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Review

Consequence of neo-antigenicity of the 'altered self'

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Post-translational modifications play a central role in determining the function of proteins. Such protein modifications come in a great variety of guises, and include phosphorylation, proteolysis, glycosylation, citrullination and oxidative modifications. In relation to inflammatory autoimmune diseases, some post-translational modifications appear to result in the generation of new antigens, and hence autoantibodies. Examples include: the induction of peptide immunogenicity by the spontaneous conversion of aspartic acid residues to isoaspartic acid; granzyme B-mediated cleavage of SLE autoantigens; the oxidative modification—on the surface of apoptotic cells—of lipids and proteins, rendering them immunogenic; and the presence of antibodies to oxidatively modified type II collagen and C1q in RA and SLE patients, respectively. The measurement of autoantibodies to citrullinated proteins has been verified as a very useful diagnostic tool in RA. Proteomics techniques, in principle, allow the detection of all types of *in vivo* protein modifications, and the increasing application of such technologies to the study of rheumatological diseases will further our understanding of autoantigenicity.

Key words: Post-translational modification, Protein oxidation, Citrullination, Oxidized low-density lipoprotein, Inflammation, Rheumatic diseases, Anti-phospholipid syndrome, Neo-antigenicity, Autoantibody, Apoptosis.

Background

The management of patients with autoimmune disease remains a significant challenge. Often the rheumatologist is restricted to treating and relieving the symptoms and consequences and not the underlying cause of the disease. Dramatic advances in the management of patients with rheumatic diseases have resulted from improved understanding of the disease mechanisms, e.g. the introduction of anti-TNF-α therapy in RA. The unravelling of an extremely complex disease mechanism requires the combined efforts of clinical and laboratory-based research scientists alike. An example of this synergy was observed on 8–9 May 2007, in Birmingham, UK, at the British Society for Rheumatology Annual Scientific Meeting, when special scientific sessions were organized to address the role of protein modifications in autoimmunity. New discoveries related to our understanding of immune recognition and attacks on host tissue were integrated with their relevance to clinical implications in the pathology and diagnosis of autoimmune disease.

Concepts of 'self', 'non-self' and post-translational modifications

The ability of the immune system to distinguish 'self' from 'non-self' has always been a key element in our understanding of how immune recognition works. However, occasionally the immune system becomes incapable of differentiating between host molecules and what appears as 'foreign'. During immune system development in an individual, lymphocytes that react to self-antigens in the thymus and bone marrow are deleted. However, host molecules, in particular proteins and nucleic acids, are constantly being modified in the course of normal physiological events—for example, protein phosphorylation. The potential array of post-translational modifications to proteins is vast (Table 1) and many of the listed modifications are currently under investigation [1–3]. Some of these post-translational modifications occurring in the thymus may lead to immune tolerance, whilst other modifications do not. The understanding of what is accepted by the host immune system as 'modified self' and what is not—and the consequence of such a shift in tolerance—will determine how we understand the aetiology of autoimmune disease. Post-translational modifications have been a focus of autoimmune disease research for a number of years. For example, a key post-translational modification in autoimmunity appears to be the citrullination of arginine amino acid residues, by the enzymatic deimination of arginine to citrulline. In multiple sclerosis and RA, citrullinated isoforms of myelin basic protein [4] and fibrin [5] have been found in the brain and synovium respectively, and later in this article we will return to the significance of citrullination for clinical rheumatology.

Role of protein modifications in neo-antigenicity

Doyle and Mamula [6] have demonstrated that even 'minor' post-translational modifications, such as the spontaneous conversion of an aspartic acid to isoaspartic acid (isoAsp), causes ignored self-antigens to become immunogenic. For example, synthesis of a cytochrome c peptide 88–104 (PCC 88-104) with and without an isoaspartyl residue, digested with cathepsin D (an enzyme involved in antigen processing), produces dramatically different isoforms of self-protein in terms of their immunogenicity. This suggests that cryptic self-peptides may be revealed to the immune system by natural modifications to self-proteins. Presentation of these peptides to T cells (recognizing PCC 88-104) reveals that the cells proliferate to a greater extent in response to the isoaspartyl PCC peptide, as compared with the unmodified form of the PCC peptide [7].

