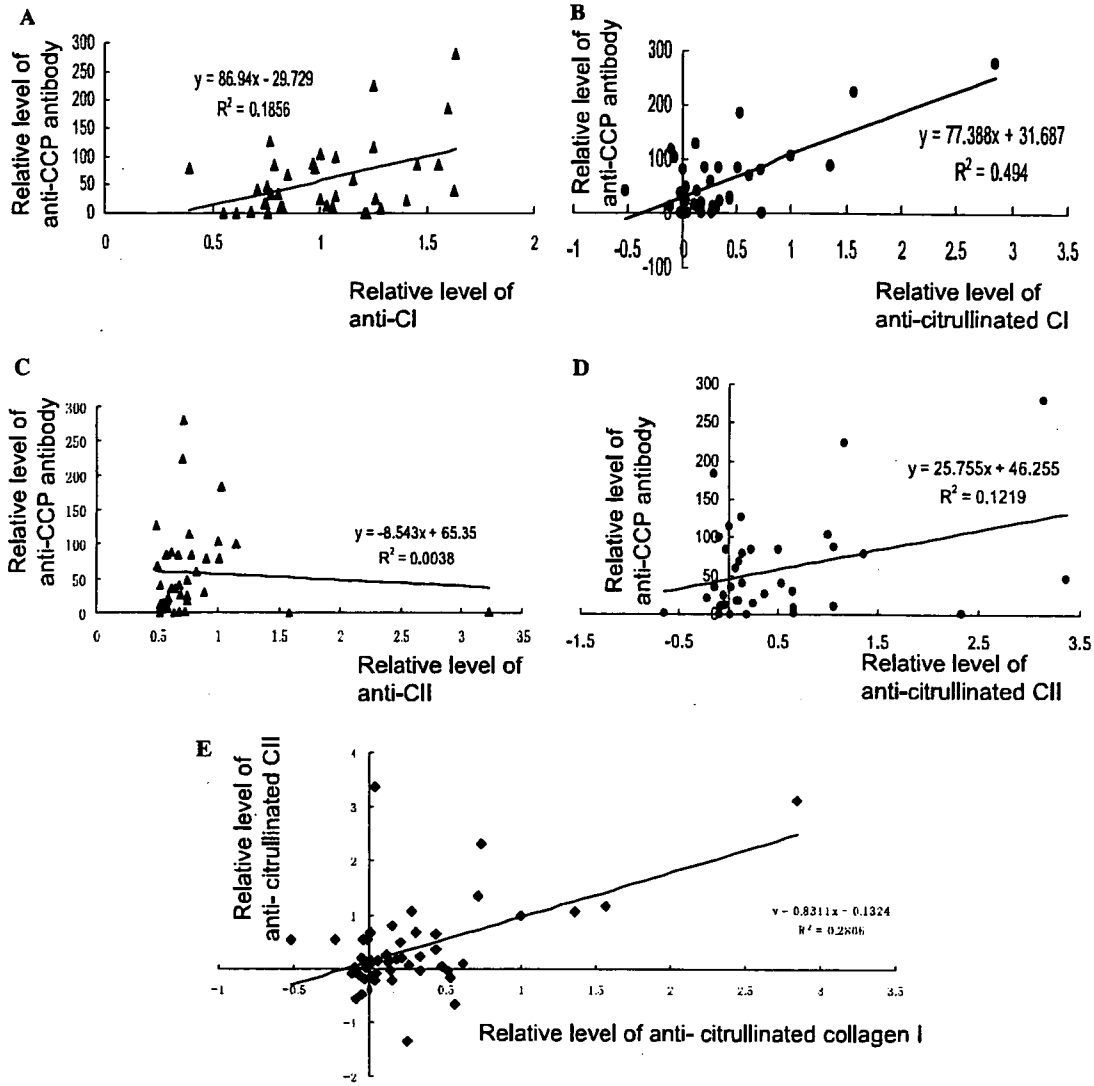

Fig. 4. Comparison of titers of anti-CCP and relative levels of anti-citrullinated collagen in RA sera. Comparison of (A) anti-huCI and (B) anti-citrullinated huCI levels with anti-CCP levels in RA sera. Comparison of (C) anti-huCII and (D) anti-citrullinated huCII levels with anti-CCP levels in RA sera. (E) Comparison of anti-citrullinated huCI levels with anti-citrullinated huCII levels in RA sera. There were no significant differences in any of these comparisons for any of the 37 RA samples. Regression line and correlation coefficient (R^2) are shown.

The present sensitivity and specificity of anti-citrullinated huCI antibody were 32% and 99%, respectively, and they correlated strongly with those of anti-CCP. The specificity of anti-citrullinated huCI antibodies was nearly equal to that of anti-CCP antibody, but the sensitivity of anti-citrullinated huCI antibodies was significantly less than that of anti-CCP antibody. Although almost all subjects who were positive for anti-CCP antibody were also positive for anti-citrullinated huCI antibody, a few were positive for anti-citrullinated huCI antibody but not for anti-CCP antibody. Because anti-CCP recognizes a mixture of synthetic peptides containing citrulline, and because huCI molecules contain multiple arginine residues that are citrullinated, it appears likely that epitopes of anti-CCP antibodies comprise the majority of those of anti-citrullinated huCI, but not all of them.

In conclusion, we found that huCI is a substrate of PADIs and that citrullinated huCI strongly correlates with RA. However, the present results indicate that CI can become an autoantigen via citrullination by PADIs, and citrullination as post-translational modification appears to be an important factor in RA. In addition, the present results suggest that anti-citrullinated collagen antibodies comprise a subclass of anti-CCP. To produce autoantigens in RA patients, PADIs must modify their substrates, but the mechanisms of this modification are unclear. However, PADIs are also clearly present in

