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Review

Citrullination of autoantigens: Upstream of TNFα in the pathogenesis of rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial inflammation and destruction of joints. Over 20 years ago, tumour necrosis factor alpha (TNFα) was identified as a key player in a cytokine network, whose multifunctional effects could account for both the inflammation and destruction in RA. The remarkable efficacy of TNF inhibitors in the treatment of RA has resulted in extensive research addressing the regulation of TNFα production responsible for this excessive production.

The discovery of autoimmunity to citrullinated protein/peptide antigens (ACPA) has led the concept that ACPA may be the essential link between disease susceptibility factors and the production of TNFα, which ultimately accounts for the disease phenotype. In this review we will consider (1) the mechanisms of citrullination, both physiological and pathological, (2) how known genetic and environmental factors could drive this peculiar form of autoimmunity and (3) how the immune response could lead to excessive production of TNFα by the synovial cells and ultimately to the disease phenotype (Fig. 1).

1. Protein citrullination

Citrullination (also commonly referred to as deimination) is the post-translational modification of the positively charged amino acid arginine, to a neutral citrulline. Citrullination in the context of a peptide backbone is catalysed by peptidylarginine deiminase (PAD) enzymes. The conversion of a positively charged peptidylarginine sidechain to a comparatively neutral peptidylcitrulline can alter the three-dimensional structure of the protein and its solubility in water. This is important in generating structural proteins, but in the context of RA, may lead to the breaching of immunological tolerance as the neo-epitopes that could be generated may not be expressed in the thymus or bone marrow during lymphocyte selection.

Citrullination has numerous essential physiological roles in a variety of cells and tissues in the body. Citrullination of structural proteins such as pro-filaggrin and keratin in the skin facilitate proteolysis and cross-linking of the proteins which contributes to cornification [2,3]. In the nervous system, citrullination of myelin basic protein (MBP) is essential for the electrical insulation offered by myelin sheaths [3,4]. When trichohyalin, a structural protein that bundles cytokeratin filaments, is citrullinated, it contributes to the maturation of hair cuticle cells [2,5]. Citrullination also controls the functions of histones as support structures and transcriptional control elements for DNA [6,7]. Furthermore, hypercitrullination of histones is needed for formation of neutrophil extracellular traps, part of the innate immune system response to bacterial infection [8,9].

The activity of PAD enzymes is dependent on high concentrations of calcium (Ca^{2+}) [10]. As the Ca^{2+} concentrations required for PAD activity are 100 fold higher than those present in intact unstimulated cells, citrullination is likely to occur in conditions which lead to mobilisation of free intracellular calcium, such as chemokine receptor ligation, apoptosis, necrosis and cellular differentiation [11]. The high calcium concentration needed in vitro also suggests activating mechanisms that may modulate this requirement in vivo [12]. However peptidylarginine deiminases may preferentially deiminate peptidylarginine bearing extracellular substrates such as cytokines, collagen, fibronectin and fibrinogen in the extracellular environment, where calcium concentrations are more optimal for their activity.

Five PAD enzymes (PAD1–4 and PAD6) have been identified in humans, which are all encoded by a single gene cluster on chromosome 1p35–36. PAD homologs for some or all of these enzymes have also been found in other eukaryotes, with similar genomic organisation across species [4,13]. However, a prokaryotic enzyme with PAD activity has only been described in one bacterium to

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date, *Porphyromonas gingivalis* [14,15], which is a major pathogen in periodontitis. This enzyme shows very limited sequence homology with human PADs, but shares a common membership of the guanidino-group modifying enzyme superfamily [16]. Unlike the human PAD enzymes, *P. gingivalis* PAD (PPAD) is not Ca^{2+} dependent, can convert free l-arginine [16] and only citrullinates carboxy-terminal arginines [14,15].

Human PAD enzymes show a characteristic tissue distribution. PAD1 is predominantly expressed in the epidermis. PAD2 is the most widely expressed, with the highest levels found in skeletal muscle, secretory glands, brain and spleen. PAD3 is expressed in hair follicles and the upper epidermal layer. PAD4 is expressed in white blood cells and most well characterised in granulocytes. PAD6 is only found in eggs, ovaries and in early embryos [4,17]. With the exception of PAD4, which has a nuclear localisation sequence, the intracellular presence of PAD enzymes is restricted to the cytoplasm.

PAD2 and 4 are the family members that have been studied most extensively in the context of RA. This is because their tissue distribution would predict that both enzymes would be found within the joint, which is enriched for myeloid cells. Indeed, both PAD2 and PAD4, together with citrullinated proteins, have been demonstrated in the synovial fluid and the synovial membrane [18,19]. Therefore, at present, PAD2 and PAD4 are the strongest candidates for generating the citrullinated antigens that are targeted in the rheumatoid joint. Several reports have demonstrated that citrullinated proteins accumulate at sites of inflammation [20–22]. Citrullinated fibrin and vimentin have been observed in the synovium (outlined in [23]). We demonstrated citrullinated α-enolase detectable at similar levels in the synovial fluid from patients with RA and spondyloarthritis [19], but undetectable in non-inflamed osteoarthritis samples. Importantly the autoantibody response to the citrullinated α-enolase was restricted to patients with RA.