Multi-systemic autoimmune diseases, such as SLE, provide further examples of immune responses directed towards post-translationally modified proteins. The detection of autoantibodies against specific antigens, which might improve both early diagnosis and monitoring of disease activity, would be of immense clinical benefit. Unfortunately, over one hundred antigens have been described in SLE patients [8]. These include autoantibodies that target nuclear antigens, cytoplasmic antigens, cell membrane antigens, phospholipid-associated antigens, blood cells, endothelial cells and nervous system antigens, plasma proteins, matrix proteins and other miscellaneous antigens.

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TABLE 1. Two of the many examples of autoimmune diseases and some of their associated post-translational modifications

Disease	Modification	Antigen modified
RA	Hydroxylation	Type II collagen
	Glycosylation	
	Oxidation	
	Citrullination	Fillagrin
		Fibrin
		Vimentin
	Glycosylation	IgG
SLE	Phosphorylation	Multiple
	Deamidation	snRNP D, H2B
	Mannose modification	Multiple
	Methylation	SM D1, D3
	Oxidation	Cardiolipin, ox LDL, C1q, calreticulin

Rosen and co-workers [9] have proposed that the generation of autoantigens in systemic autoimmune disease must satisfy a stringent set of biological criteria in order to be involved in initiating a primary immune response against a previously non-tolerized structure. Rosen has reasoned that many candidate autoantigens are conveniently packaged into apoptotic bodies when cells undergo the normal process of apoptosis. However, in SLE, there is evidence that the recognition and removal of apoptotic debris is either delayed or does not occur at all. The accumulation and enrichment of potential host material makes it a potential candidate for post-translational modification and, in particular, proteolytic cleavage. The process of apoptosis requires the activation of a family of cysteine protease enzymes, called the caspases. Analysis of the common autoantigens detected in SLE patients has revealed that many autoantigens [including poly (ADP-ribose) polymerase, laminins, U1-70 kDa, NuMA, topoisomerase I, Nor 90, fodrin, hnRNP C1/C2, PMS2, La] are cleaved by caspases early in the process of apoptosis [10–12].

The caspase cleavage of proteins during apoptosis, however, is a ubiquitous constitutive process occurring during anti-inflammatory and non-immune processes. In contrast, systemic autoimmunity occurs infrequently in the population. Rosen and colleagues [13] have speculated that a more specific protease—which is restricted to certain tissues that might themselves be influenced by genetic and environmental factors—could provide the necessary conditions for generating novel autoantigenic structures. Granzyme B is a serine protease in the cytoplasmic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, which could be the proteolytic cleavage enzyme integral to this process. Rosen's group has demonstrated that granzyme B can generate unique peptide fragments that are recognized as disease autoantigens. The proteins from which these fragments arise do not apparently produce autoantigens when cleaved by caspases [9, 14]. But what makes host proteins in patients with autoimmunity susceptible to granzyme B cleavage? It is possible that, in order for host proteins to become autoantigenic, they need to be post-translationally modified by one or more of the biochemical pathways (e.g. oxidative modifications, phosphorylation, glutathionylation, transglutamination, citrullination, or formation of protein–nucleic acid complexes), which makes them more susceptible to granzyme B cleavage. A deeper appreciation of how normal physiological processes alter the immunogenicity of self-molecules will enable the mechanisms that propagate systemic autoimmunity to be understood.