the extracellular region [36]. Also, PADIs may be activated in the extracellular region, because the calcium ion concentration is sufficiently higher in the extracellular region than in the cytoplasm or intracellular region [21]. We speculate that autoantibodies for citrullinated collagens react or cross-react with other citrullinated proteins that are locally produced at the site of rheumatoid inflammation of synovial tissue. We believe that anti-citrullinated huCI plays important roles in the development of RA. More study of the mechanisms of citrullination in vivo may provide findings that are applicable to RA therapy.

Acknowledgments

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関節リウマチに対する インフリキシマブによる寛解導入療法

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Remission Therapy by Infliximab for Rheumatoid Arthritis

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Abstract

To analyze the course of remission in RA patients, after treatment with infliximab, we investigated 8 patients with previously established RA who had gone into remission and discontinued infliximab. Infliximab was discontinued when a negative CRP level, below 2.6 points of DAS 28, was continuously obtained for more than 6 months. Of the eight cases, one case (Stage Ⅲ) showed increased CRP levels 18 months after discontinuing infliximab. Another case (Stage Ⅱ) underwent complete remission without need of further infliximab or MTX 12 months after discontinuing infliximab. The other cases showed negative CRP, negative RF, and negative MMP-3 levels for 7.9 months on average after discontinuing infliximab. The MMP-3 levels significantly decreased to within normal range compared with those before treatment. Therefore, in remission after infliximab for early RA, not only prevention of further bone destruction but also increase in CRP levels were observed.

