Commentary
Citrullination: a small change for a protein with great consequences for rheumatoid arthritis
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Abstract

A new autoantibody activity, which is almost 100% specific for rheumatoid arthritis (RA), has been found. The essential part of the B-cell epitope is a modified form of arginine (ie citrulline). The conversion of protein-contained arginine to citrulline is an enzymatic process that is carried out by peptidylarginine deiminase (PAD), an enzyme that appears to be hormonally controlled. Because of its remarkable specificity, citrullination and related processes might open new possibilities for studying the aetiology of RA.
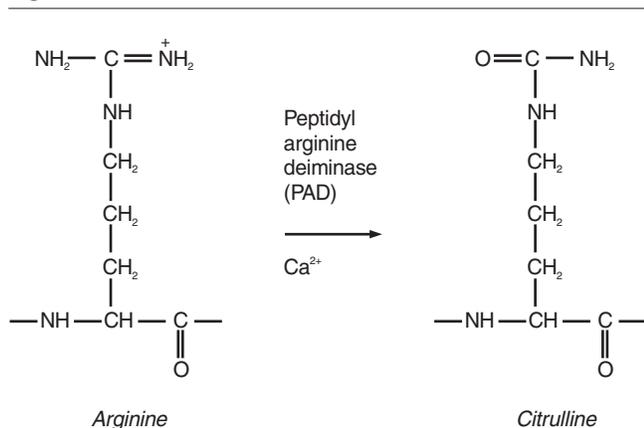
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Introduction
In many systemic immunoinflammatory diseases the presence of the so-called antinuclear autoantibodies is a dominant serological feature [1–3]. Although many autoantigens are ubiquitously expressed, often they are only autoantigenic in certain autoimmune diseases. A growing number of studies seem to indicate that post-translational modifications could be responsible for the initial triggering of autoimmunity and the breaking of tolerance (for review [4]). During the past 5 years much attention has been paid to the fact that many environmental factors known to be involved in the onset of autoimmunity lead to enhanced apoptosis, and to the finding that during apoptosis many autoantigens are uniquely modified. Such modifications on self-proteins may uncover cryptic epitopes and/or create novel epitopes to which no tolerance exists, and could therefore provoke an autoimmune response in susceptible individuals. One such modification that creates novel epitopes might be the citrullination of an as yet undefined RA-specific autoantigen.

Rheumatoid arthritis autoantibodies and citrullinated substrates
RA is diagnosed primarily on clinical disease manifestations, and serological supporting evidence has, up to now, been restricted to the determination of IgM rheumatoid factor. This antibody occurs, however, in many inflammatory diseases and in healthy elderly individuals. One RA-specific autoantibody that has been characterized reasonably well is the antiperinuclear factor, which was described in 1964 by two Dutch scientists [5]. During the past decade it has been shown convincingly that the perinuclear factor is identical to filaggrin [6,7]. Filaggrin (filament-aggregating protein) is produced during the late stages of terminal differentiation of epithelial cells in mammals, and is synthesized as a heavily phosphorylated precursor protein (profilaggrin) that consists of 10–12 homologous, but not identical filaggrin repeats. These repeats are released by proteolytic cleavage during differentiation of the cells. During this process, which resembles a sort of programmed cell death because fully

AKA= anti-keratin antibody; PAD = peptidylarginine deiminase; RA = rheumatoid arthritis.

Figure 1

$$NH_2 - C = \overset{+}{N}H_2$$

$$O = C - NH_2$$

$$NH$$

$$CH_2$$

$$CH_2$$

$$CH_2$$

$$-NH-CH-C-$$

$$\underset{O}{\overset{||}{}}$$

Peptidyl arginine deiminase (PAD)

$\longrightarrow$

Ca^{2+}

Arginine   *Citrulline*

The enzymatic conversion of protein-contained arginine to citrulline.

differentiated epithelial cells are dead cells, the filaggrin polypeptides are dephosphorylated and about 20% of the arginines are citrullinated by the enzyme PAD (Fig. 1). This process also occurs in cells of the stratum corneum of the oesophagus, and this explains why autoantibodies in RA also stain this tissue in the so-called anti-keratin antibody (AKA) test. The AKA test, which is less sensitive than the antiperinuclear factor test, thus depends on the same substrate – filaggrin [8].

It has recently been shown [7,9] that citrullinated residues are essential parts of the antigenic determinants recognized by autoantibodies that are present in RA patients. Using a selected set of citrullinated peptides in an enzyme-linked immunosorbent assay, a sensitivity of more than 70% and an impressive specificity of better than 96% was obtained [7,10]. In an early arthritis clinic study in Leiden, The Netherlands, [10] (Visser H *et al*, unpublished data), it was shown that these anti-citrullinated peptide antibodies can be present very early in disease, and, because of their extreme specificity, they have the potential to become one of the criteria for the early diagnosis of RA. Masson-Bessière *et al* [11] have recently shown that these anticitrullinated protein antibodies are locally produced by plasma cells in the synovium, and thus are likely to be triggered by a citrullinated substrate that is present in the RA synovium. At a recent workshop organized by Panayi at Oxford, UK [12], the same group presented the very interesting and intriguing finding that the α and β chains of fibrin are major citrullinated proteins in the inflamed synovial tissue. This important finding opens new and fascinating possibilities for study of the aetiology of this enigmatic disease.

