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Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis: As Good as it Gets?

Zoltán Szekanecz · Lilla Soós · Zoltán Szabó · Andrea Fekete · Anikó Kapitány · Anikó Végvári · Sándor Sipka · Gabriella Szücs · Sándor Szántó · Gabriella Lakos

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Abstract Anti-citrullinated protein antibodies (ACPAs) have recently emerged as sensitive and specific serological markers of rheumatoid arthritis (RA), providing superior alternative of the rheumatoid factor (RF) test in the laboratory diagnostics of RA. The first members of this autoantibody family were anti-perinuclear factor (APF) and anti-keratin antibodies (AKA). It became evident that both APF and AKA recognize citrullinated epitopes of filaggrin. Citrullination is a post-translational modification of arginine by deimination, physiologically occurring during apoptosis, inflammation or keratinization. The presence of several citrullinated proteins has been demonstrated in the RA synovium. The identification of citrullinated epitopes as targets for anti-filaggrin antibodies led to the development of the first and later second generation anti-cyclic citrullinated peptide (anti-CCP) antibody assays. The widely used anti-CCP2 assays have high diagnostic sensitivity and specificity, and they also show important predictive and prognostic value in RA. The anti-Sa antibody has been identified a decade ago; however, recent studies confirmed that anti-Sa is directed against citrullinated vimentin, hence it is a new member of the family of ACPAs. The newly developed anti-mutated citrullinated vimentin (anti-MCV) assay has similar diagnostic performance than the anti-CCP2 ELISA; however, the diagnostic spectrum of the anti-MCV test is somewhat different from that of anti-CCP2. It's especially useful in the diagnosis of RA in RF and anti-CCP2 seronegative patients. The combined application of anti-CCP2 and anti-MCV assays can improve the laboratory diagnostics of RA. The family of ACPAs is expected to expand; there is an increasing need for developing new diagnostic strategies after careful evaluation of the characteristics of the available assays.

Keywords Rheumatoid arthritis · Anti-mutated citrullinated vimentin · Anti-CCP2

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation that may eventually lead to joint destruction [1]. Genetic factors, autoimmunity, and environmental factors are all involved in the pathogenesis of the disease [2]. In recent years, very sensitive imaging and laboratory diagnostic modalities have become available along with the introduction of new and very effective therapeutic approaches including biological therapy [3].

Regarding laboratory approaches, several autoantibodies have recently been associated with disease activity and/or prognosis of RA [4–6]. However, until recently, rheumatoid factor (RF) of the IgM isotype has been the only laboratory marker routinely used in RA. The assessment of IgM RF has rather low specificity for RA as it can also be detected in sera of patients with other autoimmune diseases, infectious disorders, as well as in the healthy elderly population [7–9].

Antibodies to citrullinated antigens have recently been identified as potential diagnostic and prognostic markers in

Zoltán Szekanecz and Lilla Soós with equal contribution.

Z. Szekanecz (✉) · L. Soós · Z. Szabó · A. Végvári · G. Szücs · S. Szántó
Division of Rheumatology, 3rd Department of Medicine, Medical and Health Science Center, University of Debrecen, 22 Moricz Street, Debrecen H-4004, Hungary
e-mail: szekanecz@iiibel.dote.hu

A. Fekete · A. Kapitány · S. Sipka · G. Lakos
Laboratory of Immunology, 3rd Department of Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

RA [10–12]. They belong to the family of antibodies directing to epitopes containing the nonstandard amino acid citrulline. Anti-citrullinated protein antibodies (ACPAs) include anti-perinuclear factor (APF) [6], anti-keratin (AKA) [13] and anti-filaggrin antibodies [14–16]. In the last few years, an emerging amount of data has been collected on the superior diagnostic and prognostic value of anti-cyclic citrullinated peptide (anti-CCP) autoantibodies [10–12] among ACPAs. Very recently, the diagnostic performance of anti-Sa antibody [(detected by ELISA utilizing mutated citrullinated vimentin (MCV) as antigen], has also been partly elucidated. The newest members of the family of ACPAs are anti-citrullinated fibrinogen antibodies (ACF).

In this paper, we review recent data on ACPAs with special attention to anti-MCV. We will discuss the pathogenic, diagnostic and prognostic value of these antibodies in RA.

