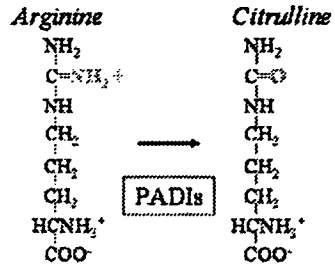

FIGURE 1. Citrullination of peptidylarginine by PAD.

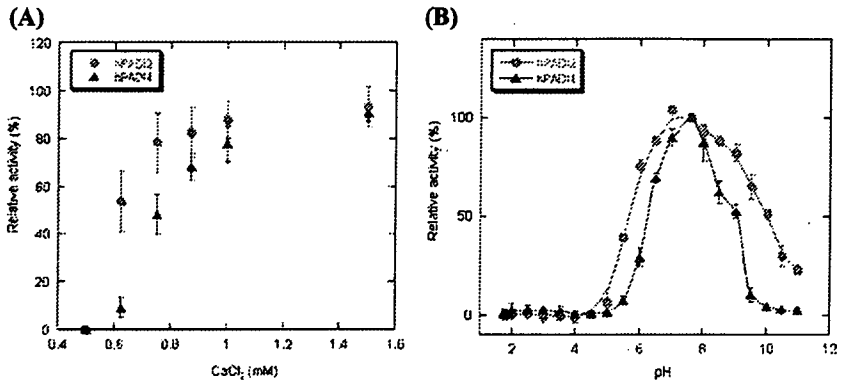

FIGURE 2. Characteristics of hPADI2 and hPADI4: (**A**) Effect of calcium; (**B**) dependence on pH. Values represent means ± SD of triplicate experiments.

(a basic coding amino acid) to citrulline via hydrolysis of the guanidino group of arginine yields a ureide group and free ammonia. This reaction results in the loss of the peptidylarginine charge and peptidyl-citrulline brings about significant biochemical and antigenic changes to the peptide.[4]

ENZYMATIC PROPERTIES OF PEPTIDYLARGININE DEIMINASE

Ca^{2+} and pH Dependence of Activity

The enzymatic PAD reactions are dependent on the Ca^{2+} concentration and pH. Ca^{2+} dependence seems common to all PAD isotypes and the kinetics of PAD4 have been intensively investigated.[5–8] Because the required Ca^{2+} concentration is far higher (~1 μM) than that available in the cytosol (~200 nM), the conversion of arginine to citrulline residues should influence the movement of calcium ions from the extracellular to the intracellular milieu. However, the intracellular Ca^{2+} concentration is tightly regulated. Besides Ca^{2+} dependence, the activity of PAD isotypes might depend on pH (FIG. 2).

TABLE 1. Summary of exonic SNPs in PADI4

	SNP ID: PADI4_#				Haplotype frequency (%)	
	89	90	92	104	RA	Control
Susceptible	**G**GC (Gly)	G**T**G (Val)	G**G**G (Gly)	**T**TG (Leu)	0.32	0.25
Nonsusceptible	**A**GC (Ser)	G**C**G (Arg)	G**C**G (Arg)	**C**TG (Leu)	0.52	0.60
Allele frequency (%)						
RA	0.45	0.50	0.45	0.47		
Control	0.40	0.40	0.39	0.41		
P-value	0.06907	0.00697	0.00046	0.00051		

NOTE: Bold type indicates actual SNPs.

Structure

Arita *et al.* determined the crystal structure of human PAD4, which is encoded by the PADI4 gene.[7] Head-to-tail contact between the N-terminal domain of one molecule of PAD4 and the C-terminal of another results in dimerization. Five Ca^{2+}-binding motifs have been identified in PAD4 and after binding to Ca^{2+}, the conformational changes that generate an active cleft and substrates can remain intact to PAD4 enzyme. The specificity of substrate peptide sequences recognized by PAD4 is broad and Arg374 of PAD4 plays an essential role in substrate recognition.[9] The structures of PAD1, PAD2, PAD3, and PAD6 are obscure, but because the amino acid sequences of the C-terminal of PADs are highly conserved, the C-terminal domains of all PADs might be structurally similar.

PEPTIDYLARGININE DEIMINASES AS GENETIC FACTORS IN RA

RA-Susceptible Variant in PADI4

A large-scale linkage disequilibrium study has revealed an RA-susceptible variant in PADI4 in a Japanese population.[2] The PADI4 gene has two major haplotypes, one of which is RA susceptible and the other is not (TABLE 1). The two haplotypes consist of four single nucleotide polymorphisms (SNPs) in exonic regions. The relative risk of RA in individuals with two copies of the susceptible haplotype is 1.97 compared to those without a copy of the susceptible haplotype.[2] Subsequent independent Japanese,[10] Korean,[11] British,[12] French,[13] German,[14] and Spanish[15] genetic studies of PADI4 polymorphisms and RA have suggested ethnic variation in the susceptibility of PADI4 variants. These studies indicated an association of PADI4 and RA in Asians, but not in European descendants. However, meta-analysis of one Japanese and five Caucasian populations[16] has confirmed an association between PADI4 and

