Citrulline and anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by the destruction of multiple joints along with multiple organ involvement. The etiology of RA is still unknown. Various autoantigens have been proposed as targets of pathogenic T and B cells in RA, although none of their precise mechanisms or significance has been confirmed. Recently, autoantibodies recognizing citrullinated self-proteins (anticitrullinated peptide antibodies) were established as one of the most specific autoantibodies with high sensitivity in RA. In addition, a gene that codes for an enzyme producing citrullinated proteins (peptidylarginine deiminase [PADI] Type 4 gene) was found to be associated with RA. These findings strongly suggest that citrullinated proteins and anticitrullinated peptide antibodies have a pathogenic role in autoimmunity in RA. This article reviews recent findings on citrulline, citrullinated proteins, citrullinating enzyme PADI and anticitrullinated peptide antibodies from biochemical, histological, immunological, genetic and clinical standpoints.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown etiology. The disease is characterized by the destruction of multiple joints, as well as multiple organ involvement. Various autoantigens have been proposed as targets of pathogenic T and B cells in RA, although the mechanisms or significance of these have not been confirmed. However, autoantibodies recognizing citrullinated self-proteins (anticitrullinated peptide antibodies) have been recognized recently as one of the most specific autoantibodies with high sensitivity in RA [1] and the peptidylarginine deiminase (*PADI*) Type 4 gene, which encodes an enzyme that produces citrullinated proteins, was identified as being associated with RA [2]. This strongly suggests that citrullinated proteins and anticitrullinated peptide antibodies have a pathogenic role in autoimmunity in RA. Here, recent findings on citrulline, citrullinated proteins, the citrullinating enzyme PADI and anticitrullinated peptide antibodies from biochemical, genetic, histological, immunological and clinical standpoints are reviewed.

Citrulline

Free citrulline, peptidyl citrulline & antibodies to recognize them

Citrulline is a noncoding native amino acid. It is a deiminated form of arginine (Figure 1). Citrulline is present in two distinct forms in mammals – a free amino acid form and a peptidyl form – and the metabolic pathways of these two forms are independent. Free citrulline is a member of the citric acid cycle and its metabolism is tightly regulated. Hypercitrullinemia, an innate metabolic disorder, is a result of an abnormality in the metabolism of free citrulline. Citrulline has a ureide group that leads to specific chemical reactivity and biological functions of citrulline. The ureide group is reactive owing to its strong electrophilic carbon atom. Peptidyl citrulline residues in proteins are produced only by post-translational deimination of arginine residues because of the lack of transfer RNA for citrulline. The enzyme responsible for the conversion of peptidyl arginine to peptidyl citrulline is PADI. Although the chemical reactivity of peptidyl citrulline is not equal to that of free citrulline, peptidyl citrulline appears to have characteristic chemical features similar to amino acid substitution from arginine – the most basic coding amino acid – to citrulline, having the ureide group without arginine's charge, which brings significant biochemical and antigenic changes to the peptide [3].

There are multiple terms for antibodies that recognize citrulline and/or citrullinated peptides: anticitrullinated peptide antibodies, anti-cyclic citrullinated peptide (CCP) antibodies, anticitrulline antibodies and antimodified citrulline antibodies, which are confusing. In rheumatology clinics, 'anti-CCP antibodies' have been in use and the majority of articles regarding their clinical utility have used this term, although the more generic term 'anticitrulline antibody' has appeared recently. However, 'anticitrulline antibody' has a different meaning to researchers of citrulline and citrullinated peptides: in other words, an antibody that detects cirtulline as a free amino acid as well as peptidyl citrulline. 'Anticitrullinated peptide antibodies' are defined as antibodies that recognize