The Pathogenic Role of ACPAs: Citrullination, Genetics, Autoimmunity and Environmental Factors

Citrulline is a nonstandard amino acid originating from an enzymatically modified, deiminated arginine residue present on certain human proteins. Citrullination is a physiological process during keratinization of epithelial cells, inflammation, and apoptosis [17–20]. Citrulline is generated from arginine by the peptidylarginine-deiminase (PAD) enzyme, which has five isoforms in mammals designed as PAD1, 2, 3, 4, and 6 [20–22]. Inflammatory leukocytes including synovial T and B cells, macrophages, neutrophils, as well as fibroblast-like synoviocytes express two isoforms of this enzyme, PAD2 and PAD4 [21, 22].

The normal, uninflamed synovium do not contain high amounts of citrullinated proteins [22]. However, there is an augmentation of citrullination under inflammatory conditions [22]. Citrullinated epitopes have been observed in extravascular fibrin deposits and extracellular fibrinogen aggregates in the inflamed synovium [22, 23]. Citrullinated proteins were identified in the synovial lining layer, the sublining layer, and in the extravascular fibrin deposits in the RA joint [23]. Citrullinated fibrinogen was detected as a soluble autoantigen in RA synovial fluids [24]. Moreover, citrullinated vimentin is also present in inflammatory macrophages [25, 26]. The major synovial targets of the RA-specific antifilaggrin autoantibodies have been identified as deiminated forms of fibrin α and β chains [27]. This finding is supported by the demonstration of cross-reactivity between autoantibodies to filaggrin and citrullinated fibrin [28]. However, although a few studies suggest that the presence of citrullinated proteins in the synovial tissue is specific for RA [29, 30], there is a general agreement now indicating that citrullination commonly occurs in various types of arthritides [23, 31].

Synovial citrullinated proteins described above may trigger autoimmunity in RA. Antigen-driven maturation of citrullinated protein-specific B cells has been observed at sites of inflammation, such as in the RA synovium. Synovial fluid $CD38^+$ B cells from RA patients are characterized by somatic hypermutation and clonal selection [32]. ACPAs constitute a 7.5-fold proportion of IgG in the rheumatoid pannus than in paired sera [33], and culture supernatants from synovial tissue fragments obtained from anti-filaggrin antibody positive RA patients contain significant amount of antibodies [33]. Moreover, B cells isolated from the synovial fluid of anti-CCP positive RA patients produce IgM anti-CCP antibodies [34]. Taken together, APCAs are present in the synovial fluids of RA patients [33, 34], and they are produced by local plasma cells in the inflamed joints [34]. ACPAs may be directly involved in the pathogenesis of RA [35, 36], as locally produced autoantibodies generate immune complexes and may contribute to the initiation and sustaining of synovial inflammation by triggering monocyte and granulocyte activation and cytokine production. These data, together with the efficacy of B-cell depletion therapy using the anti-CD20 antibody rituximab in RA [37] suggest an important role for B lymphocytes and ACPAs in the pathogenesis of RA.

The development of an autoimmune response against citrullinated epitopes is facilitated by a specific genetic predisposition namely, the presence of particular HLA-DRB1 alleles ("shared epitope," SE) [17–19]. As recently published, the presence of SE and anti-CCP antibodies are not independent risk factors for RA, but the presence of HLA-DRB1 SE alleles in RA patients are primary risk factors for the generation of anti-CCP antibodies [38]. The presence of one or two SE alleles has been associated with anti-CCP antibody positivity [18, 39]. Moreover, unfavourable disease progression has been related to anti-CCP production and SE positivity [12, 39, 40], whereas HLA-DR3 has been related to anti-CCP negative disease [39]. In a recent study, we also found significant correlation between SE and both anti-CCP positivity and absolute serum concentrations of anti-CCP [41]. HLA-DR genes exert a major influence on the $CD4^+$ αβ T-cell repertoire, and SE alleles are thought to efficiently present self-peptides to $CD4^+$ T cells in the thymus [42]. The conversion of arginine to citrulline significantly increases peptide-MHC affinity and leads to the activation of $CD4^+$ T cells [43]. Thus, a genetic predisposition may lead to the perpetuation of the production of anti-CCP antibodies in RA.

Recently, smoking, as an environmental factor, has been implicated in the pathogenesis of RA and has been associated with protein citrullination under certain genetic contexts. Smoking may facilitate citrullination and anti-CCP production in patients carrying the SE. This effect is only seen in anti-CCP positive RA patients. Thus, smoking

may trigger autoimmunity, possibly by affecting citrullination, in SE positive patients [44].

