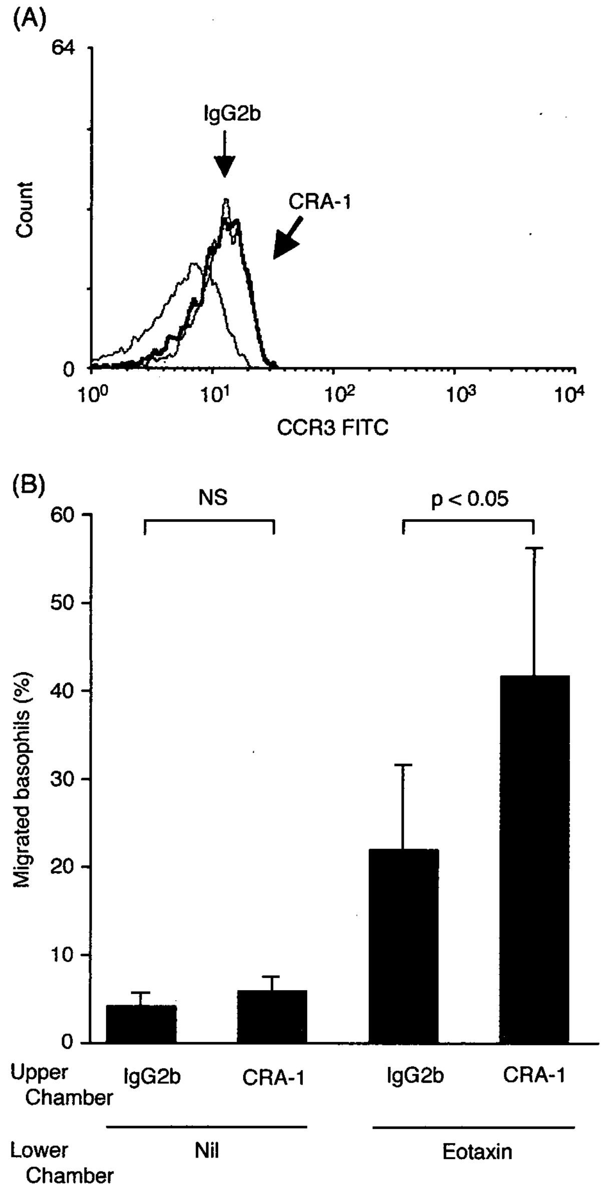


**Fig. 3.** CRA-1 enhances basophil migration induced by eotaxin. (A) After stimulation with either CRA-1 or control IgG2b at 1 ng $ml^{-1}$ for 2.5 h, surface CCR3 expression on basophils was assessed by flow cytometry. Basophils stained with FITC–control mouse IgG1, in place of FITC–anti-CCR3 mAb, are shown as shaded area. Data are representative of two separate experiments, showing similar results. (B) Basophils were mixed with either CRA-1 or control IgG2b (1 ng $ml^{-1}$) and then placed in the upper chamber. Eotaxin at 10 nM was added to the lower chamber. Spontaneous migration of untreated basophils in the absence of eotaxin in the lower chamber was 4.8 ± 0.8%. The bars represent the SEM ($n = 4$).

as mast cells and basophils (16, 21, 22). Recent reports have shown that IgE itself can regulate apoptosis of mouse mast cells (23, 24). With regard to cell motility, IgE aggregation has been demonstrated to induce rodent mast cell migration (11, 12). Ishizuka *et al.* (12) recently reported that sensitized mouse mast cell line MC/9 cells and bone marrow-derived cultured mast cells migrate toward a specific antigen and that the migration is chemotactic. Our results presented herein are basically similar to their mast cell findings. And, importantly, we found that eotaxin-induced migration of basophils is augmented by treatment of the cells with CRA-1 mAb at a concentration as low as 1 ng $ml^{-1}$, although this concentration is unable to evoke significant degranulation. It is noteworthy that such weak stimulation can affect basophils; our results coincide with a previous report that concentrations of stimulus lower than those required for histamine release enhance basophil adherence to vascular endothelium (25). Since treatment with CRA-1 failed to increase the level of basophil surface CCR3 expression, the intracellular signal pathway following eotaxin and CCR3 interaction may be up-regulated. Such a migration-enhancing action arising from FcεRI cross-linkage might be similar to that known in mast cells (26). Thus, previous reports and the present study collectively imply that the effect of IgE- and FcεRI-dependent stimulation on cell locomotion, in both direct and indirect (enhancing) ways, might be a phenomenon common to both FcεRI-abundant basophils and mast cells.