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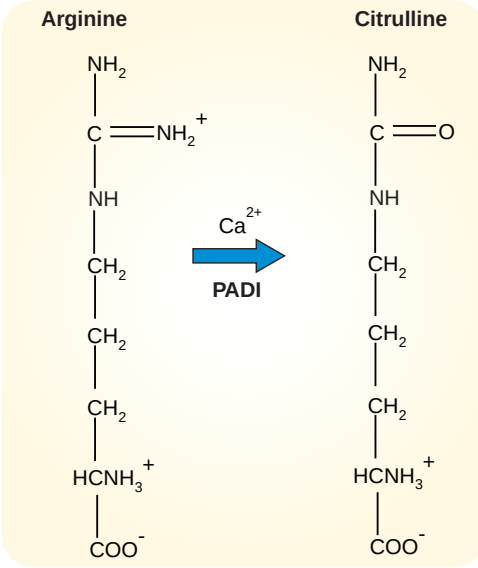
future medicine

Figure 1. Enzymatic conversion of arginine to citrulline by peptidyl arginine deiminase.



This reaction requires a high concentration of Ca^{2+}.

citrullinated peptide(s) in the most generic way. Therefore, in this review, 'anticitrullinated antibodies' is used as a generic term for antibodies that recognize citrulline-containing peptides; 'anti-CCP antibodies' for antibodies measured as a clinical marker of RA; and 'anticitrulline antibodies' for the antibody to detect free citrulline and peptidyl citrulline. Antimodified citrulline antibody is another research antibody that is a specific antibody for detecting peptidyl citrulline with specific chemical procedures for its use.

Peptidyl arginine deiminase

Isotypes & tissue distribution of PADIs

As mentioned previously, there is no tRNA or codon for citrulline. All of the citrulline residues in the protein sequence are products of enzymatic substitution from an arginyl residue by PADI (PADI, enzyme commission [EC]: 3.5.3.15). PADI enzymes catalyze the conversion of arginine residues to citrulline residues in proteins. Five isotypes of PADI (PADI1, 2, 3, 4 and 6) have been cloned from several mammals and their peptide sequences are well conserved. This conservation of PADI isotypes appears to implicate their importance in the physiology of humans but their fundamental function is unclear. All of these isotypes are believed to be intracellular enzymes. PADI4 is unique in that it possesses a putative monopartite nuclear localization signal and is at least partially localized in the nucleus. Tissue distribution of PADI isotypes varies. PADI1 is expressed mainly in the epidermis and uterus. PADI2 is expressed in neuronal tissue and macrophages, as well as in many other tissues. PADI3 is expressed in hair follicles and PADI4 is expressed mainly in white blood cells, especially in neutrophils and eosinophils. PADI6 is the most recently identified isotype and is expressed in oocytes. These differences in tissue distribution among PADI isotypes are thought to be related to their physiological functions. Two of the five PADIs (PADI2 and PADI4) were reported to be present in RA synovial tissue. PADI4 was detected in the nucleus [4] and cytoplasm, and PADI2 was detected only in cytoplasm. Mouse counterparts of PADI2 and PADI4 were also detected in inflammatory joints in the RA model [5].