Similar perturbations in protein modification may be important in RA. For example, a recent study [15] has demonstrated that post-translational modification of the matrix protein collagen can dramatically alter its immunogenicity. The human joint contains abundant type II collagen (CII), and collagen-induced arthritis is a common experimental animal model of RA. Previously, it has been difficult to substantiate the involvement of autoimmunity to CII in the pathogenesis of RA, perhaps because sera from patients have mainly been used to probe native CII rather than post-translationally modified CII. Is there evidence that post-translationally modified CII exists within the joint? Certainly, the inflamed joint is a site of activation of the many infiltrating leucocytes that arrive during inflammation. This results in these cells generating an influx of reactive oxidants which can damage lipids, protein and/or DNA. Nissim and co-workers [15] exposed CII to biochemical conditions which simulated those found in an inflamed joint, resulting in chemical modifications of native CII. They treated CII with hydroxyl radical ($^{\cdot}OH$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{-}$), and in addition carried out glycation of CII with ribose. Patient antibodies within serum did not usually recognize unmodified CII. However, following experimental modification of CII, 50% of RA patients' anti-sera had antibodies specifically recognizing various CII-modified forms. This evidence demonstrates that in conditions typically found within the inflamed joint, CII is an autoantigen in RA.

The question remains whether post-translationally modified forms of CII are relevant to the pathogenesis of the disease of RA itself. One possibility is that these antibodies target the larger fragments that are capable of being retained in the joint, and ultimately can provoke an inflammatory response. Certainly, post-translationally modified CII-antibody immune complexes can activate and complement other inflammatory pathways within the joint and surrounding tissue [16, 17]. Other important proteins such as the first component of complement—C1q—and the collectins have 'collagen-like' structures that are also vulnerable to post-translational modification. Indeed, previous studies have shown that oxidized C1q, but not native C1q, has the capacity to induce antibodies reactive with both C1q and CII [18]. This is an example where an autoantibody against an extracellular matrix collagen structure in one molecule, which cross-reacts with a similar structure in another molecule, can have profound implications in autoimmune pathology. If anti-collagen antibodies are shown to impede C1q functions, such as immune complex clearance, complement activation and removal of apoptotic cells, then this would be a case in which the generation of autoantibodies against an apparently innocuous protein (CII) influences the functions of other proteins—without any obvious connections at first glance. Clearly, there are therapeutic advantages in preventing the generation, or binding, of potentially pathological autoantibodies to the extracellular matrix collagen and collagen-like structures. This can be done by either suppressing the inflammatory process (and thus reducing the oxidation processes) or by blocking the autoantigen/autoantibody interaction.

Reservoirs of 'altered self' in the dying cells of autoimmune disease patients

Our understanding of autoimmune processes has been greatly helped by studying the mechanisms involved in the apparently non-inflammatory process of apoptosis, and the consequence of not effectively clearing cell constituents and potentially exposing neoantigens to the immune system. Dead and dying cells are normally removed by apoptosis in a rapid and efficient manner. In some autoimmune diseases, this clearance mechanism is defective [19–21], resulting in the accumulation of dead and dying cells for long periods in the circulation. This leaves such cells as targets of autoantibodies in a large variety of autoimmune diseases. Furthermore—in line with the 'danger model' of immunity [22]—these unremoved, dying, cells may release 'danger signals' which trigger the immune system response.

As stated earlier, in both human and experimental models of SLE, many 'autoantigens' have been identified, which cluster in the surface blebs of apoptotic cells. The blebs of these concentrated autoantigens are surrounded by phospholipids that share

