CITRULLINATED PROTEINS IN RHEUMATOID ARTHRITIS

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1. ABSTRACT

Citrullinated proteins that are produced by enzymatic deimination of arginine residues in proteins by peptidylarginine deiminases (PADIs) are of particular interest in the pathogenesis of rheumatoid arthritis (RA). First, peptidylarginine deiminase type 4 (PADI4) gene, which codes one of the PADI enzyme isotypes, has a genetic variant that increases susceptibility to RA. The RA-susceptible variant of PADI4 seems to increase the risk of RA by increasing its enzymatic activity. Second, this post-translational protein modification unfolds proteins by loss of a positive charge in arginine residues, with a subsequent change in antigenicity of the self-proteins. Third, these citrullinated proteins are recognized by anti-citrullinated peptide antibodies that are the most RA-specific autoantibodies. Fourth, the expression of the PADI enzyme, citrullination of proteins, and production of anti-citrullinated protein antibodies occur in synovium. These data suggest that citrullination of proteins by PADI is related to alteration of antigenicity of peptides and very closely linked to pathogenesis of RA autoimmunity.

2. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder with autoimmune features that affects 0.5% to 1.0% of the world’s population. RA is characterized by the destruction of multiple joints along with multiple organ involvement. The etiology of RA is still unknown. Several causes have been proposed, including 1) genetic factors, 2) immune and inflammatory responses against microbial pathogens, 3) autoimmune responses directed against components of the synovium and cartilage, 4) autoantibodies and autoreactive T cells, 5) disordered regulation of production of proinflammatory and tissue-destructive cytokines, and 6) autonomous, tissue-invasive cells and tissues. Although the above-mentioned causes seem to interact to develop RA, it is likely that autoimmune factors play a central role in the pathogenesis of RA. However, the precise autoimmune mechanism in the pathogenesis of RA is still controversial.

Cellular immune mechanisms have been implicated to have an important role in RA. First, many T cells and antigen-presenting cells are present in RA synovial tissues. (1) Second, synovial T cells are known to be activated in RA. (2) Third, T cells in RA synovial tissue are not accumulated randomly. (3) Fourth, the HLA DRB gene has been shown to be strongly associated with RA. (4) Fifth, therapy against T cells seems effective in RA. Finally, T-cell cytokines are present in RA synovium. (5) Various evidences also support the involvement of B cells in RA. For example, in RA synovium, B cells differentiate and plasma cells produce antibodies. In addition, B cells in patients with RA show oligoclonality and an autoantibody-producing subset of B cells have been seen to increase in patients with RA. (6)

Although various autoantigens were proposed as targets of pathogenic T cells and B cells in RA, none of their pathogenic mechanisms or significance has been confirmed. Recently, autoantibody recognizing citrullinated self-proteins (anti-citrullinated antibodies) were reported to be the most specific autoantibody in RA (7) and a gene that codes an enzyme producing citrullinated proteins (peptidylarginine deiminase type 4 (PADI4) gene) was identified to be associated with RA. (8) These findings strongly suggest that citrullinated proteins and anti-citrullinated peptide antibodies have a pathogenic role in autoimmunity in RA.

3. CITRULLINATION: POST-TRANSLATIONAL DEIMINATION OF ARGININE RESIDUES

3.1. Citrulline and arginine

Citrulline is an amino acid that is not directly
They possess a Ca-binding motif, and they depend on high concentrations of Ca\(^{2+}\). None of PADI peptide sequences contain a secretory signal. Isotypes are believed to be intracellular enzymes because of historical reasons and 6—have been cloned from several mammals, including humans. Amino acid sequences are well conserved among the isotypes of PADI—PADI1, 2, 3, 4 (or sometimes called 5 (Figure 1). The biggest difference between the isotypes is that arginine is one of the most basic amino acids and citrulline lacks the charged feature. Citrulline is a member of the citric acid cycle, and its metabolism is tightly regulated. Metabolism of protein-bound citrulline is independent from metabolism of free-form citrulline. Because no citrulline tRNA exists, all citrulline residues in proteins are the result of post-translational deimination of arginine residues. The enzyme responsible for the conversion of peptidyl arginine to peptidyl citrulline is peptidylarginine deiminase (PADI) (9).