The Evolving Story of ACPAs

The historically first ACPA was APF, which has been known since 1964 [6]. APF can be detected on buccal mucosal epithelial cells using indirect immunofluorescence. Only 10% of buccal epithelial cell donors express enough antigen to detect APF, therefore the assessment of APF has been limited to certain specialized laboratories. APF has been detected in 50–70% of RA patients, even in the early phase of the disease. It has a high specificity (90%) but rather low sensitivity [45]. The autoantigen detected by APF has long been unidentified, however, after the discovery of AKA it was soon determined that APF and AKA both recognize the same antigen [46].

AKA has been described in 1979 [13] as an antibody reacting with the stratum corneum epithelial cells of rat esophagus. AKA has also been determined by indirect immunofluorescence. AKA has a somewhat lower sensitivity (40–60%) in comparison to APF [45]; however, its specificity is similar to that of APF. Furthermore, AKA can also be detected in early RA, as well as in RF negative patients [46]. The antigen recognized by AKA has been identified as filaggrin, a short designation for filament-aggregating protein [47], whereas APF has been shown to be directed against profilaggrin [46].

After the discovery of the antigenic specificities of APF and AKA, purified filaggrin has been used in more sensitive assays [48, 49]. It has soon been demonstrated that all these antibodies recognize antigenic epitopes containing citrulline [20]. Citrulline is an essential constituent of all epitopes recognized by APF, AKA, and anti-filaggrin antibodies [10], hence they all belong to the family of ACPAs. The first ELISA systems detecting antibodies against citrullinated antigens utilized synthetic, filaggrin-derived linear peptides [10]. However, the sensitivity of these tests could be increased by using cyclic rather than linear antigenic peptides, which then lead to the development of first generation anti-CCP (anti-CCP1) assays [12]. Nowadays even more sensitive, second generation anti-CCP2 assays are used.

The Diagnostic Properties of Anti-CCP Antibodies

Anti-CCP antibodies, the most important members of the family of ACPAs so far, are specific diagnostic and prognostic markers in RA [57]. The determination of anti-CCP helps to distinguish RA from other arthropathies [12]. Anti-CCP2 antibody ELISA testing has shown a specificity of 98% in sera from patients with established RA and 96% in sera from subjects with early RA [12]. The overall sensitivity of anti-CCP2 assays is similar to that of RF (60–80%), but importantly, anti-CCP2 antibody is positive in 20–30% of RF seronegative patients [50]. Anti-CCP antibodies have significant predictive value, as they can be found very early, sometimes even during the preclinical phase of RA [51, 58]. Anti-CCP positivity may precede clinical symptoms by years [51, 58]. The presence of anti-CCP antibodies also has been associated with more destructive joint damage and aggressive course of the disease [52–59]. The anti-CCP status only rarely (5%) changes during the disease course in established RA [55]. The absolute serum levels of anti-CCP may slightly change during the progression of the disease, but these changes may not reflect disease activity or response to therapy [55–58].

Anti-MCV/Anti-Sa, A Recently Identified Autoantibody in RA

An RA-specific autoantibody was discovered in 1994 named after a patient as anti-Savoie (anti-Sa) [5]. This antibody was present in the sera of 43% of RA patients but not in many other autoimmune diseases and in healthy individuals. In addition, 27% of RF negative RA patients were also tested positive for anti-Sa [5]. Using immunoblot technique, anti-Sa reacted with a 50 kDa protein present in the spleen, placenta, as well as in the RA synovium [5]. Numerous studies have been performed since that time, and the overall specificity of anti-Sa was found to be 92–98%, whereas the sensitivity was about 40% [59]. The high specificity is coupled with substantial prognostic value [60, 61], as anti-Sa positivity has been associated with more active and destructive disease [59]. Thus, anti-Sa has been thought to have important diagnostic and prognostic relevance in RA.

It has recently been demonstrated that anti-Sa specifically recognizes citrullinated vimentin [62]. Vimentin is secreted and citrullinated by macrophages in response to apoptosis, or by proinflammatory cytokines, such as tumor necrosis factor-α [25, 26]. Vimentin contains 43 arginine residues, which can be potentially citrullinated by PAD. These results suggest that citrullinated vimentin may be a newly identified, promising autoantigen in RA. To detect antibodies to citrullinated vimentin, a new ELISA system has recently been developed. This assay utilizes genetically MCV to improve the performance of the test [62, 63].