TABLE 2. Expression of PADI isotypes in various tissues

	Expression sites	
	Protein	mRNA/EST
PADI1 (PAD1)	Epidermis, uterus	Brain, colon, ES cell, eye, inner ear, kidney, muscle, placenta, skin, thymus
PADI2 (PAD2)	Brain, uterus, salivary grand, macrophage, spleen, bone marrow, skin, synovial membrane, synovial fluid	Brain, breast, bone marrow, colon, lung, muscle, skin, ovary, synovial membrane, synovial fluid
PADI3 (PAD3)	Hair follicle	Muscle, skin, thymus
PADI4 (PAD5)	Eosinophils, neutrophil, granulocyte, bone marrow, synovial membrane, synovial fluid	Brain, bone marrow, eye, fetal liver, spleen, kidney, leukocyte, synovial membrane, synovial fluid
PADI6 (PAD6)	Egg, ovary, early embryo	Embryo, ovary (egg), thymus

RA with a common odds ratio (OR) of 1.14 (95% CI = 1.07–1.21) for allelic distribution.

An increased level of PADI4 might produce susceptibility to RA, because transcription from a susceptible haplotype is more stable than from the other common haplotype of the PADI4 gene.[2] In fact, more PADI4 is expressed in peripheral blood from RA patients than from normal individuals.[17] However, a U.K. study found no relationship between PADI4 haplotypes and either citrullinated protein deposition in RA synovium or levels of ACPA in sera from RA patients.[18] Although the genetic effect of polymorphisms in PADI4 genes might be more prominent in Asians than in Caucasians, higher PAD4 activity regardless of other PADs seems to play a role in RA pathogenesis despite ethnic background.

Isotypes and Tissue Distribution of PADs

The apparent physiological role of PAD remains unclear. All five isotypes are localized in the cytosol except for PAD4, which is localized in the nucleus. The tissue distribution of the PAD isotypes varies (TABLE 2). PAD1 is mainly expressed in the epidermis and uterus, PAD2 is expressed in neuronal tissue and macrophages as well as in many other tissues, PAD3 is expressed in hair follicles, and PAD4 is expressed mainly in bone marrow and white blood cells, especially in neutrophils and eosinophils. The most recently identified PAD6 is expressed in oocytes. These differences in tissue distribution among PAD isotypes might be associated with their physiological functions. PAD4 has been detected in the nucleus[19] and cytoplasm, whereas PAD2 has been found only in the cytoplasm of RA synovial tissue. In murine RA models, mRNA of mouse

PAD2 (mPAD2) and mouse PAD4 (mPAD4), the counterparts of PAD2 and PAD4, are also expressed in synovial tissues, whereas mPAD4 protein has been detected in inflammatory joints of RA models, but not mPAD2.[20]

CITRULLINATED PROTEINS AND ACPA

PAD Substrates

Several peptides can be natively and or experimentally citrullinated. Dermal citrullination seems to be the most thoroughly investigated. PAD2 catalyzes citrullination of filaggrin and K1 keratin in the epidermis. Filaggrin[21–23] is an aggregative protein of epidermal keratins.[24] Oligomeric profilaggrin is initially synthesized and forms keratohyalin granules. Then oligomeric profilaggrin is digested by proteases followed by PAD2.[25] Citrullination levels are low in the affected skin of patients with psoriasis.[26] PAD4 can also modify filaggrin and keratin *in vitro*. In addition, several proteins undergo citrullination, such as myelin basic protein,[27] vimentin,[28] fibrinogen/fibrins,[29] antithrombin III,[30] type I collagen,[31] α-enolase,[32] CapZα1,[33] and eukaryotic initiation factor-4G.[34] Also, some biological events, such as inflammation, apoptosis, trauma, and aging, increase post-translational citrullination. Although most citrullinated substrates react with RA sera, the physiological role of citrullination remains unknown.[35–38]

PAD4 plays important roles in the intranuclear citrullination of histones and in the regulation of gene expression.[39,40] In terms of biological functions, citrullination is apparently linked to other post-translational modifications, such as methylation and acetylation in the regulatory mechanism of gene expression through histone modification. Although citrullination plays a principal role in skin integrity, it might also function in other fundamental processes, such as the regulation of gene expression by protein modification.

All PAD isotypes can deiminate various proteins *in vitro*, and have different types of reactivity against various substrates.[41] When a PAD substrate has several arginine residues, some tend to become more citrullinated than others.[5,6,42] However, consensus amino acid sequences of PAD targets remain obscure.

