Putative Approaches to Bypass the Citrulline-Specific Autoimmune Response in Rheumatoid Arthritis

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ABSTRACT: The major autoantigens in the inflamed synovium in rheumatoid arthritis (RA) are citrullinated peptides. Citrullinated peptides are employed in diagnostic kits for detection of anti-citrullinated protein antibodies (ACPA), a serological marker with high specificity and sensitivity in the diagnosis of RA, and have been included in the new ACR/EULAR classification criteria for RA. ACPA-positive RA patients suffer from an erosive and more aggressive disease compared to ACPA-negative patients. In view of the mounting indications that ACPA plays a seminal role in the pathogenesis of RA, it might be valuable to pursue a specific treatment aiming ACPA as a target. We found that citrullinated peptides, which contain a unique amino acid, citrulline, alter the protein structure within the connective tissue, leading to tolerance breakdown and triggering the autoimmune response in RA. However, with different doses and routes of administration, citrullinated peptides can promote immune tolerance rather than induction of disease.

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KEY WORDS: rheumatoid arthritis (RA), anti-citrullinated protein antibodies (ACPA), tolerance, inflammation, peptides

Rheumatoid arthritis is an autoimmune disease characterized primarily by progressive synovial inflammation, resulting in irreversible joint destruction, enhanced atherosclerosis, respiratory illness and other extra-articular manifestations [1-3]. The immune response in RA is frequently dominated by the production of autoantibodies generated against citrullinated antigens (abbreviated as ACPA, anti-citrullinated protein antibodies). Citrullination is the post-translational conversion of arginine to citrulline catalyzed by the peptidylarginine deiminase enzyme that is up-regulated under inflammatory conditions [4].

Inflammation and smoking up-regulate peptidylarginine deiminase, which citrullinizes connective tissue proteins

RA = rheumatoid arthritis

Citrulline is not a natural amino acid and is not encoded by DNA. The modification of positively charged arginine to neutral uncharged amino acid may markedly influence the organization of the protein structure and interactions with macromolecules, resulting in altered protein folding. Although it is a physiological process, autoreactivity towards citrullinated residues may develop in susceptible individuals.

Genetic and environmental factors interact in the etiology of rheumatoid arthritis. ACPAs are present in most patients with RA and have a strong genetic association with certain polymorphic human leukocyte antigen molecules from the major histocompatibility complex region (i.e., HLA-DRB1 shared epitope) [5]. The SE alleles encode for a particular amino acid sequence of the P4 pocket peptide-binding site at positions 70–74 (QRRAA, RRRAA, or QKRAA). Citrullination may increase the affinity of peptide binding to MHC class II molecules on cells expressing HLA-DRB1 containing the SE alleles [4].

We and others recently showed that environmental factors, like smoking, are associated with RA susceptibility in individuals with HLA-DRB1 containing the SE motif [2,6]. Those two factors might play a seminal role in the process of the autoimmune response against citrullinated peptides. A first step in the pathogenic process is the citrullination of specific endogenous proteins, potentiated in the lungs of smokers and other locations as a consequence of tissue inflammation [7,8]. This first step is not specific to RA, but it enables binding of SE alleles to self-citrullinated epitopes that cannot be exerted in their native form [9,10]. Hence, the citrullinated self-peptides bound to the antigen pocket of the SE alleles are then presented to autoreactive T cells [9]. The antigenic stimulation of naive CD4+ T cells to differentiate into distinct functional T cell subsets, including T helper 1, Th2 and Th17, is characterized by the production of different cytokines [11,12]. T regulatory cells are another distinct subset of Th cells and play a critical role in immune homeostasis [13]. The tolerance breakdown against citrullinated peptides leads to

ACPA = anti-citrullinated protein antibodies
SE = shared epitope
MHC = major histocompatibility class
Th = T helper cells

imbalance of pathogenic T cells and Treg cells and eventually to the autoimmune response in RA.

AUTOREACTIVE T CELL RESPONSE TO CITRULLINATED PEPTIDES

Since PAD has not been shown to be expressed in the thymus, the likelihood that citrullination occurs in the thymus is low [14]. Subsequently, T cells reactive to citrullinated antigens are not eliminated, resulting in a possible immune reaction against citrullinated antigens. However, T cells specific for citrullinated proteins have not been well characterized. The accumulation of citrullinated peptides at the inflammation suggest their putative potential to serve as a target for tolerance induction.