### 3.2. Peptidylarginine deiminase (PADI)

PADI enzymes catalyze the conversion of arginine residues to citrulline residues in proteins. Five isotypes of PADI—PADI1, 2, 3, 4 (or sometimes called 5 for historical reasons) and 6—have been cloned from several mammals, including humans. Amino acid sequences are well conserved among the isotypes of PADI (50%-55%) and among mammals (70%-95%). All these isotypes are believed to be intracellular enzymes because none of PADI peptide sequences contain a secretory signal. They possess a Ca-binding motif, and they depend on high concentrations of Ca\(^{2+}\) for their enzymatic activity. Because the required concentration of Ca\(^{2+}\) is much higher than the cytosolic concentration, 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 enzyme from dying cells. Mammalian PADIs are unable to convert free L-arginine to free L-citrulline. It appears that all PADI isotypes can deiminate many proteins in vitro, although some combinations of PADI isotypes and particular proteins tend to react more rapidly than others.

Several molecules have been reported to be natively and or experimentally citrullinated. Among the known citrullinated proteins are myelin basic protein, (11) filaggrin, (12-14) keratin, (15) histones, (16) vimentin, (17) and fibrinogen/fibrins. (18)

The biggest difference between the isotypes is their tissue-specific expression. Tissue distribution of the isotypes of PADIs varies. PADI1 is mainly expressed 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. PADI4 is unique in that it possesses a putative monopartite nuclear localization signal (NLS) and is localized in the nucleus; other PADIs do not have an NLS sequence and are detected in cytosol. (9) PADI6 was most recently identified and it is expressed in oocytes. These differences in tissue distribution among PADI isotypes are expected to be related to their physiologic functions.

### 3.3. Citrullination: Physiologic function and effects on protein structure

Although some biological events, such as inflammation, apoptosis, trauma, and aging, increase post-translational citrullination, the precise physiologic role of citrullination is still unknown. (19-22) What is known about citrullination and its consequences is that a variety of proteins are citrullinated and subsequently changes their conformation. Because the enzymes are only active in high concentrations of Ca\(^{2+}\), citrullination should occur outside of cells or in a tightly regulated intracellular condition. A basic group of arginine residue contributes by forming hydrogen bonds and determining secondary and tertiary protein structure (Figure 2). 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 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 charges, as well as their conformation. The biochemical changes caused by citrullination resemble denaturing proteins with detergents. (23) Citrullination is also reported to change the immunologic recognition by antibodies. This antigenic alteration of proteins by citrullination was confirmed by the finding that experimentally designed anti-citrullinated peptide antibodies and autoantibodies from sera of RA patients recognized deiminated proteins but not non-citrullinated forms. (7) More interestingly, citrullination of peptides increases peptide-MHC affinity and activates CD4+ T cells in HLA-DR4 transgenic mouse. (24) This finding supports the idea of alteration of antigenicity by citrullination and also implicates that a change in antigenicity by peptidyl citrullination has a role in the context of HLA-DR4-dependent antigen recognition. Citrullination and its effect on antigenicity are illustrated in Figure 3.

### 4. RA-RELATED PHENOMENA REGARDING CITRULLINATION AND PADI

#### 4.1. Anti-citrullinated peptide antibody

Various autoantibodies have been detected in sera from patients with RA, including rheumatoid factor (RF). RF
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Figure 2. Deimination of arginine residues disrupts intramolecular non-covalent bonds and alters tertiarily structure. Basic arginine residues participate in non-covalent intra-molecular bonds. Substitution of the arginine residues loses such bonds and tertiarily structure of the molecule is changed.