In a recent study, we utilized anti-MCV ELISA to assess the performance of this test in comparison with anti-CCP2 and RF assessments. Using cut-off levels recommended by the manufacturers, anti-MCV showed 9% higher sensitivity than anti-CCP2 and 4% higher sensitivity than IgM RF; however, its diagnostic specificity was somewhat lower than that of anti-CCP2 (91.5 vs 98.8%). According to receiver operating characteristic curve analysis, the diagnostic performance of the anti-MCV ELISA for the diagnosis of RA was similar to

that of the anti-CCP2 test (area under the curve: 0.853; 95% CI 0.801–0.905 for anti-MCV and 0.910; 95% CI 0.873–0.946 for anti-CCP2), whereas it was significantly higher than that of IgM RF. When introducing a cut-off level resulting in 95% specificity for both tests, we observed 69.7% sensitivity for the anti-MCV assay and 74.8% for anti-CCP2 ELISA. Our data confirms the results of Dejaco et al. [64], who found that using a cut-off value for the anti-MCV ELISA to obtain identical specificity than that of the anti-CCP2 test resulted in decreased sensitivity of the assay compared to anti-CCP2. The agreement rate between anti-MCV and anti-CCP2 tests in our hands was 88.2%. More than 10% of anti-CCP2 negative RA patients and approximately one third of IgM RF negative RA subjects were anti-MCV positive. We also found strong correlation between anti-MCV and anti-CCP2 levels in RA sera suggesting potential cross reactivity. However, the presence of anti-MCV positive patients in the anti-CCP2 negative group suggests that these antibodies may be directed to citrullinated epitopes present only in the anti-MCV assay. Our study suggests that the anti-MCV test is able to identify a number of RA patients who are tested seronegative by IgM RF or anti-CCP2 assays. Moreover, double positivity for anti-MCV and anti-CCP2 may provide a very high (98.3%) specificity in diagnosing RA. Thus, determination of anti-MCV antibodies may have additional value to the anti-CCP test in RA patients [63], and both tests are especially valuable in IgM RF negative arthritis. Other investigators have recently confirmed that anti-Sa positivity is associated with higher serum anti-CCP levels [65]. In addition, in a recent study, we found significant association between HLA-DRB1 SE positivity and the production of anti-MCV antibodies in RA patients (Lakos et al., unpublished observations).

Anti-Citrullinated Fibrinogen Antibodies

Whereas filaggrin is not present in the RA joint, this protein itself cannot account for the autoantibody response against citrullinated antigens. Citrullinated fibrinogen, however, is abundant in the inflamed synovium [22–24]. Using in-house assays, several studies confirmed the presence of ACF antibodies in the sera of RA patients [66–68]. The diagnostic performance of the assays was very similar to that of anti-CCP2 ELISA, with a very high agreement rate [68]. ACF antibodies were good predictors of diagnosing RA at 1 year in patients with early arthritis, and for radiographic progression after 1 years [68]. An association was observed between HLA-DRB1*0404 alleles and the production of ACF antibodies [69].

Anti-CCP, Anti-MCV or ACF...?

The diagnostic properties of anti-CCP2, anti-MCV and ACF assays are very similar, and on the other hand, different from that of IgM RF. Their production is associated with the presence of HLA-DRB1 SE alleles, and their presence is highly specific for RA. Anti-CCP2 and ACF antibodies have been ranked in the same cluster by a recent study [67]. In agreement with our findings, however, this same paper also confirmed that disagreement between ACPA tests might occur. The increasing number of potential autoantigens, the cross-reactivity between antibodies to filaggrin and citrullinated fibrin [28], as well as the demonstration of individual reactivity patterns of sera from RA patients against several citrullinated peptides [12] suggest that citrullinated epitopes, rather than a single citrullinated molecule, may be involved in the induction of ACPAs.

In summary, the identification of citrullinated proteins as autoantigens and the development of new assays detecting ACPAs is a major breakthrough in the laboratory diagnostics of RA. The high predictive and prognostic values of APCAs for the evolution and progression of the disease course strongly support early diagnosis and tailored treatment.

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