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Variable domain glycosylation of ACPA-IgG: A missing link in the maturation of the ACPA response?

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ABSTRACT

Anti-citrullinated Protein Antibodies (ACPA) are excellent markers for Rheumatoid arthritis (RA) and are postulated to have a pathogenic role in the disease process. A multistep model for the evolution of the ACPA response in RA was proposed in which an initial break of tolerance causes, as "first hit", "silent" production of ACPA without any clinical symptoms. The model further proposes that the ACPA immune response matures upon a certain (unknown) trigger, a "second hit", which leads to epitope spreading, an increase in ACPA titres and extended isotype usage before clinical RA manifestations. These occurrences are indicative of an expansion of the citrulline-specific B cell response, though ACPA remain of low avidity even in established disease. This persistence of low avidity is puzzling, as the typical signs of maturation of the immune response seem to be uncoupled from the classical process of affinity maturation. In fact, it suggests that B cells expressing ACPA could bypass selection mechanisms that otherwise control the expansion of auto-reactive B cells. In the established, chronic phase, we recently found that ACPA-IgG are extensively glycosylated in the variable (Fab) domain. More than 90% of ACPA-IgG molecules carry Fab glycans that are highly sialylated. This molecular feature is striking and may provide a missing link in our understanding of the maturation of the ACPA immune response. This review, therefore, describes the current knowledge about ACPA Fab glycosylation in the pathogenesis of RA.

1. Introduction

1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by systemic and persistent inflammation of the synovium which can lead to joint damage, bone destruction and, ultimately, may cause disability and increased mortality [1]. Around 0.5–1% of the western population is affected by RA with a higher incidence among woman and upon increased age [1,2]. Although it is not yet understood what exactly drives RA pathogenesis, genetic and environmental risk factors are thought to contribute to the development of RA.

1.2. Autoantibodies in RA

An important hallmark of RA is the presence of autoantibodies. Around 50–80% of patients with RA harbour either one or multiple autoantibodies [1]. Rheumatoid factor (RF) was the first autoantibody discovered to correlate with RA and is included in the current RA classification criteria [3,4]. RF are directed against the Fc tail of the immunoglobulin G (IgG) molecule and thought to exert a pathogenic effector mechanism via the formation of immune complexes, the activation of complement and the subsequent induction of inflammatory mediators [5]. However, RF are not specific for RA as they are also common in chronic and infectious diseases, and in healthy individuals [6]. In addition to RF, antibodies against posttranslational modifications can be found in the sera of patients with RA, such as anti-carbamylated protein (anti-CarP) antibodies and Anti-citrullinated Protein Antibodies (ACPA). Anti-CarP antibodies recognize proteins which are carbamylated, a process leading to the chemical conversion of lysine to homocitrulline [7]; ACPA, instead, recognize proteins that are citrullinated, a process in which the positively charged amino acid arginine is converted to a neutral citrulline by peptidyl arginine deiminase (PAD) enzymes [8]. Importantly, ACPA are highly specific for RA and are strongly associated with arthritis, severe joint destruction during the course of the disease and lower remission rates [9,10]. Also histologically, ACPA-positive disease differs from ACPA-negative disease [11]. Furthermore, ACPA are very useful in predicting the onset of RA as ACPA can be present years before first clinical disease manifestations [12,13]. Also ACPA are now included in the 2010 EULAR/ACR classification criteria for RA [14]. All together, these observations have led to the general assumption that ACPA may play a crucial role in RA

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pathophysiology. However, the nature of the ACPA response is still an unknown aspect. Here, we discuss how a novel molecular feature of ACPA could contribute to the pathogenic immune response in RA.

2. Maturation of the ACPA response

2.1. The two-hit model of ACPA-positive RA

The development of ACPA-positive RA is thought to be a multistep process which is described here by a two-hit model [15]. Environmental and stochastics events ("first hit") may drive the initial break of tolerance leading to ACPA production which can precede the onset of RA for years without signs of clinical symptoms [16]. Upon a certain trigger, citrulline-specific B cells may receive T cell help inducing an inflammatory autoimmune response. Because of this "second hit", the ACPA response matures, an event strongly associated with imminent onset of arthritis.