Figure 3. Citrullination of a molecule changes its tertiarily structure. The change in tertiarily structure seems larger when the molecule is citrullinated more (1 and 2). The citrullinated molecule with altered antigenicity is presented by an antigen presenting cell (APC) to T cell and the stimulated T cell signals B cell to produce anti-citrullinated peptide antibody (a-cit pep Ab) (3). In the condition where the molecule is self protein and PADI reacts on the protein, the antigenic citrullinated peptide is continuously supplied and anti-citrullinated antibody production continues (4).

recognizes the Fc portion of immunoglobulin G. Discovery of RF and its association to RA was an important step in the investigation of RA. RF is now routinely measured in clinical laboratories and its presence in serum is one of the American College of Rheumatology’s classification criteria for patients with RA. (25) However, using RF levels to diagnose RA is inadequate. Its sensitivity was reported to be 75% to 90% even in very selected population with low specificity (26, 27).

Besides RF, several autoantibodies have been reported to be more specific and to have higher positive predictive value for RA. Antiperinuclear factor (28) and anti-keratin antibody (29) have a sensitivity of 43% to 52% and a specificity of 97% to 99%. (30, 31) Anti-Sa antibody was reported to have sensitivity of 27% to 50% and specificity of 99%. (32) These highly RA-specific autoantibodies have better positive predictive values due to their high specificity, and all of them have been found to recognize citrullinated peptides. (12-14, 18, 33) Based on these findings, enzyme-linked immunosorbent assay (ELISA) systems that quantitate antibodies recognizing citrullinated antigens have been developed for use in RA. One such system uses an artificial citrulline-containing peptide designed to be circular to expose as much citrulline residue as possible. This was done because investigations of the properties of citrullinated arginine residues in various peptides that were recognized by antibodies derived from RA sera revealed that more exposed residues of citrullinated arginines were better recognized. (14, 34) The diagnostic performance of anti-citrullinated peptide antibody seems to be promising based on multiple reports. (34-37) It was found that such autoantibodies not only are very specific for RA (up to 98%), but they are detected very early in the disease or even several years before the disease onset (41) and their titre tends to correlate with an erosive subtype of RA. The anticitrullinated peptide antibodies are believed to be produced in inflamed RA antibodies is increased among synovial immunoglobulins compared to serum. Table 1 summarizes reports on sensitivity and specificity of anti-
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Table 1. Sensitivity and specificity of anti-citrullinated peptide antibody assays for rheumatoid arthritis

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year of publication</th>
<th>Subjects</th>
<th>Antigen and assay method</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simon et al.</td>
<td>1993</td>
<td>48 cases and 56 controls</td>
<td>human dermal filagrin (IB)</td>
<td>75%</td>
<td>89%</td>
</tr>
<tr>
<td>Shellekens et al.</td>
<td>1998</td>
<td>134 cases vs. 154 controls</td>
<td>CCP* (ELISA)</td>
<td>76%</td>
<td>96%</td>
</tr>
<tr>
<td>Shellekens et al.</td>
<td>2000</td>
<td>134 cases vs. 154 controls</td>
<td>CCP (ELISA)</td>
<td>68%</td>
<td>98%</td>
</tr>
<tr>
<td>Goldbach-Mansky et al.</td>
<td>2000</td>
<td>106 early cases vs. early non-RA arthritides</td>
<td>human dermal filagrin (ELISA)</td>
<td>33%</td>
<td>93%</td>
</tr>
<tr>
<td>Bizzaro et al.</td>
<td>2001</td>
<td>98 cases vs. 232 controls</td>
<td>CCP (ELISA)</td>
<td>41%</td>
<td>98%</td>
</tr>
<tr>
<td>Suzuki et al.</td>
<td>2003</td>
<td>549 cases vs. 208 controls</td>
<td>CCP (ELISA)</td>
<td>88%</td>
<td>89%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>human recombinant-citrullinated-filaggrin</td>
<td>69%</td>
<td>95%</td>
</tr>
</tbody>
</table>

CCP*: cyclic citrullinated peptide

citrullinated antibodies.