Citrullinated Proteins in RA and Other Diseases

Although RA sera recognize citrullinated auto-antigens, true auto-antigens containing citrulline residues in RA are unknown. Evidence indicates that arginine residues undergo local citrullination in the RA synovium.[43,44] Filaggrin is a citrullinated self-peptide recognized by RA-specific sera,[21] but it is not an articular component. Therefore, it might be recognized by ACPA as

a consequence of cross-reactivity.[29] Fibrin(ogen) was initially identified as a citrullinated protein in RA synovial tissue,[29] and it is recognized by anticitrullinated antibodies in RA sera. Matsuo *et al.* detected 51 citrullinated proteins in RA synovial tissues and 30 of 51 citrullinated proteins were autoantigenic. PAD enzymes citrullinate many proteins including true autoantigens.[33]

Further studies of RA synovial tissue have also revealed both extracellular and intracellular citrullinated proteins. Because the intracellular physiological Ca^{2+} concentration is too low to activate PAD according to studies *in vitro* (details of PADs are reviewed in the PAD section), PAD does not citrullinate intracellular proteins under physiological conditions. Studies have suggested that apoptosis or terminal differentiation is the key event for Ca^{2+} influx to activate PAD.[7,45,46]

A recent report has indicated that intracellular, but not extracellular citrullinated proteins are associated with high titers of ACPA in blood and synovial fluid, although their presence is independent of local disease activity.[47] The distribution of intracellular citrullinated proteins is co-localized with PAD2,[47] and extracellular citrullinated protein deposits, including fibrin, are overlapped by PAD4 distribution.[48] In mice with collagen-induced arthritis (CIA) and in those with streptococcal cell wall induction, PAD2 mRNA is present in the synovium but not translated to PADI2 protein. In contrast PAD4 mRNA, although absent from healthy synovial tissues, is rapidly transcribed and translated in neutrophils of the inflamed synovium.[49] Consequently, PAD4 is more specifically expressed during inflammation in mice. In the rat model of arthritis induced by collagen, protein citrullination induces a breakage of immunological tolerance against self-antigens and potentiates the arthritogenicity of type II collagen.[50] In the same model, PAD4 was induced in inflammatory joints and the severity of joint inflammation correlated with the appearance of PAD4.[50] However, ACPA were undetectable in these[20] and in other autoimmune and/or arthritic animal models.[51]

The presence of citrullinated proteins including fibrin is not a specific symptom of RA, as these proteins have also been detected in other arthritides, including ankylosing spondylitis, psoriatic arthritis, undifferentiated spondyloarthropathy, and joint involvement by multiple myeloma, as well as osteoarthritis, gout, and pseudogout.[52–54] The severity of arthritis is correlated with citrullinated protein deposition in the synovial tissues of an animal model, but not in human RA.[47]

In addition, citrullinated proteins are also associated with the affected organs of non-arthropathic pathologies, for example, in plaque interfaces of patients with secondary progressive multiple sclerosis,[55] in myelin basic protein of murine experimental autoimmune encephalomyelitis,[56,57] in the hippocampus of patients with Alzheimer's disease,[58] and in the glomeruli of patients with obstructive nephropathy.[59] These findings must be understood along with increased comprehension of the physiological and pathological roles of citrullination and the autoantigenicity of citrullinated proteins.

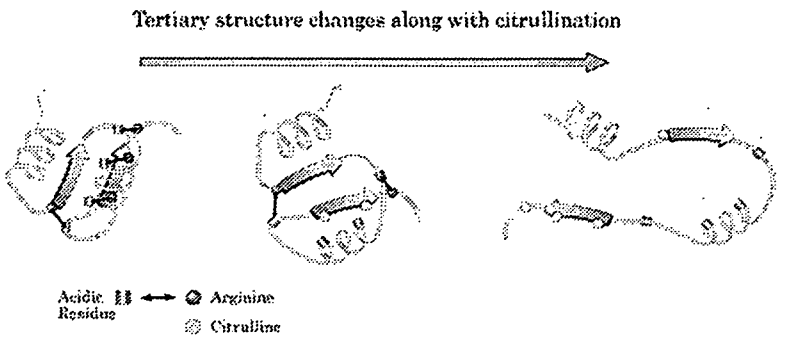

FIGURE 3. Structural changes induced by citrullination. Positive charge on arginine interacts with negative charge on acidic residues. Loss of positive charge induced by citrullination breaks secondary and tertiary protein structures via intermediate form generated by partial modification.

Antigenicity and Citrullination

All the post-translational changes in self-peptides seem to influence antigen recognition within the immune system.[60] Many autoimmune diseases produce autoantibodies that recognize post-translationally modified self-peptides.[35,61–64] Meanwhile, the absence of the normal post-translational modification of self-proteins is also associated with autoimmune diseases.[62,65] These findings suggest that post-translationally modified peptides can induce a break in tolerance.[66]

Arginine residues are positively charged and contribute to the tertiary structure of proteins by forming hydrogen bonds and by determining secondary and tertiary protein structures (FIG. 3). The charge on arginine is lost after citrullination, which results in the disruption of intracellular interactions. Therefore, the conversion of arginine to citrulline residues affects protein folding although the produced difference in mass is very slight (~1 Da). Protein citrullination decreases mobility in SDS-PAGE because of changes in molecular weight and charge, as well as conformation.[8] The biochemical changes caused by citrullination resemble those of detergent-induced protein denaturation.[67] Such mobility changes might be associated with modifications in both structure and charge.