Enzymatic properties of PADIs

Ca^{2+} & pH dependence of activity

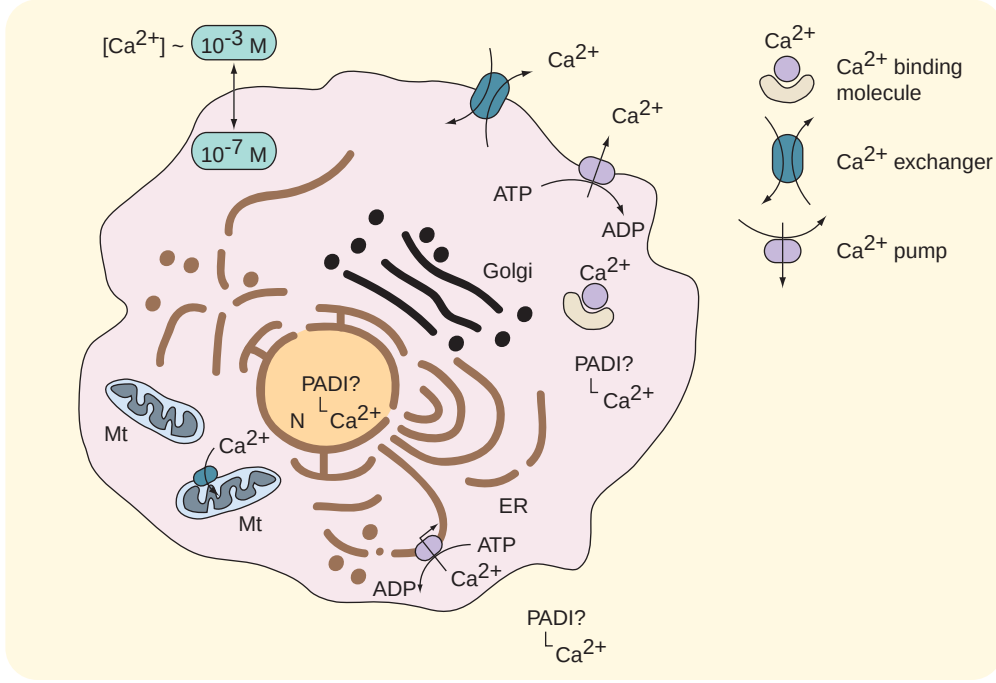
The activity of PADI enzymes depends on Ca^{2+} concentration and pH. They possess Ca-binding motifs and depend on high concentrations of Ca^{2+} for their enzymatic activity. Ca^{2+} dependency seems common among all PADI isotypes and PADI4 has been the best investigated for its kinetics. As the required concentration of $Ca^{2+}$to activate PADIs is much higher (~1 μM) than the cytosolic concentration (~200 nM), conversion of arginine residues to citrulline residues should be carried out in a microenvironment where extraordinarily high concentrations of Ca^{2+} are achieved in tightly regulated conditions or in an extracellular environment along with leakage of enzymes from dying cells (Figure 2). Besides Ca^{2+} dependency, the activity of PADI isotypes depends on pH [6–8].

PADIs & RA

RA-susceptible variant in PADI4

A large-scale linkage disequilibrium study revealed an RA-susceptible variant in PADI4 in a Japanese population [2]. The *PADI4* gene has two major haplotypes: RA-sensitive and RA-nonsensitive. The two haplotypes consist of four exonic single nucleotide polymorphisms. The relative risk of RA in individuals with two copies of the susceptible haplotype is 1.97 compared with individuals without a copy of the susceptible haplotype [2]. As transcripts from a susceptible haplotype are more stable than the other common haplotype of the *PADI4* gene, it is hypothesized that an increased activity of *PADI4* produces susceptibility to RA. Follow-up genetic studies on PADI4 polymorphisms and RA

Figure 2. Molecules that regulate Ca^{2+} concentration.



Ca^{2+} concentration is tightly regulated and its change is used to regulate the activity of various intracellular enzymes and biological phenomena. Although enzymatic activity of PADI enzymes depends on Ca^{2+} concentration, the *in vitro* mechanism of the regulation is still unknown. The figure describes molecules that regulate Ca^{2+} concentration in cellular compartments and the potential location where PADI enzyme molecules encounter Ca^{2+} in adequate concentrations to be activated.
ADP: Adenosine-5'-diphosphate; ATP: Adenosine-5'-triphosphate; ER: Endoplasmic reticulum; Golgi: Golgi apparatus; Mt: Mitochrondrion; N: Nitrogen; PADI: Peptidylarginine deiminase.

have suggested ethnic variations in susceptibility of PADI4 variants. Another independent Japanese cohort study [9] and a Korean study [10] replicated the initial association; although British [11], French [12] and Spanish [13] studies have not replicated the association in European descendants. Relations among genetic variants, expression of PADI4, presence of citrullinated proteins, anti-CCP antibodies and diagnosis of RA have also been investigated. A British study revealed a possible association between *PADI4* polymorphisms and RA, and reported a higher expression of *PADI4* in peripheral blood from patients with RA than from controls [14]. Another study of Caucasian subjects demonstrated a positive association between deposition of citrullinated protein in RA synovium and anticitrullinated peptide antibodies, as well as human leukocyte antigen-D related (HLA-DR) shared epitope and anticitrullinated peptide antibodies, without any association with PADI4 haplotypes [15]. Although the genetic effect of polymorphisms in PADI4 genes might be more prominent in Asians than Caucasians, a higher activity of PADI4 with or without other PADIs seems to play some role in RA pathogenesis, regardless of ethnic background.