はじめに

関節リウマチ（以下RAと略す）の治療で関節変形をいかに抑制あるいは改善できるかは臨床上極めて重要である。関節変形は主に関節破壊から起こり，RAが発症してから約5年で75％が始まるとされている[1]。すなわち関節破壊を抑制するには早期治療が重要である。またRAには自然寛解があるとされているが実際のところ長期の詳細な報告はない。メトトレキサート（以下MTXと略す）を使用しても約3年で手指の変形は確実に進行することをしばしば経験する（図1）。このような内服薬のみの治療においてはRAの関節破壊は止められず，現在，

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生物学的製剤をもってこの効果が発揮されることが明らかにされてきた[5]。RA発症早期に生物学的製剤を使用することにより関節破壊が防止可能である（Windows of Opportunity）[9]。2003年より日本に導入された生物学的製剤インフリキシマブによってその効果と副作用は明らかにされてきているが，日本人の寛解については不明である。インフリキシマブによって最も改善を示す関節は手指の関節であり，患者はインフリキシマブ投与後手指関節の疼痛，腫脹がとれるということが多い（図2）。寛解とはDisease Activity Score（以下DASと略す）28が2.6以下になるものを臨床的寛解とされているが[8]，RAの治療において薬剤中止可能すな

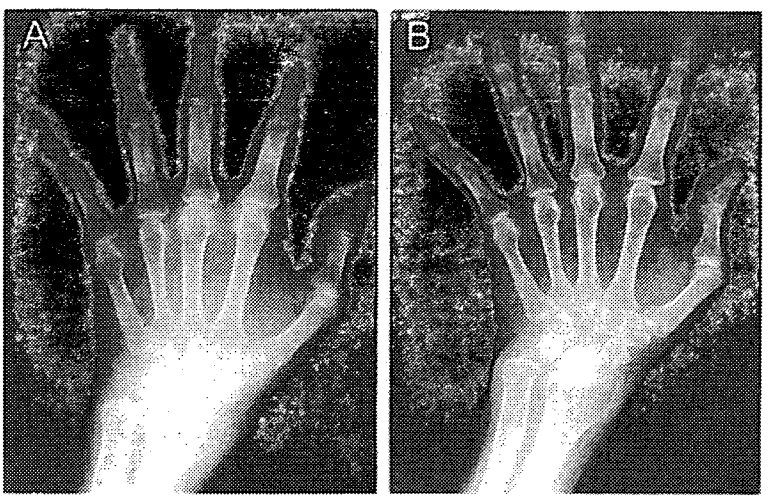

図1　RAにおける関節破壊の進行，65歳　女性，
MTX 6mgにて治療した。A：治療前，B：治療後1年

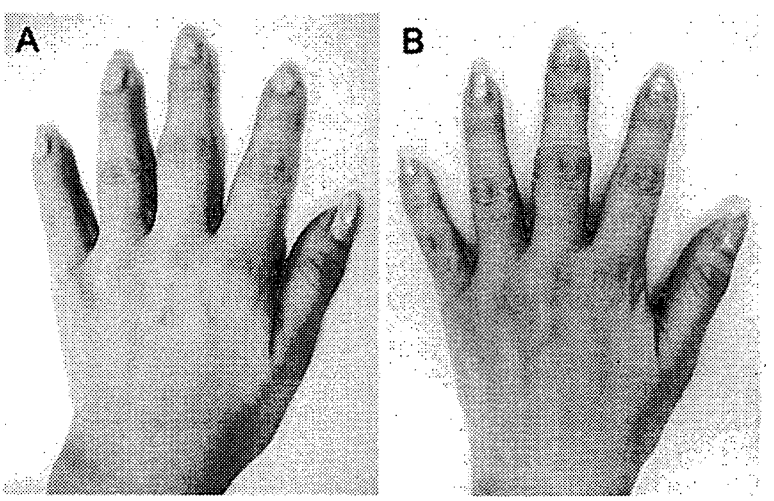

図2　インフリキシマブによる手指関節腫脹の改善，43歳　女性，
A：投与前，B：投与後1ヵ月

わちインフリキシマブ投与中止できた寛解は患者にとってより治癒に近い寛解とも言える。われわれはRAに対してインフリキシマブを使用した193例の患者背景とインフリキシマブを中止できた8例の臨床追跡評価とXP所見を検討したので報告する。