The effects of citrullination on the autoimmune response have been confirmed *in vitro* and *in vivo* using citrullinated and unmodified peptides.[1,50] Citrullinated proteins break immunological tolerance in the rat and antibodies against citrullinated protein cross-react with native protein, implying that the antigenicity and arthritogenicity of citrullinated proteins is altered. In mice with CIA, anticyclic citrullinated peptide (anti-CCP) appears early after immunization as well as antitype II collagen and these antibodies bind to citrullinated filaggrin and citrullinated fibrinogen.[68]

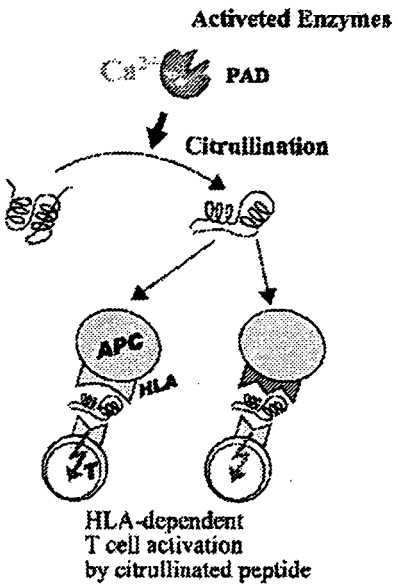

FIGURE 4. Effect of citrullination by PAD in immunological response.

These data suggest that citrullinated proteins and antibodies against citrullinated proteins are associated with the development of inflammatory arthritis in model animals. Furthermore, peptide citrullination increases peptide-major histocompatibility complex affinity and activates $CD4^+$ T cells in mice that integrate histocompatibility locus antigen DR beta (HLA-DRB)1∗0401, which is a shared positive epitope (FIG. 4).[69] This finding supports the notion that citrullination alters antigenicity and also predicts that such change plays a role in antigen recognition of shared-epitope (SE)-positive HLA-DRB.[5,50,69,70]

Anti-CCP Antibody and Other Autoantibodies

Although various autoantibodies can be detected in sera from patients with RA, several of them are highly specific and sensitive, and some of have a higher positive predictive value for RA. Antiperinuclear factor[71] and antikeratin antibody[72] are 43–52% sensitive and 97–99% specific.[73,74] The sensitivity and specificity of Anti-Sa antibody are 27–50% and 99%, respectively.[75] These highly RA-specific autoantibodies recognize citrullinated peptides.[21–23,29,76] Collagen types I[77] and II[78] as well as fibrinogen[79] are more likely to be recognized by RA sera when in the citrullinated, than in the noncitrullinated form.

In particular, autoantibodies to citrullinated proteins such as part of citrullinated filaggrin and its circularized form (CCP) are remarkably specific and

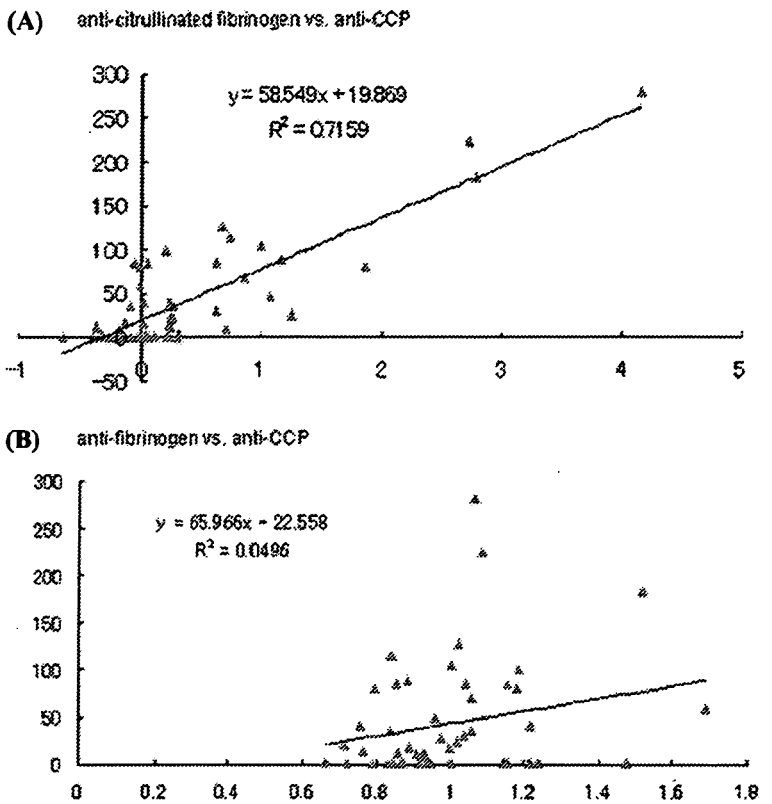

FIGURE 5. Comparison of anticitrullinated fibrinogen (**A**) and antifibrinogen levels (**B**) with anti-CCP level in RA sera. Results are shown as regression lines and correlation coefficients (R^2).

sensitive in RA patients and these autoantibodies can also serve as early diagnostic markers and as a prognostic factor of joint destruction. Several clinically useful anti-CCP assay kits have been commercially developed based on these findings. A second-generation anti-CCP antibody assay system (anti-CCP2; INOVA Diagnostics, Inc., San Diego, CA) utilizes a mixture of synthetic peptides containing citrulline, because ACPA are heterogeneous and the epitopes containing citrulline that are recognized by individual patients with RA vary.[23] Such autoantibodies are not only very specific (up to 96%), but also sensitive (up to 74%) for RA. Anti-CCP2 can detect very early in the disease and can predict the disease before onset[80,81] and the titer tends to correlate with an erosive RA subtype. These anti-CCP test series serve as a clinical diagnostic marker of RA. However, antigens of anti-CCP *in vivo* were not clear, because CCP did not physiologically present *in vivo*. Recently, we found that levels of anti-CCP and anticitrullinated fibrinogen antibodies correlated (FIG. 5). These data suggested that antigens of anti-CCP are mixtures of citrullinated

proteins. A third-generation anti-CCP antibody assay system (anti-CCP3; INOVA Diagnostics, Inc.) is currently available.