Although RA sera recognize citrullinated auto-antigens, the auto-antigens that function as immunogens for the anti-citrullin responses in RA are unknown. Filaggrin, which was identified for the first time as a citrullinated self-peptide recognized by RA-specific sera, (12) is not an articular component. Therefore, it seems to be recognized by anti-citrullinated peptide antibodies as a consequence of cross-reactivity. A study of synovial tissues revealed that fibrin (ogen) in RA synovium were citrullinated, (18) and they were recognized by anti-citrullinated antibodies in RA sera. Although fibrins might be the true self-antigens that trigger a break in tolerance as a causative event of RA, further studies need to be performed. It was reported that anti-citrullinated protein antibodies were polyclonal and a restricted set of variable region genes were used by the clones. (42) Since production of antigens that are recognized by anti-citrullinated antibodies is affected by genetic variants of PADI4 gene and epitope recognition of citrullinated peptides are influenced by HLA-DR types, it seems that there are genetic predispositions to develop anti-citrullinated antibodies.

4.2. RA-susceptible variant in the PADI4 gene

Another genetic contribution to peptidyl citrullination in RA has been reported. One of the PADI genes, PADI4, was identified to be associated with RA. (8) All the genes coding isotypes of PADI enzymes are located in a single cluster. The human PADI gene cluster spans a 350kb segment in chromosome 1p36.1 (Figure 4). The PADI4 gene has two major haplotypes: one is RA-sensitive and the other is RA-non-sensitive. The two haplotypes consist of four single nucleotide polymorphisms (Figure 5). 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. (8) Because transcription from a susceptible haplotype is more stable than the other common haplotype of PADI4 gene, it is hypothesized that increased activity of PADI4 produces susceptibility to RA.

Both macrophages and granulocytes are present in RA synovial tissue. The expression of PADI4 was also reported in RA synovial tissue. (8) The mouse counterpart of PADI2 and PADI4 was also detected in inflammatory joints in mouse RA models. (45) Citrullinated proteins were also detected in RA synovium. One of the citrullinated proteins in RA synovium was shown to be fibrin (ogen), and it was recognized by anti-citrullinated protein antibody from RA patients. (18) Although citrullinating enzymes and citrullinated proteins are present in RA synovium, they were also detected in inflamed joints in diseases other than RA (unpublished data). Therefore citrullination and citrullinated proteins are not specific to RA synovium.

5. SUMMARY AND PERSPECTIVE

Much data exist regarding peptidyl citrulline and RA. (1) The presence of autoantibodies recognizing citrullinated self-proteins is highly specific to RA. (2) The production of anti-citrullinated peptide autoantibodies precedes the development of RA, or at least precedes the manifestation of clear symptoms of RA. (3) T cell recognition of citrulline-containing peptides is accelerated by RA-susceptible HLA-DR4 epitopes. (4) In addition, anti-citrullinated peptide antibodies are produced in inflammatory RA synovium. (5) Furthermore a genetic polymorphism that seems to increase the enzymatic activity of one of the citrullinating enzymes increases the susceptibility to RA.

Although all these data suggest that citrullination and citrullinated proteins play key roles in the pathogenesis of RA, they do not explain all of the relationship between citrulline and RA. First, neither the expression of PADI nor the presence of citrullinated proteins in the synovium is restricted to RA. Second, the fact that the production of anti-citrullinated peptide antibodies precedes disease onset contradicts the simple hypothesis that citrullination and/or anti-citrullinated peptide antibodies are pathogenic on their own.

Figure 6 describes the processes that are present or absent in symptomatic RA, non-RA arthritides, and the preclinical period of RA. When inflammation is established in RA joints, inflammatory cells accumulate in the synovium and inflammatory cytokines are produced. The PADI enzyme is activated with an appropriate Ca$^{2+}$ concentration.
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Association Plots in the PADI Cluster

Figure 4. Result of linkage disequilibrium mapping of the PADI-cluster region. a indicates distribution of genes and SNPs. The vertical axis of b represents negative value of logarithm of P in case-control association test on each SNP. PADI1, 2, 3 and 4 genes cluster and association with RA was detected in PADI4 gene.