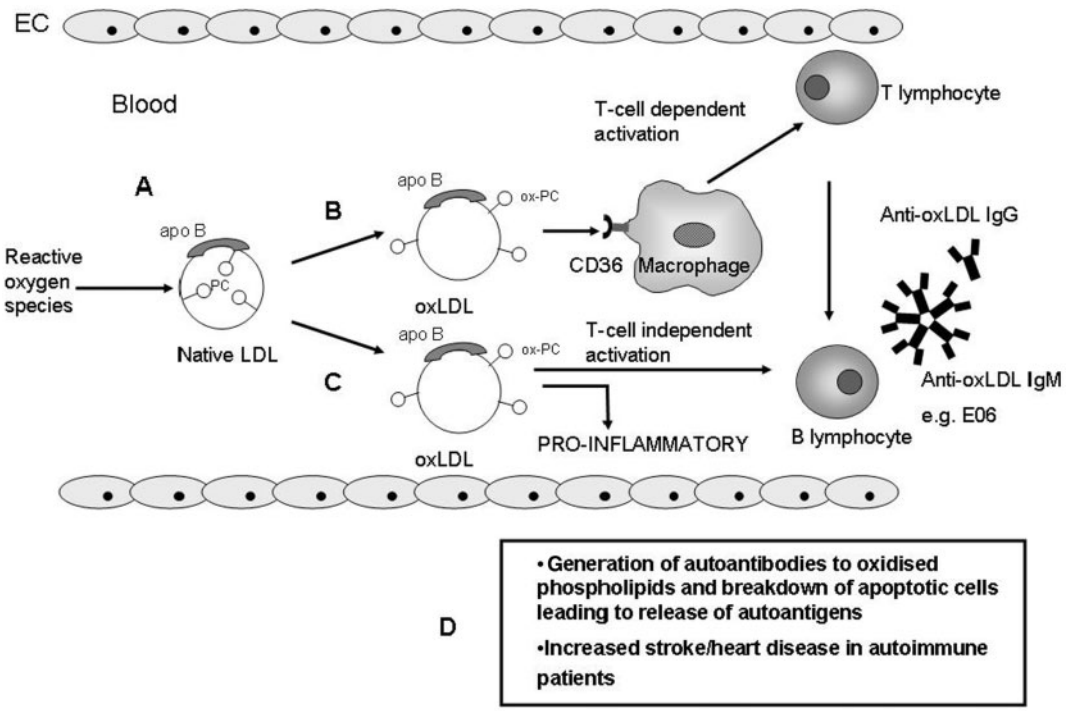

FIG. 1. The oxidation of phospholipids—and the ensuing post-translational modification of proteins by lipid peroxidation products—may play a role in autoimmunity by activating both adaptive and innate immunity. (**A**) In the blood circulation, native LDL can become oxidized in the presence of free radicals, which generate oxidized lipids that are toxic and pro-inflammatory. (**B**) oxLDL (which contains the apoB protein in a form which has been modified by reactions with lipid peroxidation products) is taken up by macrophages via the scavenger receptors, for example, CD36. This leads to foam cell formation and promotion of atherosclerosis. In addition, uptake of oxLDL can activate macrophages to release cytokines such as IL-1β and activate T cells possibly via antigen presentation of oxLDL components, which promote B-cell activation and anti-phospholipid IgG production. (**C**) oxLDL can also activate B1 lymphocytes independently of T-cell help resulting in the production of natural IgM autoantibodies. Moreover, oxLDL can also bind directly to the vascular endothelial cells promoting the surface expression of adhesion molecules and release of chemotactic molecules for the recruitment of T cells and monocytes. (**D**) The generation of anti-phospholipid autoantibodies, particularly E06 IgM, which bind to apoptotic cells and/or oxidized membranes, may modulate the recognition and removal of apoptotic debris. This could result in further antigen–immune complex formation, and increased inflammation leading to further pathological complications in the immune interactions during the course of atherosclerosis in autoimmune patients. OX-PC, oxidised phosphatidylcholine.