ACPA are apparently produced in the inflamed RA synovium because a fraction of ACPA is increased in synovial fluids rather than in serum. Anti-CCP positivity is also associated with the copy number of the HLA-DR SE alleles, but not in RA patients who are anti-CCP negative.[47,69,82–86]

Further studies are warranted to identify true self-antigens that trigger a break in tolerance as a causative event of RA. Anticitrullinated protein antibodies are polyclonal and a restricted set of variable region genes are utilized by the clones.[87] Genetic variants of the PADI4 gene affect the production of antigens that are recognized by anticitrullinated antibodies and HLA-DR types influence the epitope recognition of citrullinated peptides. These facts indicate that a genetic predisposition is involved in the development of anticitrullinated antibodies.

CONCLUSIONS

Although studies have suggested that citrullination is related to various physiological phenomena, the functional significance of this process has remained obscure. Isoforms of PAD were thought to play important roles because they are highly conserved in vertebrates and their products are also involved in several human diseases. However, ACPA that react with the PAD products in RA are specifically detected in RA sera and their clinical utility has been established. Mixtures of various modified citrullinated peptides perform better in the clinical environment, probably because sets of citrullinated epitopes are heterogeneous among patients.

PADI4 seems to be associated with the development of RA. Although the high specificity of anti-CCP antibodies and RA-susceptible genetic variants in PADI4 suggest that an autoimmune reaction to citrullinated peptides is one cause of RA, whether citrullinated proteins/peptides constitute a cause or an effect remains unknown. If citrullination is indeed the cause of RA, how immunological tolerance toward citrullinated proteins is broken and why the breakage is highly specific to RA are key questions that should be addressed.

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Peptidylarginine deiminase 4 (PADI4) identified as a conformation-dependent autoantigen in rheumatoid arthritis

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Objective: Peptidylarginine deiminase (PADI) catalyses the post-translational modification of arginine to citrulline, which is specifically recognized by sera from rheumatoid arthritis (RA) patients. The PADI4 gene has recently been identified as a risk factor for RA. We aimed to determine whether PADI4 constitutes an autoantigen in RA.

Methods: Serum samples were obtained from 42 patients with RA, 19 patients with systemic lupus erythematosus (SLE), 23 patients with other rheumatic diseases, and 40 normal individuals. The presence of antibodies against recombinant human PADI4 (anti-PADI4) was examined using enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Results: For ELISA, the prevalence of anti-PADI4 among RA patients (50%) was significantly higher than that of normal individuals (2.5%), SLE (10.5%), and other rheumatic diseases (4.3%), while for Western blot analysis, PADI4 was recognized only by a portion of the ELISA-positive serum samples.

Conclusions: PADI4 is an autoantigen in some RA patients, and its conformational epitope(s) may be important.

Antibodies against citrulline-containing peptides, such as anti-filaggrin antibodies (AFA) and anti-cyclic citrullinated peptide antibodies (anti-CCP), are useful serological markers for the diagnosis of rheumatoid arthritis (RA) (1–5). Citrulline is formed by the post-translational modification of arginine residues by peptidylarginine deiminase (PADI). Five different types of PADIs have been identified in human tissues, including PADI1, PADI2, PADI3, PADI4, and PADI6 (6–11). Using single nucleotide polymorphism (SNP) analysis, we previously found that a functional haplotype of PADI4 is associated with susceptibility to RA and also with the production of anti-CCP, indicating that PADI4 is important for the pathogenesis of RA (9).

Nissinen et al showed recently that sera from patients with RA and other collagen diseases, including SLE and primary Sjögren syndrome, recognize PADI2 purified from rabbit muscle (12). In this study, we developed an enzyme-linked immunosorbent assay (ELISA) system to detect antibody against recombinant human PADI4 (anti-PADI4), and examined whether PADI4 constitutes an autoantigen in RA.

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Material and methods

Serum samples

Serum samples from 42 patients with RA (33 females and nine males) who fulfilled the American College of Rheumatology (ACR) criteria for RA, 19 patients with SLE (16 females and three males), 23 patients with other rheumatic diseases (19 females and four males), and 40 normal individuals (23 females and 17 males) were collected after obtaining the informed consent of all participants (Table 1). The median disease duration of RA was 10.1 years with a range of 1.1 to 38 years.