Local influx of basophils at inflammatory sites is an important aspect of allergen-induced late-phase reactions as well as allergic diseases such as asthma (3–5). In normal conditions, basophils reside only in circulating blood; thus, there must be some mechanism(s) that induces basophil migration into local tissues during allergic reactions. Since the first description of *in vitro* basophil chemotaxis by Kay and Austen (27), various agents have been identified as basophil chemoattractants, including complement (8), bacteria-derived peptides (9, 28), cytokines (9), chemokines (10), enzymes such as urokinase (28) and, in this study, specific antigens. Our results showing that allergens can induce basophil migration may need to be taken into account when we try to identify potential chemoattractant(s) in clinical allergy. Moreover, our findings that eotaxin-induced migration is up-regulated in basophils treated with low levels of CRA-1 mAb might explain, at least in part, the pathogenesis of basophil accumulation at inflammatory sites in allergic diseases, where prolonged antigen exposure and various pro-inflammatory mediators co-exist (29).

Recent studies have shown that FcεRI-positive cells include not only mast cells and basophils but also eosinophils, macrophages, dendritic cells, neutrophils and platelets in humans (30–34). In this context, it will be of great interest to assess whether IgE- and FcεRI-mediated migrations occur in all of these $FcεRI^+$ cells, and, if so, to analyze to what extent this mechanism can account for the clinical efficacy of the IgE-targeting approach to treatment of allergic diseases.

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## Abbreviations

| | |
|---|---|
| $ED_{50}$ | effective dose of 50% |
| FcεRI | high-affinity receptor for IgE |
| MCP-1 | monocyte chemoattractant protein-1 |
| MESF | molecules of equivalent soluble fluorochrome unit |
| $PGD_2$ | prostaglandin $D_2$ |

PIPES piperazine-*N,N'*-bis-2-ethanesulfonic acid
RAST radioalloergosorbent test

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# Citrullination by Peptidylarginine Deiminase in Rheumatoid Arthritis

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ABSTRACT: **Rheumatoid arthritis (RA) is a complex, multifactorial disease with genetic and immunological aspects. Because RA is an autoimmune condition, dysregulation of the immune system is implied. Many linkage and association studies have also indicated that multiple genetic factors are associated with RA. Although the contribution of each genetic factor is small, the combination of these factors affects RA development. Previous studies have suggested that genetic changes affect the internal immunological environment, which results in autoimmune diseases. More recent genetic studies indicate that the HLA-DRB gene is the predominant cause of RA and that other non-HLA genes are also involved. We reported that peptidylarginine deiminase (gene name abbreviated to PADI, protein name abbreviated to PAD) type 4 is the one of the non-HLA genetic factors involved in RA via citrullination. Antibodies against citrullinated proteins/peptides are highly specific to RA, but the physiological roles of PADI gene, PAD proteins as their products and citrullinated proteins/peptides are obscure. However, levels of anticitrullinated protein antibodies are apparently also increased and were involved in the pathogenesis of autoimmune arthritis in mice with collagen-induced arthritis (CIA). These data suggested that citrullinated protein and anticitrullinated protein antibodies play important roles in the development of RA. This review summarizes the relationship between RA and citrullination, as well as the role of PADI4 genetics.**

KEYWORDS: **rheumatoid arthritis (RA); peptidylarginine deiminase (PADI); anti citrullinated peptide antibody; single nucleotide polymorphism (SNP)**

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## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by the destruction of many joints accompanied by multiple organ involvement. The disorder is an autoimmune disease and many autoantibodies that react against various autoantigens are detectable in the serum of RA patients. However, the etiology of RA remains unknown. Autoantibodies that recognize citrullinated self-proteins (anticitrullinated peptide antibodies [ACPA]) have recently been established as highly specific autoantibodies in RA,[1] and PADI4, a gene that encodes an enzyme producing citrullinated proteins is associated with RA.[2] These findings suggest that protein citrullination by peptidylarginine deiminase (PAD; gene name abbreviate to PADI, protein name abbreviate to PAD) is essential for the development of RA and thus citrullinated proteins and ACPA should play a pathogenic role in the autoimmunity associated with RA. We review recent findings of citrulline, proteins citrullinated by PAD enzymes, and ACPA from genetic, biochemical, histological, immunological, and clinical aspects of RA.