Variants of rheumatoid arthritis-susceptible gene,

663 amino acids, 4 SNPs with 3 amino acid substitutions

Gly 55 Ser, Val 82 Ala, Gly 112 Ala

Figure 5. PADI4 molecule is consisted of 663 amino acids. There is a nuclear leading sequence and EF-hand (calcium-binding motif) and its C-terminal is conserved among PADI isotypes. There are two variants in PADI4 gene with 4 SNPs and 3 amino acid substitutions.
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Joint

Inflammatory cytokines

Autoantibody production against citrullinated peptides

Antigen recognition in conjunction with HLA molecules

Citrullinated proteins

PADI + Ca²⁺ (Intracellular or extracellular)

Deimination of self proteins

Antigen-antibody reaction

Augmentation

HLA-DR4

Susceptible variant of PADI4

Citrullinated proteins

Antigen recognition in conjunction with HLA molecules

Deimination of self proteins
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Figure 6. Citrullination, production of anti-citrullinated peptide antibodies, and inflammation are illustrated for inflammatory RA joint (A), inflammatory non-RA joint (B), and pre-clinical RA condition (C). Descriptions of each panel are in the main text.
Deimination of arginine residues occurs and citrullinated proteins are produced in synovial tissue. Because anti-citrullinated peptide antibodies and citrullinated peptide-reactive lymphocytes are present in inflammatory RA synovium, an autoimmune reaction against the citrullinated proteins occurs, and subsequently inflammation is accelerated. Thus, a cycle of reactions is established. It appears that ongoing chronic inflammation of RA could be explained by this circular reaction. In this scenario, RA-susceptible HLA-DR alleles may promote the process that citrulline-containing peptides activate autoreactive lymphocytes, but they are not indispensable. In the same way, genetic predisposition to higher expression of PADI4 can work to promote the production of citrullinated proteins, but the step can be still active without the variant. On the other hand, in non-RA arthritis, inflammatory cells, and cytokines accumulate in the joints and subsequently the PADI enzyme is activated and citrullinated proteins are produced. The citrullinated proteins might be presented to T cells by antigen-presenting cells, but somehow, autoimmunity against citrullinated peptides does not develop. In this case, a circular reaction does not occur. Thus, some other factor(s) that keeps arthritis continuous must be present.

Because anti-citrullinated peptide antibodies are detected in sera several years before the diagnosis or even before the initial manifestation of RA symptoms, production of citrullinated proteins and anti-citrullinated peptide antibodies should occur under different conditions from symptomatic RA. What differentiates symptomatic RA from the preclinical condition of RA is presence of inflammation and possibly a local reaction between citrullinated proteins and anti-citrullinated peptide antibodies in the inflamed synovium. What is common between preclinical RA and symptomatic RA is presence of anti-citrullinated antibodies in the serum. In addition, genetic predisposition to RA (HLA-DR and PADI4) must be common in preclinical and clinical RA. In the preclinical phase of RA, synovial inflammation is absent or negligible. Therefore autoimmunity against citrullinated peptides should be established in joints without inflammation or only with negligible inflammation, or the autoimmunity should be started somewhere else such as lymph node or bone marrow.

Although it is very likely that citrullination has an important role in RA pathogenesis, several questions still need to be answered: (1) How is the immunologic tolerance for citrullinated and non-citrullinated forms of self-proteins achieved and maintained? (2) What is the physiologic role of citrullination? (3) What is the difference between physiologic citrullination and pathogenic citrullination? (4) What is the initial citrullinated protein that triggers the citrulline-related autoimmunity and where does it start? (5) Is there any pathogenic citrullinated protein that induces RA? If there is, what is it? (6) Because joints are the primary loci of disease activity of RA, citrulline-related autoimmune reactions should have some joint-specific components. What are they? Investigations to answer these questions will provide us with a better understanding of RA.

6. ACKNOWLEDGMENTS

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