Citrullination
The process of citrullination in mammalian cells has been studied by only a few groups, and involves the enzymatic conversion (deimination) of protein-contained arginine residues (Fig. 1). The result of this conversion is a very

small change in molecular mass (somewhat less than 1 Da) and the loss of one positive charge. The consequence of the latter might be a change (loss or gain) in its ability to interact with neighbouring proteins [13]. The enzyme responsible for the citrullination is PAD. Today several different human PAD enzymes (at least five) have been identified, but not much is known about their tissue distribution, their cellular localization, and how and when these enzymes are activated. Very interesting in the context of autoimmunity is the finding that PAD activity (ie PAD mRNA levels) appears to be strongly influenced by a variety of oestrogenic compounds [14,15].

At present there are only a few citrullinated proteins known in mammalian cells. It is unlikely that one of these (ie myelin basic protein, filaggrin and trychohyalin) would be the citrullinated RA-specific autoantigen, because none of these proteins appears to be present in, for example, synovial tissue. Therefore, it seems misleading to refer to these autoantibodies as antifilaggrin antibodies [11]. We propose to name them anticitrullinated protein antibodies, because it is very likely that many more citrullinated proteins exist, including in the synovium, as has recently been shown [12]. An intriguing possibility is that some proteins may become citrullinated under pathological conditions, as might be the case for fibrin in the synovial tissue [12]. It is also interesting to note that during apoptosis some cellular proteins become citrullinated.

Apoptosis and autoantigen modification
During apoptosis the morphology of the cell changes dramatically. Membrane ruffling occurs, followed by the formation of apoptotic blebs, cytoplasmic and organelle condensation/shrinkage, and nuclear contraction. The resulting cellular fragments, or apoptotic bodies, under normal circumstances are subject to rapid receptor-mediated ingestion by neighbouring cells and resident tissue phagocytes. In a widely cited publication [16] the group of Rosen showed that many nuclear and cytoplasmic autoantigens translocate to the membrane and can be detected in the large and small apoptotic blebs. It has also been shown [4] that such autoantigens often are modified by cleavage, (de)phosphorylation, ubiquitination or cross-linking. Citrullination of cellular proteins also occurs during apoptosis [17,18]. In particular the apoptotic citrullination of vimentin [17] is interesting because such modifications may contribute to the morphological changes of the apoptotic cell. The citrullination lowers the positive charge of the protein (Fig. 1), which may lead to a destabilization or even loss of intermolecular and intramolecular interactions. In the case of vimentin filaments, citrullination can induce almost complete depolymerization, disrupting the cytoskeletal network [19].

Apoptosis, citrullination and rheumatoid arthritis
Although the presence of apoptotic cells in, for example, synovial tissue is not obvious [20], it is possible that

environmental factors (including pathogenic processes) may locally induce abnormal cell death or disturb the clearance of apoptotic cells. Subsequently, citrullinated (or otherwise modified) protein fragments may be presented to the immune system. We postulate that such modifications may take place only at certain sites in the body, and thus represent unique epitopes to which no effective tolerance exists. A primary and specific immune response will then develop. The resulting autoantibodies will recognize epitopes on apoptotic cells that express autoantigenic molecules at the cell surface, and such opsonized apoptotic cells will generate further proinflammatory responses, and finally induce epitope spreading to nonmodified regions of the autoantigen(s).

Conclusion

Many more studies on apoptosis in pathological conditions are necessary to prove or refute the hypothesis presented above. We know that autoimmunity in general is influenced strongly by genetic, hormonal and environmental factors. The control of apoptosis and the regulation of the immunological processes (the quality of the self–nonself discrimination) are very much dependent on the genetic load of the patient. The fact that autoimmune diseases occur much more frequently in women is a clear indication that sex hormones play an important role. Interestingly, the activity of citrullinating enzymes (PAD) appears to be dependent on hormonal influences. Finally, it has been shown repeatedly that environmental factors (eg viral/bacterial infections) may lead to enhanced cytotoxic T lymphocyte-induced apoptosis, and that apoptotic cell fragments can be processed and presented by dendritic cells. It is also evident that citrullination occurs in dying cells and that citrullinated antigens are specifically recognized by autoantibodies in RA patients.

What we need to show is that citrullination of relevant self-proteins induces the production of autoantibodies. Such studies may also shed light on whether anticitrullinated peptide antibodies have pathological effects.

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