## CITRULLINE AND CITRULLINATION

### *Citrulline*

Citrulline is a noncoding, native, deiminated form of arginine (FIG. 1) that in mammals assumes free amino acid and peptidyl forms with independent metabolic pathways. Citrulline is part of the citric acid and ornithine cycles, and its metabolism is tightly regulated. Hypercitrullinemia is an innate metabolic disorder that results from the abnormal metabolism of free citrulline.

Citrulline might have a pathological function in inflammatory diseases because it induces nitric oxide (NO), and serum nitrite and citrulline, in addition to urinary citrulline levels are higher in patients with systemic lupus erythematosus (SLE) than in controls.[3] Citrulline also has a ureide group, which is reactive because of a highly electrophilic carbon atom.

### *Citrullination*

Peptidyl-citrulline residues in proteins are produced only through posttranslational modification of arginine residues catalyzed by PAD, which is encoded by the PADI gene, because the tRNA for citrulline is unknown. This enzymatic reaction is called citrullination or deimination. The PADI 1, 2, 3, 4/5 (human PADI5 is orthologous to mouse PADI4 and has been renamed human PADI4), and 6 isozymes with highly conserved peptide sequences have been identified in several mammals. Although the chemical reactivity of peptidylcitrulline and free citrulline differs, amino acid substitution from arginine

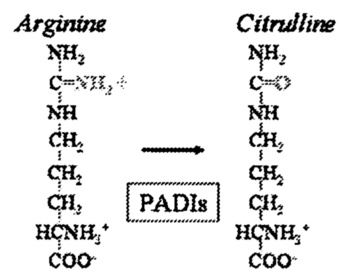


**FIGURE 1.** Citrullination of peptidylarginine by PAD.

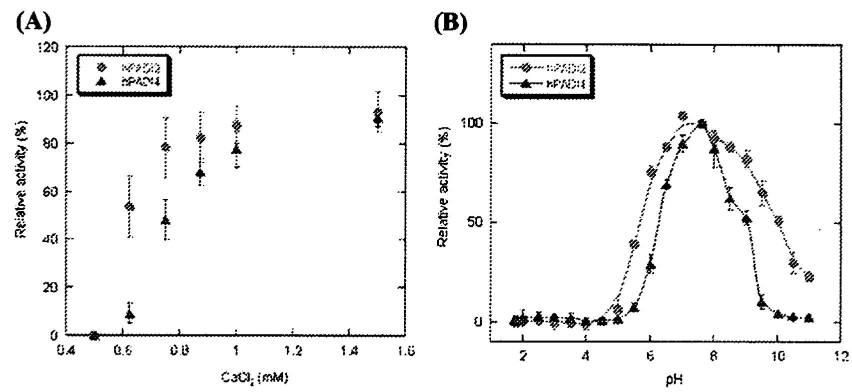


**FIGURE 2.** Characteristics of hPADI2 and hPADI4: (**A**) Effect of calcium; (**B**) dependence on pH. Values represent means ± SD of triplicate experiments.

(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 | GGC (Gly) | GTG (Val) | GGG (Gly) | TTG (Leu) | 0.32 | 0.25 |
| Nonsusceptible | AGC (Ser) | GCG (Arg) | GCG (Arg) | CTG (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.