Enzyme-linked immunosorbent assay (ELISA) for anti-PADI4

Full-length human PADI4 cDNA was amplified by the reverse transcriptase-polymerase chain reaction (RT-PCR), and cloned into the prokaryotic expression vector pDONR201. The sequence-verified plasmid was then introduced into *Escherichia coli* BL21-SI. After inducing expression by sodium chloride, recombinant PADI4 with a His-tag was purified by a cobalt-chelate column.

One hundred microlitres of PADI4 (5 μg/mL) was incubated in a 96-well ELISA plate at 4°C overnight.

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Table 1. Characteristics of patients with RA, other rheumatic diseases, and normal individuals.

	Total number (male, female)	Age (years) median (range)
RA	42 (9, 33)	56 (31–79)
SLE	19 (3, 16)	43 (28–66)
Other rheumatic diseases	23 (4, 19)	60 (27–73)
Systemic sclerosis	5	
Behçet's disease	4	
Primary Sjögren syndrome	4	
Vasculitis syndrome	3	
Polymyositis	2	
Mixed connective tissue disease	1	
Polymyalgia rheumatica	1	
Ulcerative colitis	1	
Pustulosis palmaris et plantaris	1	
Weber Christian disease	1	
Normal control	40 (17, 23)	41 (28–77)

The wells were then washed with phosphate-buffered saline with 0.05% Tween-20 (PBS-T) and blocked with 300 μL of 4% BlockAce, a blocking reagent made from purified milk proteins (Dainippon Pharmaceutical, Japan), for 1 h at room temperature (RT). Wells without PADI4 were simultaneously set up for non-specific background examination. Patients and normal sera were diluted at 1:250 with PBS-T and 2% BlockAce, and were preincubated with *E. coli* lysate for 30 min to remove antibodies against *E. coli*. The optimal concentration of the *E. coli* lysate was determined to be 100 μg/mL by extensive titration. One hundred microlitres each of the diluted sera was added to each well. After incubation for 3 h at RT, the wells were washed three times with PBS-T. Then 100 μL of horseradish peroxidase-conjugated goat $F(ab')_2$ antibody against human immunoglobulin G (IgG; Biosource, Camarillo, CA, USA) diluted at 1:100 000 was added to each well and incubated for 2 h at RT. After washing five times with PBS-T, the bound antibodies were detected with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The reaction (30 min) was stopped by the addition of 100 μL of 1 N sulfuric acid/well. The plates were read at a wavelength absorbance of 450 nm (A450). A representative serum pool was used as positive control. The titre of anti-PADI4 was expressed as an arbitrary index calculated as [A450 of sample – A450 of the non-specific background of the sample]/[A450 of the positive control – A450 of the non-specific background of the positive control] × 100. All the samples were tested in duplicate.

Western blotting

Electrophoresis of recombinant PADI4 (200 ng/lane) was performed on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel. After transblotting onto a PVDF membrane, the membrane was blocked with Tris-buffered saline (TBS) and 0.1% Tween20 (TBS-T) containing 10% skimmed milk. The membrane was then cut and incubated with serum samples, diluted at 1:150 in TBS-T with 5% skimmed milk containing *E. coli* lysate. After washing, the membrane was incubated with alkaline phosphatase-conjugated goat $F(ab')_2$ antibody against human IgG (KPL, Gaithersburg, MD, USA) at a dilution of 1:5000. Colour development was performed using BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitro blue tetrazolium).

Statistical analyses

The Mann–Whitney *U*-test and the χ^2-test were used to compare the distribution and the prevalence of anti-PADI4, respectively, between groups.

Results

Distribution of anti-PADI4 measured by ELISA

The distribution of anti-PADI4, expressed as an index, is shown in Figure 1. The median values and ranges were 80.1 (15.0–341) for RA, 58.5 (6.0–87.5) for SLE, 52.5 (1.5–75.0) for other rheumatic diseases, and 38.8 (4.5–87.0) for normal control, respectively. Significant differences were observed between RA patients and normal individuals ($p<0.001$), RA

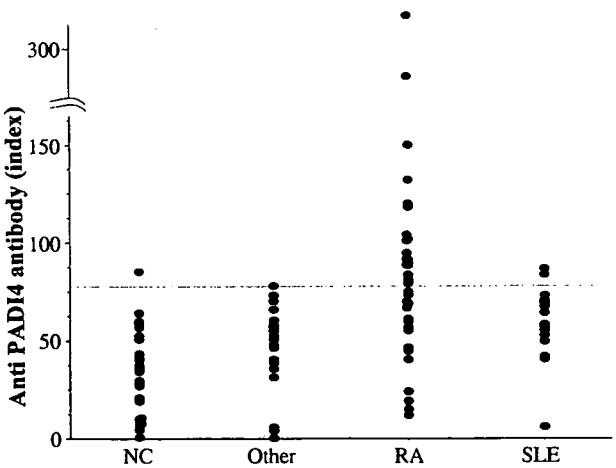

Figure 1. Distribution of IgG class antibody against human PADI4 (ELISA). Anti-PADI4 was measured by ELISA using recombinant human PADI4 as a coating antigen. The titre is expressed as an arbitrary index calculated as [A450 of the measured serum sample – A450 of the non-specific background of the measured serum sample]/[A450 of the measured positive control sample – A450 of the non-specific background of the positive control sample] × 100. The cut-off level [median plus 2 standard deviations (SD) of normal sera] is shown by the dotted horizontal line. NC, normal controls; Other, other rheumatic diseases.

patients and SLE patients ($p=0.02$), and RA patients and other rheumatic diseases patients ($p=0.007$).