Citrullinated proteins & their antigenicity

Substrates of PADIs

All of the citrulline residues in proteins are products of PADI catalysis. It appears that all PADI isotypes can deiminase various proteins *in vitro*, although some combinations of PADI isotypes and particular proteins tend to react more rapidly than others [16]. Among multiple arginine residues in a PADI substrate protein, some residues tend to be more frequently citrullinated than others [6,7,17]. Several molecules have been reported to be natively and/or experimentally citrullinated. Dermal citrullination appears to be the most thoroughly investigated. PADI2 catalyzes filaggrin and K1 keratin in the epidermis. Filaggrin is an aggregative protein for epidermal keratins [18–21]. Initially, profilaggrin, a protein oligomer, is synthesized and forms keratohyalin granules. It is then catalyzed by proteases followed by

PADI2 [22]. Inadequate citrullination was reported in the affected skin of patients with psoriasis [23]. Intranuclear citrullination of histones by PADI4 and its role in gene expression regulation have been reported [24–26]. Although citrullination plays a principal role in the integrity of the skin and may also play a part in other fundamental processes, such as the regulation of gene expression by reversible protein modification, these few examples of citrullination only indicate its role in limited organs or compartments of cellular structures. It does not explain the significance of the wide distribution of PADIs and several proteins that have been reported to be citrullinated, such as myelin basic protein [27], vimentin [28], fibrinogen/fibrins [29] and antithrombin III [30]. Although some biological events, such as inflammation, apoptosis, trauma and aging, are known to increase post-translational citrullination, the precise physiological role of citrullination has yet to be elucidated [31–34].

Citrullinated proteins in RA synovial tissues & other pathologies

Although RA sera recognize citrullinated autoantigens, pathogenic autoantigens containing citrulline in RA are unknown. Filaggrin, which was identified for the first time as a citrullinated self-peptide recognized by RA-specific sera [18], is not an articular component. As there is evidence that citrullination of arginine residues occurs locally in RA synovium [35,36], it is believed that there are citrullinated peptides besides filaggrin in joints and the intra-articular components are more likely to participate in autoimmune reactions in synovial tissues [29]. Citrullinated proteins have been detected in RA synovium. Fibrin(ogen)s were identified initially as one of the synovial citrullinated proteins in RA synovial tissue [29] and have been recognized by anticitrullinated antibodies in RA sera. Further studies of RA synovial tissue have also revealed citrullinated proteins, both in extra- and intracellular regions. Recently, it was reported that intra- but not extra-cellular citrullinated proteins were associated with high titers of anticitrullinated peptide antibodies in blood and synovial fluid, although their presence was independent of local disease activity [37]. The distribution of intracellular citrullinated proteins was colocalized with PADI2 [37] and extracellular citrullinated protein deposits, including fibrin, were reported to be overlapped by PADI4 distribution [38]. As PADI4 is believed to be intranuclear, PADI2 appears in cytoplasm and because intracellular physiological Ca^{2+} concentration is too low to activate PADIs based on *in vitro* experiments (see previously), the distribution of PADI2 and PADI4, along with their substrates and regulatory mechanism of catalysis, have to be elucidated.

In some arthritis models, PADI and citrullinated proteins have also been investigated. Collagen-induced arthritis (CIA) in mice and streptococcal cell-wall-induced arthritis in mice have been shown to express PADI2 and PADI4 in affected joints. In addition, PADI4 was expressed more specifically in inflammation in mice and citrullinated proteins, including fibrin, were also detected [5]; although no anticitrullinated peptide antibodies were seen in these models [5] or other autoimmune and/or arthritic animal models [39]. In the CIA rat model, citrullination of protein induced breakage of immunological tolerance against self-antigens and potentiated arthritogenicity of Type II collagen [40]. In the same model, induction of PADI4 in inflammatory joints was observed and the severity of joint inflammation correlated with the appearance of PADI4 [40].