2.2. The HLA association of ACPA-positive RA

The strongest genetic risk factor associated with RA is found within genes encoding certain HLA class II molecules, specifically HLA-DR. Within the third hypervariable region of the HLA-DRB1 chain there is a common amino acid sequence, known as the shared epitope, identified as susceptibility epitope for RA [17]. It is hypothesized that the positively charged amino acids of the shared epitope favour binding to negatively or neutrally charged amino acids thereby preferring binding of citrullinated antigens over arginine residues [18]. Importantly, the shared epitope predisposition is only associated with ACPA-positive RA, not with ACPA-negative disease [19–21]. As HLA is involved in the interaction of T- to B-cell responses, it is conceivable that T cells contribute to the maturation of the ACPA response, possibly via T cell help to citrulline-specific B cells. Supporting this hypothesis, a recent paper identified unexhausted, activated T 'peripheral helper' cells in the joint of RA patients which expressed factors enabling help for B cells, directed migration to the inflamed site and induced plasma cell differentiation [22].

2.3. Expansion of the ACPA response

While HLA shared epitope alleles are associated with ACPA-positive RA, they are not associated with the presence of autoimmunity/ACPA in health [21,23]. Therefore, it was suggested that HLA molecules contribute to the "second hit" rather than the "first hit" of the two-hit model, thereby increasing the range and intensity of the ACPA immune response. A role for T cells as drivers of the activation of citrulline-specific B cells and of the maturation of the ACPA response is supported by the increase in ACPA titre, isotype switching and antigen recognition profile shortly before clinical onset of RA [13,24–26]. However, ACPA are highly cross-reactive towards different citrullinated proteins and, unlike antibodies against recall antigens, ACPA are of low affinity. This indicates that citrulline-specific B cells might not primarily be selected on affinity for their cognate antigens, but on different selection mechanisms in or outside of germinal centers [27–30].

3. Variable domain glycosylation of ACPA

3.1. Introduction of IgG glycosylation

The stability and biological activity of antibodies are influenced by *N*-glycosylation of the antibody Fc region [31,32]. *N*-glycans can be incorporated within the IgG molecule when the *N*-glycosylation consensus sequence Asn-X-Ser/Thr (N-X-S/T, where X ≠ P) is present. IgG molecules have two conventional biantennary N-linked Fc glycans attached to asparagine at position 297 in the C_H2 region of the heavy chain [33]. Glycans are highly dynamic and may display a modified oligosaccharide composition dependent on physiological and pathological conditions. Previously, we and others have demonstrated that ACPA-IgG in RA have a pro-inflammatory Fc glycosylation pattern with reduced galactosylation and sialylation levels, if compared to non-ACPA IgG [34,35]. Interestingly, this ACPA phenotype started to develop around three months before clinical onset of RA [36].

In addition to Fc glycosylation, however, few human genes encoding V-segments of the antigen-binding fragment (Fab) also harbour *N*-glycosylation consensus sequences [37]. These allow for the presence of *N*-glycans in the Fab domain. Moreover, Fab glycosylation can be found in the antibody variable domain as a consequence of somatic hypermutation which, during antibody maturation, can generate de novo *N*-glycosylation sites [38]. These *N*-glycosylation motifs are infrequently found in normal B cells, but are commonly observed in immunoglobulins of patients with follicular lymphoma, especially in diffuse large B-cell lymphoma and Burkitt's lymphoma [39,40]. Interestingly, also IgG of RA patients were observed to have an increased presence of Fab glycans compared to IgG from healthy donors [41].