The cut-off value was determined to be 74.8 as the median value plus 2 standard deviations among normal individuals. Twenty-one out of the 42 patients with RA (50%), two of the 19 SLE patients (10.5%), one of the 23 other collagen diseases patients (4.3%), and one of the 40 healthy controls (2.5%) were regarded as positive for anti-PADI4. Significant differences in the occurrence of anti-PADI4 antibodies were observed between RA patients and normal individuals ($p<0.001$), RA patients and SLE patients ($p<0.001$), and RA patients and other rheumatic diseases patients ($p<0.001$). No statistical significance was observed among other combinations.

Western blotting

To further characterize anti-PADI4, we performed Western blotting analysis using sera that were positive for anti-PADI4 by ELISA. This revealed that only three ELISA positive sera recognized PADI4. Representative data using nine serum samples, including six samples that were anti-PADI4 positive by ELISA and three negative samples, are shown in Figure 2. The indices of anti-PADI4 (ELISA) for serum samples that were positive by Western blotting (lanes 1 and 2) were 80.8 and 129.0, respectively.

Discussion

Recent genomic analysis has revealed that PADI4 is related to the pathogenesis of RA (9). Nissinen et al have shown that anti-rabbit muscle PAD can be detected in sera from patients with RA, SLE, and primary Sjögren syndrome (12), suggesting that the arginine–citrulline converting enzyme PAD is a novel autoantigen in inflammatory rheumatic diseases. This prompted us to search for anti-PADI4 in RA. In this study, we developed an ELISA system using recombinant human PADI4 and found for the first time that the prevalence and the titres of anti-PADI4 are significantly higher in RA patients than in other rheumatic disease patients, including SLE, and healthy individuals.

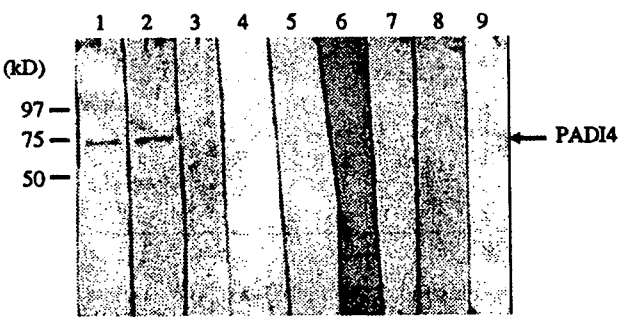

Figure 2. Western blot analysis of anti-PADI4 antibodies. Recombinant human PADI4 (200 ng/lane) was electrophoresed on a 10% SDS–polyacrylamide gel and blotted onto a PVDF membrane. Each lane was then cut and incubated with diluted (1:150) serum samples. The bound antibodies were detected by alkaline phosphatase-conjugated goat $F(ab')_2$ antibody against human IgG, using BCIP/NBT as substrate. Lanes 1–6: serum samples positive for anti-PADI4 by ELISA; lanes 7–9: serum samples negative for anti-PADI4 by ELISA.

To characterize the epitope(s) targeted by anti-PADI4, we performed Western blotting analysis using sera that were found to be anti-PADI4 positive by ELISA. However, only three ELISA positive sera were found to be positive by Western blotting. As the results of the Western blotting did not correlate with the titres of anti-PADI4 measured by ELISA, this discrepancy cannot be explained by the difference in sensitivity between ELISA and Western blotting. Of note, Nissinen et al reported that the epitope(s) of anti-rabbit muscle PAD is conformation dependent, as it was not detected by Western blotting analysis. Previous studies have described such conformation-dependent autoantibodies that are detectable by radioimmunoassay or ELISA but not by Western blotting, including anti-glutamic acid decarboxylase autoantibody in insulin-dependent diabetes mellitus and anti-tissue glutaminase autoantibody in coeliac disease (13, 14). Therefore, it is possible that the conformational epitope(s) expressed by PADI4 may be the targets of anti-PADI4. It should also be noted that, as shown by Western blotting analysis, there exist RA sera that recognize the conformation-independent linear epitope(s) of PADI4, although at a low frequency, suggesting the presence of diverse reactivity against PADI4 in RA.

Although its sensitivity in RA was lower in this study, the specificity of anti-PADI4 was higher than that of anti-rabbit muscle PAD, raising the possibility that the breakdown of immunological tolerance to PADI4 is a specific phenomenon of RA. As the PADI4 gene is a susceptible locus of RA and the mRNA transcribed from the susceptible haplotype is more stable, we believe that PADI4 is overexpressed in RA. This in turn leads to the breakdown of immunological tolerance to PADI4 in addition to the generation of citrullinated autoantigen(s) targeted by anti-CCP. The prevalence of anti-rabbit muscle PAD decreases with the progression of RA as mentioned elsewhere (12), and anti-CCP can be detected in early RA patients as well as in RA patients years before they develop the disease (15). We suggest that PADI4 is involved in the initiation phase of the pathogenesis of RA.