When the presence of PADIs and citrullinated proteins in synovial tissues of RA and its model animals are considered along with the high specificity of anticitrullinated peptide antibodies in RA, a causative string of PADI, citrullination of self-antigens, anticitrullinated peptide antibody and RA might seem straightforward. However,. this is not the case. The presence of citrullinated proteins, including fibrin, is not an exclusive feature 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 [41–43]. In an animal model, the severity of arthritis was shown to correlate with the deposition of citrullinated proteins in synovial tissues. On the other hand, in human RA, deposition of citrullinated proteins and local disease activity have been shown to be mutually independent [37]. Besides non-RA arthritides, citrullinated proteins were also reported in the affected organs of nonarthropathic pathologies, for example: in plaque interfaces of patients with secondary progressive multiple sclerosis [44]; in myelin basic protein of murine experimental autoimmune encephalomyelitis [45,46]; in the hippocampus of patients with Alzheimer's disease [47]; and in glomeruli of patients with

obstructive nephropathy [48]. These findings and arthritide-related phenomena on citrullinated proteins have to be understood along with an increased knowledge of the physiological and pathological roles of citrullination and autoantigenicity and breakdown of immunological tolerance of citrullinated proteins.

Antigenicity & citrullination

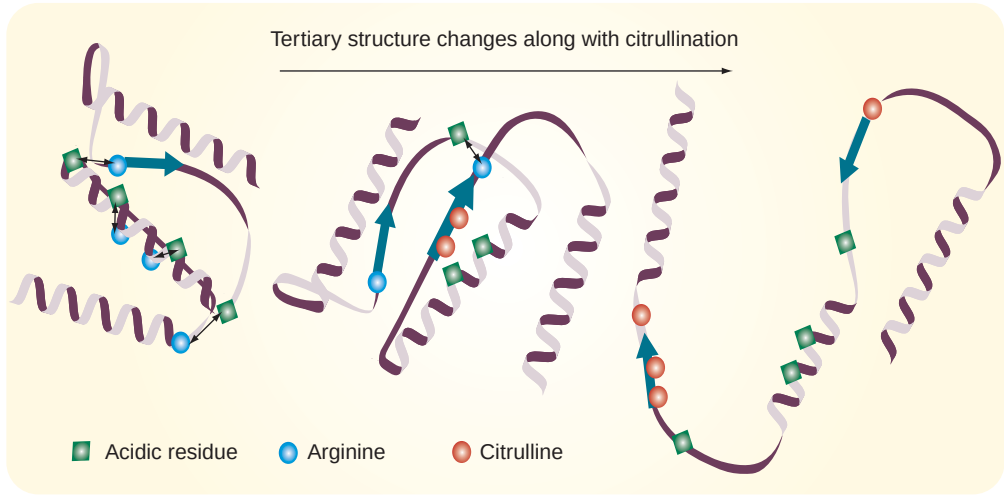
Citrullination is one of several post-translational protein modifications [49]. Phosphorylation, glycosylation, acetylation, hydroxylation and methylation are among such post-translational protein modifications [50]. Post-translational modifications are considered to play important roles in various biological events, including cell signaling and DNA replication. Although the least is known about the biological function of citrullination, it is important to note that citrullination seems to interact with other post-translational modifications, such as methylation and acetylation, in the regulatory mechanism of gene expression by modification of histones [24,25]. From the standpoint of the immune system, all of the post-translational changes in self-peptides seem to influence antigen recognition [50]. Recently, many autoimmune diseases have been reported to produce autoantibodies recognizing post-translationally modified self-peptides [29,51–54]. On the contrary, a lack of usual post-translational modification of self-proteins is also known to be associated with autoimmune diseases [52,55]. These findings might suggest that a break in tolerance to post-translationally modified peptides is a mechanism of development of autoimmunity [56].