many common biochemical features with low-density lipoprotein—LDL—which is well known for its susceptibility to oxidation, and indeed oxidized LDL (oxLDL) is known to promote chronic inflammation in atherosclerosis, which is exacerbated by immune mechanisms [23]. Some of the mechanisms involved are depicted schematically in Fig. 1. These mechanisms are consistent with the important experiments of Kagan and colleagues [24], who showed that many of the phospholipid molecules on the surface of apoptotic cells are oxidized. Again, antibodies generated against lipoproteins have the potential to attack intact cell membranes leading to the release of other potential autoantigens. It is therefore of great interest that autoantibodies against oxLDL are generated against a variety of oxidatively modified lipids (e.g. the oxidized phospholipid backbone and malondialdehyde) and protein adducts, which can be found in oxidized plasma membranes. Such antibodies target so-called 'oxidation-specific' neoepitopes that are highly immunogenic [25]. The generation of monoclonal IgM autoantibodies (Fig. 1), such as E06, to these neoepitopes has revealed that oxLDL autoantibodies also bind to the membranes of cells undergoing apoptosis [26]. The consequence of this is that such cells are inefficiently recognized for apoptotic clearance by macrophages, implying that oxidized phospholipids may act as macrophage scavenger receptor ligands. It is therefore not surprising that premature atherosclerosis is common in patients with SLE and that these patients have elevated levels of oxLDL and autoantibodies to oxLDL. Furthermore, autoantibodies to oxLDL share common features with anti-cardiolipin antibodies, suggesting that both sets of autoantibodies recognize lipid peroxidation products in general [27]. Ultimately, the inefficient removal of apoptotic cells provides an opportunity for the phospholipid membranes of the packed cell debris to be modified, leading to the release of potentially pro-inflammatory danger signals and autoantigens. This, in turn, could cause the production of autoantibodies to 'neo' self-antigens such as protein adducts with lipid peroxidation products. The generation of oxidized lipids and proteins may possibly lead to one or more complications in the autoimmune patient—for example, the premature development of cardiovascular disease. Feng *et al.* [28] have tested this possibility by generating various mouse models of accelerated atherosclerosis in lupus, including apolipoprotein E-deficient [apoE(−/−)] and Fas(lpr/lpr) [Fas(−/−)] C57BL/6 mice. Double knockout mice showed many of the phenotypes of lupus patients: apoE(−/−) Fas(−/−) mice had enlarged glomerular tuft areas, severe proteinuria, increased circulating autoantibody levels and increased apoptotic cells in renal and vascular lesions compared with either of the single knockout mice. This evidence supports the notion that lupus-like disease and atherosclerosis share a common pathway in these disease processes.

Oxidative post-translational modifications are involved in anti-phospholipid syndrome

Yet another rheumatological autoimmune condition known to involve post-translational modifications is anti-phospholipid syndrome (APS) or Hughes' syndrome, which is an important cause of vascular morbidity and mortality and pregnancy loss, not only in patients with autoimmune disease (commonly lupus), but also in patients without known associations. It is characterized by the presence of 'anti-phospholipid antibodies' (aPLs), directed against cell membrane phospholipids that have undergone lipid peroxidation. However, the autoantibodies are not solely directed

against negatively charged phospholipids, but also bind to β2-glycoproteins and prothrombin in complex with phospholipids. APS is an example of a disease pathology, involving autoantibodies to oxidized lipid in association with protein complexes, which results in patients requiring long-term therapeutic intervention [29]. The increased incidence of thrombosis and atherosclerosis observed in APS and SLE may be explained by the binding of this cocktail of autoantibodies directly to endothelial cells, or by the binding of an autoantibody complex with phospholipids, thereby provoking tissue injury and inflammation. Both SLE and APS patients generate various autoantibody isotypes (IgG and IgM) against oxLDL or cardiolipin. In humans, IgG autoantibodies correlated with risk factors for cardiovascular disease, while IgM raised against proteins that have been modified by oxidized phospholipids may be protective [25]. A subset of aPL antibodies can cross-react with oxLDL, and characterization of these antibodies may help us understand the link between APS- and SLE-induced pathologies.

Susceptibility gene for RA encodes a protein that is involved in post-translational modifications

Another key post-translational modification is phosphorylation. Intriguingly, molecular genetic studies have recently shown that—outside the MHC region—one of the most important susceptibility genes for RA (and other autoimmune diseases) is within the gene encoding a protein tyrosine phosphatase (PTPN22) [30, 31]. The PTPN22 gene contains a putative single missense nucleotide polymorphism (SNP) that leads to the disruption of the P1 proline rich motif important for the interaction of this phosphatase with proteins that function as regulators of T-cell activation. The risk allele appears twice as frequently in patients with RA than healthy individuals. However, the SNP is also present in a number of other autoimmune diseases, for example, type I diabetes, suggesting that the alteration of common pathways of post-translational modification can heighten an individual's susceptibility to autoimmune disease.