In summary, we have identified the presence of anti-PADI4 autoantibodies in RA, which presumably recognize the conformation-dependent epitope(s) of PADI4. Further studies that examine the mechanism responsible for anti-PADI4 production would be useful for a better understanding of the pathogenesis of RA.

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Hepatocyte Growth Factor Significantly Suppresses Collagen-Induced Arthritis in Mice

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Hepatocyte growth factor (HGF) plays an important role in angiogenesis, cell proliferation, antifibrosis, and antiapoptosis. Moreover, recent studies have highlighted the immunosuppressive effect of HGF in animal models of allogenic heart transplantation and autoimmune myocarditis and in studies in vitro as well. We also reported that HGF significantly suppresses dendritic cell function, thus down-regulating Ag-induced Th1-type and Th2-type immune responses in allergic airway inflammation. However, the immunosuppressive effect of HGF in many other situations has not been fully clarified. In the present study, using a mouse model of collagen-induced arthritis (CIA) and experiments in vitro, we examined the effect of HGF on autoimmune arthritis and then elucidated the mechanisms of action of HGF. To achieve sufficient delivery of HGF, we used biodegradable gelatin hydrogels as a carrier. HGF suppressed Ag-induced T cell priming by regulating the functions of dendritic cells in the Ag-sensitization phase with down-regulation of IL-10. In contrast, under continuous Ag stimulation HGF induced IL-10-producing immunocytes both in vivo and in vitro. Moreover, HGF potently inhibited the development of CIA with enhancing the Th2-type immune response. We also confirmed that HGF significantly suppressed the production of IL-17 by immunocytes. These results indicate that HGF suppresses the development of CIA through different ways at different phases. They also suggest that HGF could be an attractive tool for treating patients with rheumatoid arthritis. ***The Journal of Immunology,*** **2007, 179: 5504–5513.**

Hepatocyte growth factor (HGF),[2] originally identified and cloned as a potent mitogen for hepatocytes (1–3) and a scatter factor (4), targets various cell types (5). HGF has many functions such as induction of angiogenesis, promotion of cell proliferation and migration (5), and inhibition of apoptosis (6, 7). HGF exhibits these functions through its receptor c-Met (5). It is well established that HGF promotes tumor progression (8–12) and suppresses the development of fibrosis after injury (13–15).

The role of HGF in immune-mediated disorders has not been fully studied. HGF promotes adhesion and migration of B (16, 17) and T cells (18) and enhances dendritic cell (DC) migration (19, 20). HGF frequently counteracts TGF-β, a potent immunosuppressive cytokine (13, 14, 21). These results indicate that HGF might accelerate immune responses. In contrast, recent studies clarified an immunosuppressive effect of HGF. In a mouse model of allogenic heart transplantation, HGF reduced acute and chronic rejection of the allograft with increased expression of TGF-β and IL-10, indicating that HGF might induce allograft tolerance (22). HGF ameliorates the progression of experimental autoimmune myocarditis, a Th 1-type dominant immune response, inducing production of Th2 cytokines (23). In addition, other articles reported that HGF suppresses the development of Th2-type responses as well (24–26). HGF attenuates allergic airway inflammation (24, 25), and one article recently reported that HGF prevents lupus nephritis in a murine lupus model of chronic graft-vs-host disease through suppression of Th2-type immune responses (26). These results indicate that HGF could suppress both Th1-type and Th2-type immune responses. As to the mechanisms of immune suppression by HGF, two major possibilities have been reported. One is the down-regulation of functions of DCs, a mechanism elucidated in the case of allergic airway inflammation that was reported by us previously (24). Another mechanism is to induce the regulatory phenotype of $CD4^+$ T cells that produce IL-10 or TGF-β, which was studied in an experimental system of allogenic heart transplantation (22) and in vitro (23).

Rheumatoid arthritis (RA) is an autoimmune disorder and a systemic chronic inflammatory disease characterized by persistent synovial cell proliferation with inflammatory cell infiltration and destruction of joints (27). The mechanism and pathogenesis of RA have not been fully clarified. RA has traditionally been assumed to be a Th1-type disease (28, 29). However, recent studies revealed a new lineage of effector $CD4^+$ T cells characterized by the production of IL-17, and this Th17 lineage plays an essential role in both the development of autoimmune arthritis (30, 31) and bone destruction (32). In addition to the T cell-mediated immune responses, angiogenesis plays a very important role in maintaining and promoting RA (33).

The role of HGF in RA has been reported in a few cases. HGF and its receptor c-Met were found in the synovial tissue of patients with RA (34). HGF levels in synovial fluids were significantly higher in patients with RA than in those with arthritis of other

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[2] Abbreviations used in this paper: HGF, hepatocyte growth factor; CIA, collagen-induced arthritis; CII, type II collagen; DC, dendritic cell; EU, ELISA unit; LN, lymph node; RA, rheumatoid arthritis; rhHGF, recombinant human HGF; Treg, regulatory T.