A basic group of arginine residues contributes to the tertiary structure of proteins by forming hydrogen bonds and determining secondary and tertiary protein structures (Figure 3). Citrulline lacks this polar feature of arginine. Therefore, conversion of arginine residues to citrulline residues affects protein structures, despite the fact that it produces only a small difference in mass (~1 Da). Citrullinated proteins differ from their non-modified forms in their electrophoretic mobility because of changes in their molecular weight and charge, as well as their conformation. The biochemical changes caused by citrullination resemble denaturing proteins with detergents [57]. This antigenic alteration of proteins by citrullination was confirmed by the finding that experimentally designed anticitrullinated peptide antibodies and autoantibodies from the sera of patients with RA recognized deiminated proteins but not non-citrullinated forms [1,40]. More interestingly, citrullination of peptides increases peptide-major histocompatibility complex (MHC) affinity and activates $CD4^+$ T cells in the HLA-DR4 transgenic mouse [58]. This finding supports the idea of alteration of antigenicity by citrullination and also implicates a change in antigenicity by peptidyl citrullination with a role in the context of HLA-DR shared epitope-dependent antigen recognition. The potency of citrullination to break tolerance against an antigen and to increase the arthritogenicity of the tissue-specific protein Type II collagen [40] implies an alteration in the antigenicity and arthritogenicity of citrullinated proteins.

Anti-CCP antibodies as clinical markers of RA

Although various autoantibodies can be detected in the sera of patients with RA, several autoantibodies have been reported to be more specific and have a higher positive predictive value for RA. Antiperinuclear factor [59] and antikeratin antibody [60] have a sensitivity of 43–52% and a specificity of 97–99% [61,62]. The anti-Sa antibody was reported to have a sensitivity of 27–50% and a specificity of 99% [63]. These highly RA-specific autoantibodies have better positive predictive values owing to their high specificity and all of them have been found to recognize citrullinated peptides [18–20,29,64]. Based on these findings, multiple commercial anti-CCP assays have been developed, all of which perform well clinically. These are called second-generation anti-CCP antibody assays and they use a mixture of citrulline-containing peptides with chemical modifications [65–68]. This is because anticitrullinated peptide antibodies in RA patients are heterogeneous and citrulline-containing epitopes recognized by individual patients with RA vary [20]. It was found that such autoantibodies are not only highly specific for RA (up to 98%), but are detected early in the disease or even several years before disease onset [69] and their titer tends to correlate with an erosive subtype of RA. The anticitrullinated peptide antibodies are believed to be produced in inflamed RA synovium because a fraction of anticitrullinated peptide antibodies are increased compared with those in serum. These second-generation assays use a mixture of citrullinated peptides to achieve their excellent profiles in clinical use. The interindividual variation of

Figure 3. Tertiary structure changes with citrullination.



Deimination of arginine residues disrupts intramolecular noncovalent bonds and alters the tertiary structure. Basic arginine residues participate in noncovalent intramolecular bonds. Substitution of the arginine residues removes such bonds and the tertiary structure of the molecule is changed.

citrulline-containing epitopes could be explained by genetic heterogeneity of molecules in antigen recognition. This was suggested by the report that anticitrullinated protein antibodies were polyclonal and a restricted set of variable region genes were used by the clones [70], as well as by other studies that anticitrullinated peptide antibodies were associated with HLA-DR shared epitope status [37,58,66,71–73].

Although there is no absolute need to distinguish multiple citrullinated epitopes for diagnostic purposes, it seems imperative to identify pathological epitope(s) of RA-specific autoantibodies representing anti-CCP antibodies in order to understand the mechanism of RA-specific autoimmunity toward citrulline-containing epitopes. Although multiple molecules have been reported as candidates for a source of citrulline-containing epitopes – such as collagen Type I [74] and II [75] as well as fibrinogen [76], which were reported as more likely to be recognized by RA sera when in the citrullinated than the noncitrullinated form – details of citulline-containing epitopes and their contribution to RA-specific autoimmune reaction have yet to be investigated.

Conclusions

Anticitrullinated peptide antibodies have established their utility in RA clinics. Along with this progress in clinical medicine, there is growing evidence to support a causative string of PADI, citrullination of self-antigens, anticitrullinated peptide antibodies and the development of RA. Association between the *PADI4* gene and RA susceptibility might imply that increased activity of the PADI enzyme augments the chance to trigger events leading to the manifestation of RA. However, expression of *PADI* is not exclusively specific to RA nor the presence of citrullinated proteins. The most specific phenomenon in the proposed causative string from PADI to RA is the production of autoantibodies recognizing citrulline-containing peptides. In order to understand the mechanisms along the string, physiological and pathological roles of peptidyl citrulline as well as PADIs and autoimmunity towards citrulline-containing peptides have to be investigated further before conclusions are drawn.