ACPA plays a direct pathogenic role in rheumatoid arthritis

Stimulation of T cells originating from RA patients with citrullinated peptides resulted in the production of pro-inflammatory cytokines such as interleukin-17 and interferon-gamma [15,16]. In contrast, neither T cell proliferative response nor Th1 (IFN-γ) response by stimulation with citrullinated fibrinogen peptides were detected in the peripheral blood mononuclear cells of HLA-DR4-positive RA patients [17]. One cannot rule out that exposure of these cells (from RA patients) to citrullinated fibrinogen peptides resulted in immune tolerance induction and, therefore, the "pathogenic" Th1 and Th17 pathways were not up-regulated. In addition, it is important to test whether the Th2 and regulatory pathways are up-regulated following exposure to citrullinated fibrinogen peptides.

TOLERANCE INDUCTION WITH CITRULLINATED PEPTIDES

T cell antigen-presenting cell interactions are essential for the initiation of effector responses against foreign and self-antigens. The fate and function of T cells are mediated by the T cell receptor recognizing a peptide/MHC on the surface of APC. This critical recognition event is highly specific but paradoxically is of relatively low affinity. The immune system is normally tolerant to self-antigens; however, failure or disruption of tolerance may result in autoimmunity.

ACPA-based peptides may be a new therapeutic modality that acts through tolerance induction

One way to induce tolerance is by manipulation of the interaction between APCs and lymphocytes. The suppression of autoimmune responses can be reinforced by tolerance induction with self-antigen-derived peptides. T cell-mediated antigen presentation may be central to the mechanism of peripheral tolerance. Each mature T cell retains the potential to reinforce tolerance or mediate immunity, depending on the specific antigenic cues present in the immediate environment.

The potential of peptides to serve as targets for immune tolerance induction and for amelioration of autoimmune conditions is demonstrated in multiple sclerosis. Multiple sclerosis is an autoimmune disease characterized by an inflammatory immune response directed against myelin antigens of the central nervous system. Experimental autoimmune encephalomyelitis [18], induced by the injection of myelin basic protein and adjuvants, or other myelin components, provides a useful animal model for human multiple sclerosis. Soluble peptides representing encephalitogenic epitopes administered orally and via other routes have proved effective in suppressing EAE in mouse models during the induction phase of EAE [19-21].

ACPA PATHOGENICITY

ACPAs constitute the principal autoantibody system associated with RA. Using several citrulline-containing peptide variants in enzyme-linked immunosorbent assay, ACPA could be detected in almost 80% of RA patients with a very high diagnostic specificity, 98% [22,23]. In 2010 the American College of Rheumatology and the European League Against Rheumatism established a new set of classification criteria for RA, with positivity to ACPA considered an important criterion for identifying patients at high risk for developing a persistent and erosive disease [24]. Moreover, ACPA are a superior alternative to the rheumatoid factor test in the laboratory diagnostics of RA [25].

ACPA, the most critical autoantibodies in the sera and synovial fluid of RA patients, bind to citrullinated epithelial (pro) filaggrin, fibrin, vimentin, α-enolase, and type II collagen [reviewed in 26 and 27]. ACPA are present in the blood for a significant period prior to onset of RA disease symptoms [28]. It was also found that the presence of ACPA correlates with joint erosions in RA patients [29,30].

Recent articles show that ACPA are responsible for pathogenic mechanisms in RA. Citrullinated fibrinogen immune complexes induced macrophage tumor necrosis factor-alpha production through Fc receptor engagement [31]. ACPAs can also activate the complement system through both the classical and alternative pathways [32]. Sokolove et al. [33] further demonstrated that immune complexes containing citrullinated fibrinogen directly stimulated macrophages to produce TNFα via Toll-like receptor 4. ACPA were shown to enhance nuclear factor-κB activity and subsequently TNFα production from monocytes/macrophages via binding to surface-expressed

Treg = T regulatory cells
PAD = peptidylarginine deiminase
IFN-γ = interferon-gamma
APC = antigen-presenting cells

EAE =experimental autoimmune encephalomyelitis
TNFα = tumor necrosis factor-alpha

citrullinated glucose-regulated protein 78 (cit-GRP78) [34]. Neutrophils kill pathogens by phagocytosis, degranulation, and the release of web-like structures called neutrophil extracellular traps. NETs have been shown to play a role in autoimmune disease. Aberrant neutrophil extracellular traps-associated cell death (NETosis) occurs in RA, and ACPA levels were recently described to correlate with enhanced NETosis in RA patients [35]. Glycosylation of ACPA in the Fc portion was shown to increase the pro-inflammatory phenotype of the autoantibody [36,37].