2. Induction of the autoantibody response to citrullinated proteins

The classical paradigm for induction of autoreactivity in any autoimmune disease is the interaction of genetic susceptibility factors with environmental factors to produce a surprisingly limited repertoire of disease-specific antibodies, which cause the associated pathology. In the case of RA, characterisation of the autoimmune response to specific citrullinated proteins has done much to unravel this gene/environment/autoimmunity triad.

2.1. Autoantibodies to citrullinated proteins in RA

The presence of rheumatoid factor – RF (an antibody reactive with the Fc portion of IgG) in RA individuals contributed towards RA being termed an 'autoimmune' disease, and has been a component of the classification criteria for many years [24]. However, the presence of RF is not specific for RA, but is thought to be the consequence of immune activation [25,26]. The second-generation cyclic citrullinated peptide (CCP) assays, now widely used in diagnostic laboratories and included in the new 2010 ACR/EULAR classification criteria, evolved by selecting randomly generated citrulline-containing peptides from a large panel which were tested against RA and control serum, with the sequences giving the best discrimination in diagnostic sensitivity and specificity being adopted for clinical use. Anti-CCP antibodies have been detected prior to the development of clinically apparent RA [27] and are associated with more severe and erosive disease (reviewed by Zendman et al. [28]). In spite of being a powerful diagnostic tool, the cyclic citrullinated peptides used in the CCP assay are of limited use for understanding the disease aetiology and pathogenesis of RA as they do not correspond to in vivo generated citrullinated proteins. However, four citrullinated proteins that are targeted by anti-citrullinated protein antibodies, and are present in the joint, are now well established as autoantigens: citrullinated fibrinogen/fibrin [29], vimentin [18], collagen type II [30], and α-enolase [31], with further proteins awaiting identification and characterisation (reviewed by Wegner et al. [15]).

2.2. Genetic factors contributing to the autoantibody response to citrullinated proteins

Amongst the major and best-studied genetic risk factors identified so far for the development of RA is a group of MHC class II alleles, namely HLA-DR4, -DR1 and -DR10, principally DRB1*0401, *0404, *0408, *0405, *0101, *0102, *1001 and *1402 [32]. All share variants of the Q/R-K/R-R-A-A amino acid motif, termed the 'shared epitope' (SE), present in the third hypervariable region of the DRβ1 chain and which constitutes part of the P4 pocket of the peptide binding groove [33]. The SE has been shown by many to be associated with the ACPA positive subset of RA [34,35]. However, there is much speculation in the literature regarding the underlying mechanism of the SE-RA association. Hypotheses include a direct role of the SE on increased affinity and presentation of autoantigens and subsequent activation of self-reactive T cells [36], decreased activation of regulatory T cells [32], altered thymic T cell repertoire selection [37], and the SE being an innate immune system activator [38,39]. Another genetic risk factor for ACPA positive RA, but also for other autoimmune diseases, is the susceptibility allele *620W* of *PTPN22*, a gene which encodes a tyrosine phosphatase involved in T and B cell signaling [40,41]. A gene in the tumor necrosis factor receptor-associated factor 1-C5 (TRAF1-C5) region has also been shown to associate with ACPA-positive RA [42]. Polymorphisms in the human *PADI4* gene were first associated with RA in a Japanese cohort [43]. Patients with the susceptibility haplotype were more likely to be anti-CCP antibody positive. The association with RA has been confirmed in a number of other Asian cohorts [44–46], although independent of anti-CCP status in a large Korean cohort [47]. Conflicting findings have been reported with Caucasian cohorts [48–52] and a large case-control study of over 5500 UK Caucasian patients showed no association [53]. The reasons for this discrepancy are unclear.

2.3. Environmental factors contributing to the autoantibody response to citrullinated proteins

Environmental factors are considered to contribute to the onset of RA in a genetically predisposed individual. Evidence mainly stems from the low disease concordance rate (15%) in monozygotic twins [54] and the declining incidence of RA in genetically predisposed populations such as the Pima Indians [55,56]. Although a number of environmental exposures have been linked to RA including smoking, periodontitis, hormonal factors and exposure to silica [57,58], smoking is the most clearly established [59–62].

2.3.1. Smoking

The link between RA and smoking was first recognized in 1987 [63] as an unexpected finding in a study investigating the association between RA and the use of oral contraceptives, and later confirmed in a number of case-control and cohort studies (reviewed by Sugiyama et al. [64]). The most striking results were from the Arthritis and Rheumatism Council Twin Study, where 13 pairs of monozygotic twins, discordant for RA and smoking, were identified, and in 12 out of 13 cases the RA patient was also the smoker [60].