Post-translational modification involving citrullination gives rise to a diagnostically important autoantibody

The detection of anti-citrullinated protein antibodies (ACPA) is proving useful in the early diagnosis and assessment of prognosis in RA, and has also led to insights into gene–environment effects in autoimmune disease. As mentioned earlier, citrullination is the process of deimination of arginine residues within the polypeptide backbones of proteins. This reaction is catalysed by the enzyme peptidyl arginine deiminase (PAD) [32]. In RA, the identity of the endogenous protein(s) that become citrullinated, and hence become neoantigens, is unclear. However, fibrin, fibronectin and vimentin (as well as other proteins) do undergo citrullination *in vivo*, and the citrullinated forms of these proteins may represent the true antigens that are recognized by the ACPA detected in RA patients [33]. Whatever is the identity of the true antigen, ACPA are highly specific for RA and may have a pathophysiological significance. The diagnostic and prognostic utility of ACPA measurements, now widely available in commercial test kits (ELISA), has been confirmed in studies of early RA from both the United States and Europe showing that ACPA positivity predicts a more aggressive disease course as measured by response to DMARDs, radiological damage and development of disability [34].

An impetus, provided by proteomics technologies, for the discovery of 'non-self'

From the above discussion, it is clearly of great importance to study the nature of the post-translational modifications that occur in autoimmune disease. Proteomics techniques can be directed towards identifying the alteration of 'self' proteins observed in many rheumatological disorders, but there are a number of technical complications in studying a complex protein environment. Unfortunately, the study of subtle structural changes in the global complement of proteins, within a system such as a synovial fluid sample from an RA patient, is very complex. Currently, key techniques of proteomics include two-dimensional gel electrophoresis and a variety of mass spectrometry (MS) techniques, including matrix-assisted laser desorption/ionization time-of-flight MS (MALD/I-ToF-MS). The main issues in applying these techniques to clinical samples lie in the extreme dynamic ranges of concentrations of different proteins. This makes it technically difficult to obtain well-resolved protein separations in combination with high-sensitivity detection by MS, all in a robust and reproducible fashion. In plasma, albumin—as well as several other proteins—is present at an extremely high concentration in comparison with many potential protein antigens. This problem is compounded by the fact that it is entirely conceivable that the proportion of a given protein that actually becomes modified to a neoantigenic form *in vivo* may be very small. So far, MS has been successfully employed to study protein expression of cartilage, in relation to the pathophysiology of OA [35, 36]. The application of additional proteomics techniques such as multi-dimensional liquid chromatography, in combination with MS formats that allow the detection of post-translational modifications, will doubtless be a source of advances in the field of rheumatology research.

Conclusion

Clearly, the pathophysiological mechanisms of autoimmune disease are intricate and involve complex genetic and environmental factors. There have been no logical explanations as to why an overzealous immune system recognizes and attacks the many host molecules that are present in high abundance. The production of autoantibodies to common molecules such as proteins, phospholipids, DNA and whole blood cells should not occur if the concept of immune tolerance is accepted. Many autoantigens and subsequent pathological autoantibodies are not against rare molecules released from 'immunologically protected' tissues, but common and abundant proteins, nucleic acids, lipids and sugar molecules. Further studies, exploring the extent to which chemical modifications explain the targeting of these components in rheumatological disease, will facilitate understanding of the complex recognition pattern arrays recognized by the humoral immune system. Only then will we be able to design specific therapeutic targets to prevent tissue damage in these diseases rather than just address the often disastrous consequences of our 'altered self'.

Rheumatology key messages

- Proteins undergo post-translational modifications such as oxidation, citrullination and glycosylation.
- Sometimes protein modifications may give rise to new antigens that are recognized by the immune system as 'non-self'.

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