Future perspective

As citrullinated proteins and PADIs are an area of current investigation and will continue to be so in the coming years, substantial progresses regarding citrullination and PADIs and their roles in RA are anticipated. Citrullination has been identified in various proteins, including: structural proteins, such as dermal filaggrin; and regulatory proteins, such as histone; and biologically active molecules, such as components in the enzyme cascade. Although some substrates of PADI enzymes have been identified, the mechanism of substrate specificity and residue specificity of PADIs, as well as regulation of

expression and enzyme activity of PADIs, are not yet clear. All of these findings will be studied by *in vitro* and *in vivo* assays of proteins and enzymes, as well as transgenic animals with counterparts of human PADIs. Interindividual heterogeneity of anticitrullinated peptide antibodies will be clarified in the future and may enable us to clinically sub group anti-CCP antibody-positive patients in RA clinics based on a set of epitopes of their anti-CCP antibodies. How immunological tolerance toward citrullinated proteins is broken and why the breakage is highly specific to RA are two major questions to be answered. In addition, research on citrullination, PADI and tolerance towards citrullinated proteins would help determine whether dysregulation of citrullination is a possible causative factor for RA.

Executive summary

Citrulline

- Citrulline is present in two distinct forms in mammals – a free amino acid and a peptidyl form – and their metabolic pathways are independent.
- Peptidylarginine deiminase (PADI) produces citrulline in proteins from arginine residues.

Peptidylarginine deiminase

- PADI is an exclusive enzyme for producing peptidyl citrulline.
- PADI2 and PADI4 are present in rheumatoid arthritis (RA) synovial tissue.
- Activation of PADI enzymes is believed to be regulated by Ca^{2+} concentration.
- Higher expression of PADI enzymes might be a feature of RA.
- The *PADI4* gene has a RA-susceptible variant and the genetic effect appears to be dependent on ethnicity.

Citrullinated proteins

- Citrullinated proteins are products of PADIs.
- Filaggrin is a physiological citrullinated protein known to construct healthy skin.
- Histones seem to be citrullinated and their citrullination is part of the regulation of gene expression with other post-translational modification of histones.
- Citrullinated proteins are detected in RA synovial tissue; fibrin is one of these proteins.
- The quantity of citrullinated proteins in RA synovial tissue is thought to correlate with the amount of anticitrullinated peptide antibodies.
- Except for RA synovial tissue, citrullinated proteins are detected in non-RA synovial tissues and other organs under multiple pathological conditions.
- Substrates of PADIs and the function of citrullinated proteins are still unclear.
- Citrullinated proteins are different from precitrullinated proteins in terms of antigenicity.

Anti-cyclic citrullinated peptide antibodies

- Some autoantibodies that recognize citrullinated self-peptides are almost 100% specific to RA and the second-generation anti-cyclic citrullinated peptide (CCP) antibodies have been developed based on these RA-specific citrullinated peptides and are extremely useful for the diagnosis of RA (sensitivity ~80%, specificity ~95%).
- The anti-CCP antibody assays measure the recognition of multiple citrullinated peptides in patients' sera.
- The anti-CCP antibodies are heterogeneous among individuals.
- The pathogenic epitopes of patients' anti-CCP antibodies are unclear.

What is specific to RA?

- Anti-CCP antibodies are highly specific to RA, but not 100%.
- Some autoantibodies that trigger the identification of anticitrullinated peptide antibodies and recognize citrullinated proteins are almost 100% specific to RA.
- Citrullinated proteins in synovial tissue are not specific to RA.
- The presence of PADIs in synovial tissue is not specific to RA.
- It should be determined whether RA-pathological citrullinated antigens are present and, if so, they must be identified to understand any role of citrulline in RA.

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