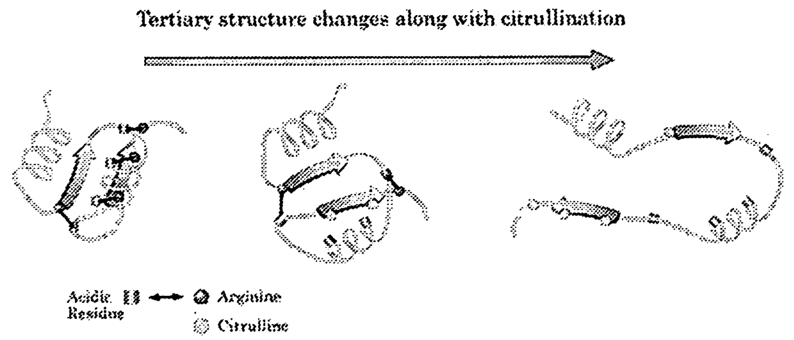


**FIGURE 3.** Structural changes induced by citrullination. Positive charge on arginine interacts with negative charge on acidic residues. Loss of positive charge induced by citrullination breaks secondary and tertiary protein structures via intermediate form generated by partial modification.

### *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]

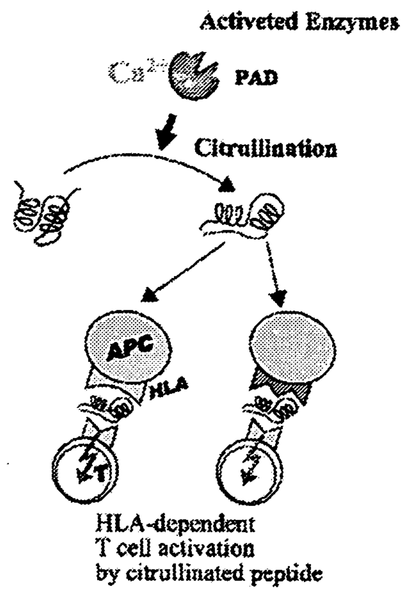


**FIGURE 4.** Effect of citrullination by PAD in immunological response.

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

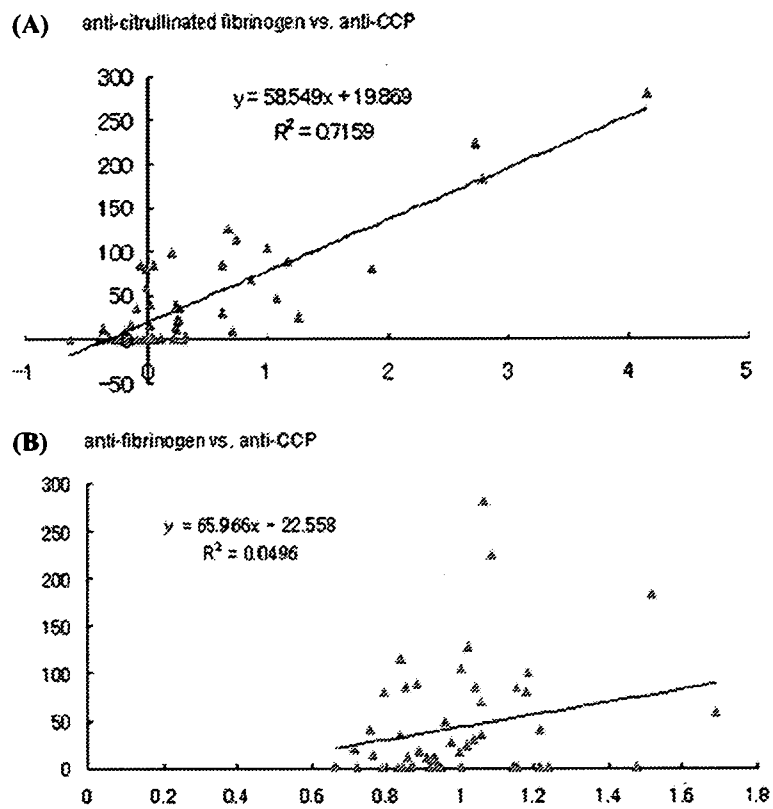


**FIGURE 5.** Comparison of anticitrullinated fibrinogen (**A**) and antifibrinogen levels (**B**) with anti-CCP level in RA sera. Results are shown as regression lines and correlation coefficients ($R^2$).

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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