3.2. ACPA Fab glycosylation

In this context, we recently identified a novel molecular feature of ACPA. ACPA of the IgG isotype were shown to be extensively glycosylated in the Fab region. These Fab glycans were found on both heavy and light chain variable domains [42]. In fact, while ~15–25% of conventional IgG antibodies harbour Fab glycans, we estimated that more than 90% of ACPA-IgG molecules carry Fab glycans [37,43]. ACPA-IgG isolated from synovial fluid, the site of inflammation, could even exceed 100% Fab glycosylation implying that multiple glycans can be attached to the variable domain [43]. Detailed analysis of the glycosylation profile further revealed that these ACPA-IgG harbour N-linked glycans that are highly sialylated. This glycosylation profile of ACPA-IgG, which comprises the full range of biantennary glycan residues, could indicate that ACPA Fab glycans are relatively easily accessible to glycosyltransferases. Although we could not identify a comparable degree of Fab glycosylation for several other autoantibodies, it is yet unknown if Fab glycans are a molecular feature that is specific for ACPA or that it represents the type of autoimmune response that is generated [42]. Therefore, further research is needed to determine the specificity of Fab glycans for ACPA or other autoantibodies in RA. To understand more about the origin of the extensive glycosylation of ACPA-IgG, ACPA-IgG variable domains were analysed by mass spectrometry. This identified *N*-glycosylation sites that were not encoded in the germline sequence, but most likely introduced by somatic hypermutation [42]. Moreover, in contrast to ACPA-IgG, we found indications that ACPA-IgM have no additional Fab glycans [44]. All together, these results strongly suggest that the extensive presence of sialylated *N*-glycans in the ACPA-IgG Fab domains results from somatic hypermutation during maturation of the ACPA response. It also provides an argument for the hypothesis that the maturation of the ACPA response is dependent on T-cell help which may contribute to the breach of tolerance checkpoints and the maturation of citrulline-specific B cells in RA.

3.3. Functionality of ACPA Fab glycosylation

So far, it is unknown what role Fab glycans play in the functionality of ACPA-IgG. Generally, glycans in the antibody variable domain have been implicated to modulate several functions in different ways. For example, Fab glycans located close to the antigen binding site may affect antigen binding [37]. Indeed, we have demonstrated that Fab glycans on monoclonal ACPA may influence antigen binding, as depletion of the variable domain glycans enhanced or decreased binding to the CCP2 antigen, albeit to a limited extent [42]. Another important functionality reported for glycans is the interaction with lectins. One hypothesis is that introduction of Fab glycosylation in the B cell receptor (BCR) via somatic hypermutation allows interaction of the BCR with

lectins in the germinal center and thereby provide survival signals to self-reactive B cells [45]. This phenomenon has been reported before in follicular lymphoma where the interaction of lymphoma cells with lectins free these cells from dependence on antigen and enhance the survival through selection based on glycan interaction [39,45,46]. Because of the abundant presence of terminally sialylated Fab glycans on ACPA-IgG and the overall low affinity of ACPA-IgG compared to conventional recall antigens [27,43], it is possible that B cells producing Fab glycosylated ACPA could have a survival advantage through selection based on the presence of N-linked Fab glycans rather than on affinity for their cognate antigen.

4. Conclusion

ACPA variable domain glycosylation is a recently reported novel molecular feature of ACPA-IgG. Almost all ACPA-IgG molecules were found to carry these highly sialylated Fab glycans whereas non-ACPA IgG and a selected group of other autoantibodies analysed thus far did not show this feature. This indicates that extensive Fab glycosylation is a specific feature of ACPA-IgG in RA and could therefore be used as a biomarker in ACPA-positive disease. The introduction of ACPA Fab glycans is likely the result of somatic hypermutation during maturation of the ACPA response. This observation, together with the finding that ACPA-IgM has no additional Fab glycans, and the strong HLA association with ACPA-positive RA implies that the introduction of *N*-glycans in the variable domain is dependent on T cell help in or outside the germinal center. Moreover, this process could contribute to the breach of tolerance checkpoints during the "second hit" of the two-hit model for the development of ACPA-positive RA. However, this does not exclude the possibility that ACPA-IgG Fab glycans accumulate over time by multiple "hits" or that some ACPA may carry Fab glycans from the start. Although the function of ACPA Fab glycosylation in the pathogenesis of RA remains to be determined, there are hints towards the involvement of Fab glycans in the selection and survival of low affinity citrulline-specific B cells. Further research is needed to determine the specificity of Fab glycosylation in RA, and to gain insight in the biological processes underlying the hyperglycosylation of ACPA-IgG.

Competing interests

None declared.

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