対象および方法

インフリキシマブ（レミケード，田辺三菱製薬）により当科で治療した関節リウマチは193例，男性31例，女性162例，Stage Ⅰ 18例，Stage Ⅱ 108例，Stage Ⅲ 61例，Stage Ⅳ 6例で，Class Ⅰ 8例，Class Ⅱ 112例，Class Ⅲ 69例，Class Ⅳ 4例であった。年齢は27歳から81歳，平均60.1歳であった。そのうち寛解症例は8例，男性3例，女性5例，Stage Ⅰ 1例，Stage Ⅱ 6例，Stage Ⅲ 1例で，Class Ⅰ 1例，Class Ⅱ 8例であった。中止できた寛解症例の罹患期間は7ヵ月から372ヵ月，平均71.4ヵ月であった。MTXは4mgから6mg，平均5.25mg，ステロイド（predonine）は0mgから6mg，平均2.125mgであった。投与時のC-reactive protein（以下CRPと略す）は1.36mg／dlから4.5mg／dl，平均2.68mg／dlであった。リウマチ因子（以下RAPAと略す）は40から160で，平均100であった。MMP-3は51.3ng／mlから167ng／mlで，平均98.6ng／mlであった。インフリキシマブ継続期間は7ヵ月から29ヵ月，平均13.1ヵ月で中止した。インフリキシマブ投与中止してから経過観察期間は症例1，6ヵ月，症例2，5ヵ月，症例3，7ヵ月，症例4，13ヵ月，症例5，18ヵ月，症例6，4ヵ月，症例7と8は5ヵ月であり，投与中止後平均経過観察期間は7.9ヵ月であった。インフリキシマブ投与中止の基準は投与開始してCRPが陰性化（当科では0.21mg／dl以下）してから最低6ヵ月以上経過し，DAS28が2.6以下の症例を中止した。中止後はMTX 4mg／週で治療し，症例4は中止後12ヵ月でMTXも中止している。インフリキシマブ投与全体の患者背景を調べるため，継続率および副作用や効果減弱にて脱落する因子をCox回帰分析を用いて解析した。投与後2週でのCRP改善を相関係数を用いて解析した。インフリキシマブ投与中止寛解症例の比率および早期RAにおける寛解比率を計算した。さらにインフリキシマブ投与中止寛解症例のMMP-3の改善をWilcoxonの符号付き順位検定を用いて比較した。インフリキシマブ投与中止寛解症例のCRPの投与前から中止後の推移とDAS28の変化および投与薬剤の変化を調べた。また寛解症例の手XP変化を比較検討した。

結　果

インフリキシマブ投与して2週間後にCRPは0.55倍に減少した。すなわちCRP＝0.5469x

表1　インフリキシマブを中止できた寛解症例

症例数	症例	性別	年齢	Stage	MTX (mg)	PSL (mg)	体重 (kg)	身長 (cm)	BMI	CRP (mg／dl) 投与前	RAPA	MMP-3 ng／ml (投与前)	罹患期間 (月)	継続期間 (ヵ月)
1	K.S.	女性	57	Ⅱ	6	0	55	152	23.81	1.36	40	64.60	47	10
2	T.A.	女性	54	Ⅰ	6	0	50	156	20.55	1.63	160	53.70	24	7
3	S.M.	女性	81	Ⅲ	4	1	46	148	21.00	1.88	160	N.A.	372	10
4	Y.H.	女性	52	Ⅱ	6	0	46	157	18.66	4.01	40	86.4	22	18
5	M.M.	女性	72	Ⅲ	4	0	53	153	22.64	1.54	160	51.3	7	29
6	M.N.	男性	69	Ⅱ	6	5	71.9	170	24.88	4.50	40	167	21	11
7	D.M.	男性	27	Ⅱ	4	6	90	160	35.16	2.64	40	157	14	9
8	S.T.	男性	56	Ⅱ	6	5	75	167	26.89	3.87	160	110	40	11