Although ACPA alone were not shown to induce arthritis in animal models, Kuhn and co-authors [38] found that when autoantibodies against citrullinated fibrinogen were co-administered with a submaximal dose of anti-CII antibodies cocktail they exacerbated disease severity in CIA mice.

Autoantibodies against citrullinated vimentin (i.e., MCV-ACPA) were shown to enhance osteoclast precursor differentiation into mature bone resorbing cells and bone loss in vitro and in vivo [39]. The observed effect was again attributed primarily to the inducible secretion of TNFα by osteoclast precursors.

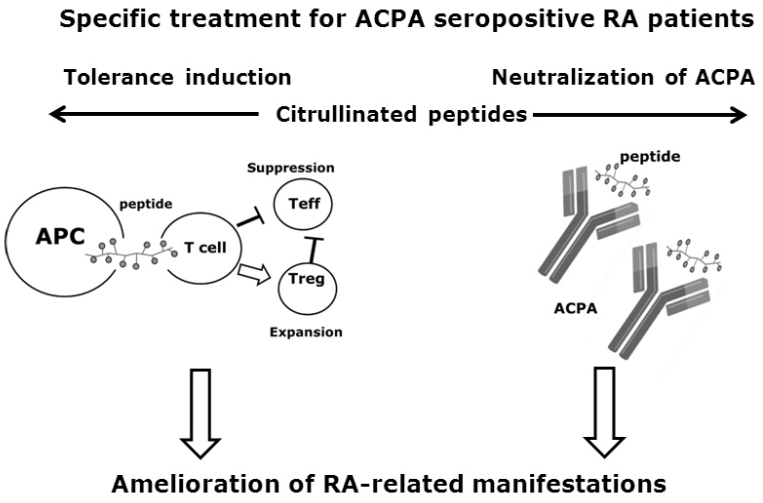
NEUTRALIZATION OF PATHOGENIC AUTOANTIBODIES BY SPECIFIC PEPTIDES

Citrullinated peptides are used in the diagnosis of RA by identifying RA-specific autoantibodies. The ability of citrullinated peptides to presumably block ACPA autoantibodies and their effects on ACPA interactions should be tested. The citrullinated peptides represent highly accurate antigenic ligands for the CCP-ELISA test. ACPA-positive RA subgroups are categorized based on the specificity and sensitivity of the peptide bearing the citrullinated natural modifications. Furthermore, the use of peptides that focus on neutralization of pathogenic autoantibodies provides a possible new therapeutic approach. Citrullinated peptides are very specific for diagnostic application. In addition, the odds for developing anti-peptide neutralizing antibodies are low given their relatively low molecular weight.

An approach termed "theranostics" – a combination of diagnostics and therapy – will identify peptides that can possibly be used as specific targets both diagnostically and therapeutically to improve a patient's treatment. An implication of this concept is demonstrated by the antiphospholipid syndrome, which is characterized by thromboembolic phenomena and recurrent fetal loss associated with elevated circulating anti-phospholipid/beta-2 glycoprotein-I antibodies. A peptide was constructed to block the β2GP-binding site to cell membrane. In vivo, the peptide significantly decreased the percentage of fetal loss in naïve mice infused with anti-β2GPI antibodies [40].

NET = neutrophil extracellular trap
ELISA = enzyme-linked immunosorbent assay
β2GP-1 = beta-2 glycoprotein-I

Figure 1. Putative mechanisms of action of the citrullinated peptides



In summary, ACPA-positive RA patients (approximately 80% of all RA patients), particularly HLA-SE positive patients, might be suitable candidates for specific treatment with citrullinated peptides. This process might turn off autoimmune responses that if left to proceed uncontrolled in susceptible individuals may culminate in RA. We suggest that the presence of ACPA positivity may open the way for RA patients to receive future treatments using citrullinated peptides with a dual purpose: induction of tolerance with the citrullinated peptides and neutralization by direct binding [Figure 1].

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Wanting to be someone else is a waste of the person you are"

Kurt Cobain (1967-1994), American musician best known as the lead singer, guitarist, and primary songwriter of the grunge band *Nirvana*. He struggled with heroin addiction, illness and depression, and eventually committed suicide